From Barrett's Esophagus Towards Adenocarcinoma:

Genetic and Clinical studies

Linetta Koppert

From Barrett's Esophagus Towards Adenocarcinoma: Genetic and Clinical studies

Van Barrett's slokdarm naar adenocarcinoom: genetische en klinische studies

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam,
op gezag van de
rector magnificus

Prof.dr. S.W.J. Lamberts

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
vrijdag 16 juni 2006 om 13.30 uur

door

Lisa Bonetta Koppert

geboren te Rotterdam

PROMOTIECOMMISSIE

Promotoren:	Prof.dr. H.W. Tilanus
	Prof.dr. J.W. Oosterhuis
Overige leden:	Prof.dr. J.W.W. Coebergh
	Prof.dr. E.J. Kuipers
	Dr. H. van Dekken
Copromotor:	Dr. W.N.M. Dinjens

Above all else, guard your heart, for it is the wellspring of life.

Proverbs 4:23

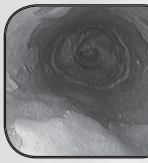
Voor mijn ouders

CONTENTS

Part I

General Introduction

Chapter 1



Adenocarcinomas of the gastro-esophageal junction

Introduction & Outline of the thesis

Partly adapted from book 'Oncologie', Chapter 16: 275-280, Bohn Stafleu van Loghum 2005

Hugo W Tilanus
Linetta B Koppert
Toni Lerut

Epidemiology

Esophageal adenocarcinoma is a highly aggressive disease from which more than 80% of patients die within 5 years after diagnosis. Worldwide almost 400,000 new patients are diagnosed annually. Herewith esophageal cancer ranks eighth on the list of most common cancers, and sixth on the list of cancer mortality causes [1]. More than 90% of esophageal cancers are either squamous cell carcinomas or adenocarcinomas [2]. Esophageal cancer incidence has been rapidly increasing in Western Europe and the USA [3-5]. This could be mainly ascribed to an increase in the rate of esophageal adenocarcinoma, which by now equals or even exceeds the rate of esophageal squamous cell carcinomas. Esophageal cancer incidence in the Netherlands is 10.2/100,000 for men and 3.2/100,000 for women with around 900 newly diagnosed patients annually [6]. Adenocarcinomas of the gastric cardia and of the esophagus, commonly located in the distal esophagus, have several similarities. They show a parallel increase in incidence. Moreover, they show similarities in epidemiological and histomorphological features as well as in patterns of comorbidity [7-9]. This thesis mainly deals with molecular biological aspects of adenocarcinomas of the distal esophagus and its precursor lesion, Barrett's esophagus, but also includes work on adenocarcinomas of the gastric cardia. Adenocarcinomas from the distal third of the esophagus plus adenocarcinomas of the gastric cardia are in this thesis altogether mentioned by the description "gastro-esophageal junction (GEJ) adenocarcinomas".

Etiology

Alcohol and/or tobacco use may confer risk for GEJ adenocarcinomas whereas they are established risk factors for esophageal squamous cell carcinomas [10-14].

Gastro Esophageal Reflux Disease (GERD) is described to be the most important etiologic factor since it can lead to the formation of Barrett's esophagus [15,16]. This precursor lesion forms the main established risk indicator for the development of carcinoma [7,15,17]. Barrett's esophagus is characterized by columnar metaplasia with intestinal differentiation that has replaced the normally present stratified squamous epithelium in the lower third of the esophagus (also see 'Pathology'). Patients with Barrett's esophagus have a 30-125 times increased risk for developing esophageal adenocarcinoma, however only about 0.2-2% of patients with Barrett's esophagus will eventually get a carcinoma [18]. The true prevalence of Barrett's esophagus in The Netherlands is difficult to establish since most patients do not undergo upper endoscopy. Weekly retrosternal pain however is present in around 10% of adults. When upper endoscopy is performed in these patients, around 12.5% of these have Barrett's esophagus. Estimated prevalence in The Netherlands is therefore around 100,000 patients.

Drugs that relax the lower esophageal sfincter and increase reflux, such as anticholinergic agents, aminophyllines and beta-blockers may contribute to the development of esophageal adenocarcinoma [19,20].

It has been postulated that Helicobacter Pylori infection (in particular strains that are positive for the CagA protein) may reduce the risk of GERD and therefore may provide protection against the development of adenocarcinoma [21-24].
By increasing intraabdominal pressure and gastroesophageal reflux, obesitas may contribute to development of esophageal adenocarcinoma, however contradictory results are provided on this subject in the literature [19,25].
Familial cases of esophageal adenocarcinoma are rare and Barrett's esophagus and esophageal adenocarcinoma have no known genetic cause to date.

Clinical manifestations & Diagnosis

Dysphagia, retrosternal pain, odynophagia (pain on swallowing food and liquids), retrosternal pain, pain during food passage, weight loss and anaemia are the symptoms that lead patients with GEJ adenocarcinoma to seek medical attention. Physical examination is usually unremarkable. Lymphadenopathy, particularly in the left supraclavicular fossa (Virchow's node), hepatomegaly and pleural effusion are indicators of metastatic disease.
Physicians should consider testing high risk patients with GERD to rule out malignancy. Upper endoscopy is performed to obtain tissue samples of suspect areas of mucosa, aiming at early detection of high grade dysplasia and early adenocarcinoma (Figure 1.1). The histological identification of dysplasia in tissue biopsies is the best marker for cancer risk and is of major importance in determining the intensity of both surveillance and treatment [26]. Patients with Barrett's esophagus are usually subjected to intensive endoscopic surveillance with biopsy sampling to identify those with neoplastic progression. The surveillance program followed at the Erasmus MC is according the guidelines from the American College of Gastroenterology and the International Society for Diseases of the Esophagus. In patients with Barrett's esophagus without dysplasia, surveillance every 2 year is considered adequate. In patients with low grade dysplasia, yearly endoscopy is advised. If high grade dysplasia is found, the diagnosis should be confirmed by an experienced pathologist. If the diagnosis high grade dysplasia is confirmed, there is no agreement on the most appropriate management of these patients. Esophagectomy is recommended to eliminate the risk of cancer or to detect and treat an unsuspected cancer at an early stage. Concerns with this approach include the potential risks of surgery, especially in older patients, and the highly variable natural history of high grade dysplasia.

Pathology

Most esophageal adenocarcinomas appear to arise from dysplastic Barrett's esophagus. Norman Ruppert Barrett (1903-1979) was born in Adelaide, Australia, and devoted his carreer to thoracic surgery. He pulished in 1950 in the British Journal of Surgery on "Chronic peptic ulcer of the oesophagus and 'oesophagitis' " supporting the view of previous observers that the affected segment was actually the gastric cardia displaced by a congenitally short esophagus.

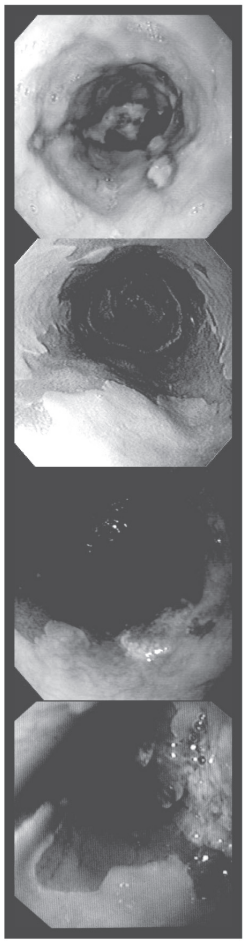


Figure 1.1 Endoscopy images. *Also see color figures page 291.*

How this concept evolved to its current understanding and Barrett's own conversion to the notion of esophageal mucosal metaplasia is told by Spechler and Goyal in Gastroenterology in 1996 [27].

Barrett's esophagus is characterized by columnar metaplasia with intestinal differentiation, as identified by Goblet cells, that has replaced the normally present stratified squamous epithelium in the lower third of the esophagus. Progression is considered to follow the 'metaplasia-low grade dysplasia-high grade dysplasia-carcinoma sequence', characterized by accumulation of cell cycle abnormalities, genetic abnormalities and aneuploidy (Figure 1.2) [28-30].

Most esophageal adenocarcinomas are located in the lower third part of the esophagus which starts at the level of the trachea bifurcation and extends to the gastro-esophageal junction ('the distal esophagus'). The gastro-esophageal junction is characterized by the Z-line, which forms the transition zone from squamous cell epithelium to cylindrical epithelium of the stomach. Carcinomas located at the gastro-esophageal junction are classified as gastric carcinomas according to the Union Internationale Contre Cancer (UICC)-TNM. With regard to epidemiology as well as histomorphology these tumors show similarities with distal esophageal adenocarcinomas [8]. Moreover the therapeutic approach is identical. In the studies described in this thesis a carcinoma was considered to arise from the distal esophagus when premalignant Barrett's mucosa was present and/or the epicenter of the mass was located in the tubular esophagus extending from the tracheal bifurcation to the gastro-esophageal junction including the intra-abdominal esophagus, according to the TNM classification (International Classification of Diseases for Oncology C15.5). The tumor was considered to be cardiac when the epicenter was immediately below the gastro-esophageal junction, extending approximately 2 cm downwards. The tumor was classified as a junction carcinoma if the epicenter was just at the GEJ, without predominance for distal esophagus or gastric cardia and if no Barrett's mucosa was present (Figure 1.3). Tumors arising from the fundus or corpus of the stomach and infiltrating the gastric cardia or distal esophagus were classified as gastric carcinomas.

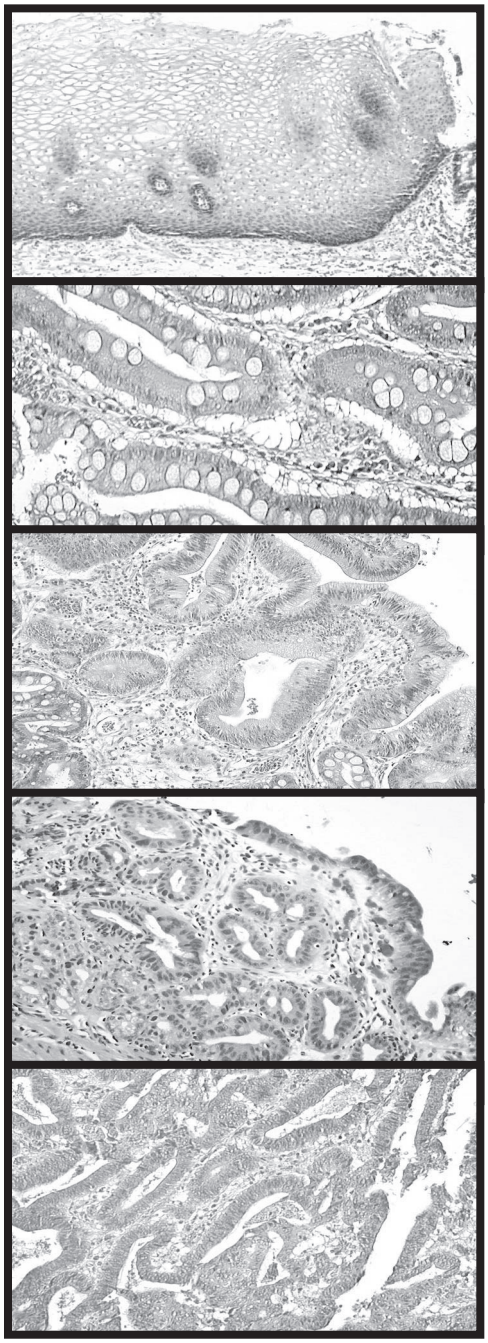


Figure 1.2 Histology of 'metaplasia-low grade dysplasia-high grade dysplasia-carcinoma sequence'. *Also see color figures page 291.*

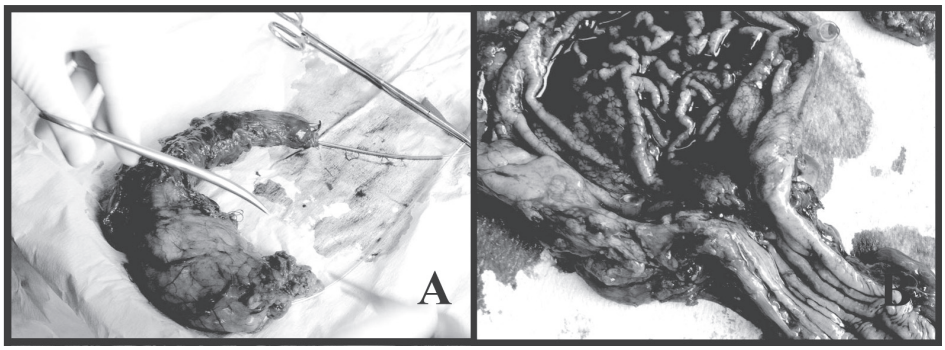


Figure 1.3 Resection specimen with GEJ adenocarcinoma. *Also see color figures page 291.*

Staging

After diagnosing esophageal carcinoma, patients' fitness for surgery needs to be established. Only in patients fit enough to undergo surgery, tumor staging is performed. Up to 50% of patients with esophageal cancer present with inoperable disease as a result of severe comorbidity or due to locally advanced or metastatic disease.

Endoscopic ultrasonography (EUS) identifies preoperative depth of tumor growth (T stadium) and involvement of lymph nodes (N stadium). Reliability of EUS in predicting T and N stadium is 84% and 77% respectively [31]. Computer tomography scanning (CT, Figure 1.4) of thorax and abdomen as well as cervical ultrasonography are used to discover possible distant metastases (M stadium). Metastases comprise most often celiac lymph nodes, supraclavicular lymph nodes and liver metastases. Cytological confirmation of distant metastases can be obtained by percutaneous biopsies [32].

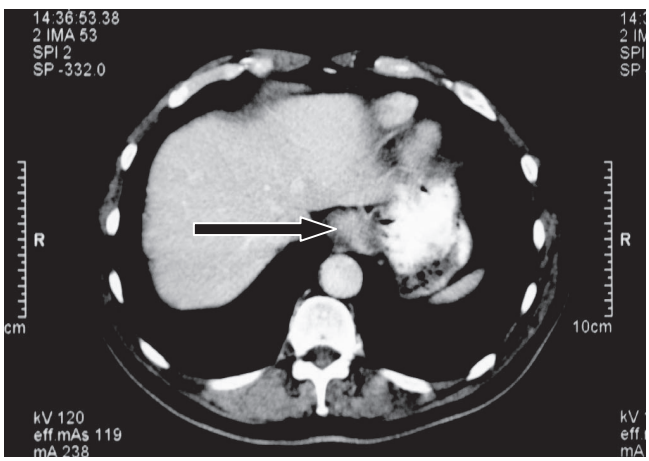


Figure 1.4 CT scan with GEJ adenocarcinoma (arrow)

AJCC-TNM (T-stage in Figure **1.5)**

Stage 0 (pTisN0M0) tumor restricted to mucosa (pTis/carcinoma in situ/high grade dysplasia) ; no regional lymph nodes (No); no distant metastasis (M0).

Stage I (pT1N0M0): tumor infiltrating in lamina propria or in submucosa (pT1); no regional lymph nodes (N0); no distant metastasis (M0).

Stage IIA (pT2-T3N0M0): tumor restricted to muscularis propria (pT2) or adventitia (pT3); no regional lymph node metastasis (N0); no distant metastasis (M0).

Stage IIB (pT1-T2N1M0): tumor restricted to mucosa or submucosa (pT1) or tumor infiltrating muscularis propria (pT2); regional lymph node metastasis (N1); no distant metastasis (M0).

Stage III (pT3N1M0 or pT4NxM0): tumor restricted to adventitia (pT3) with regional lymph node metastasis (N1); or tumor invading neighbouring structures (pT4) with or without regional lymph node metastasis (Nx); no distant metastasis (M0).

Stage IV Any T, Any N, distant metastasis M1a (i.e. metastasis in celiac lymph nodes in case of distal esophageal carcinomas) or M1b (other distant metastasis).

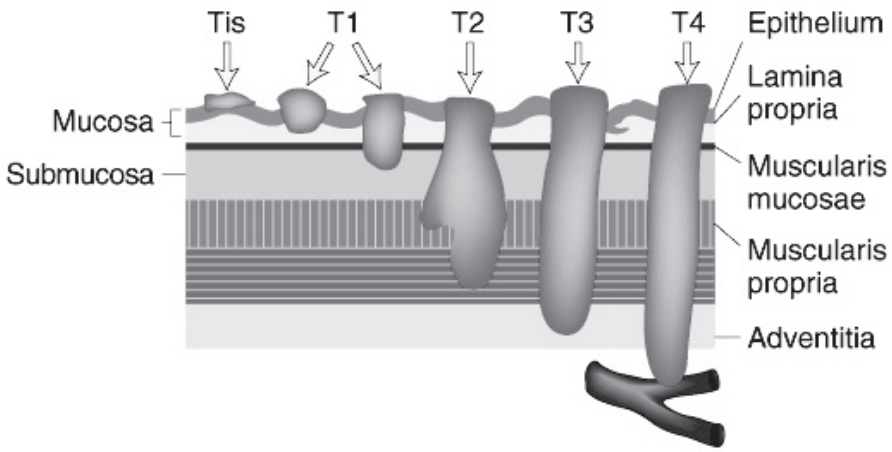


Figure 1.5 TNM classification (T-stage). *Also see color figures page 291.*

Treatment & prognosis

Surgical resection is the only curative treatment for GEJ adenocarcinoma. Survival rates mostly depend on pathological stage and are still poor despite recent achievements in surgical, anaesthesiologic and postoperative care [33]. Surgical resection is currently the preferred treatment for fit patients with tumors that are considered resectable and without distant metastases at preoperative workup (T1-3 N0-1 M0). Tumor depth restricted to the mucosa (Tis) is associated with a mean 5-year survival of 88%, whereas tumor restricted to adventitia (T3) is associated with a mean 5-year survival of 10-20% only. Tumor ingrowth in neighbouring structures (T4) and the presence of distant metastases are considered contra-indications for

surgical resection. Recent advances in non-surgical palliative procedures, such as esophageal stenting and brachytherapy have been proven safe alternatives to secure passage of food and provide a comparable quality of life [34-36].

Surgical treatment for esophageal cancer is still associated with a significant risk of mortality and a high incidence of postoperative morbidity. In the 1980s, esophagectomy was the procedure with the highest mortality risk (29%) in general surgery at that time [37]. Nowadays, the average reported mortality varies from 4% in specialized centres to more than 10% in low volume hospitals [38,39].

Esophagectomy with removal of esophagus and gastric cardia including primary tumor as well as neighboring regional lymph nodes is performed with or without opening the thorax (transthoracic and transhiatal approach respectively). Extensive surgical resection is difficult due to proximity of trachea, main bronchi, pericardia, aorta and diaphragm. Moreover tumor spread to lymph nodes occurs rather early in tumorigenesis due to the presence of a submucosal lymph plexus [40]. Extended lymph node dissection can be obtained by opening abdomen and thorax (and neck) in order to obtain long term survival. On the other hand, performing a less extensive, transhiatal, resection can decrease postoperative morbidity as well as mortality. In the literature a survival advantage has been described for distal esophageal adenocarcinoma patients when a thoracotomy with extensive lymph node dissection was performed as compared to patients that underwent regional lymph node dissection only by means of a transhiatal approach [41,42]. In our clinic, a transhiatal approach is first choice for resection of GEJ adenocarcinomas since 1989 (Figure 1.6). A 'blunt dissection' of the esophagus under direct vision through the widened hiatus of the diaphragm is performed with en-bloc dissection of tumor and adjacent lymph nodes. A gastric tube is constructed to form the new esophagus. Esophago-gastrostomy is performed in the neck.

In general, microscopically irradical resections are performed in approximately 30% of patients [41,43,44]. Several regimens of neoadjuvant therapy have been investigated in order to achieve tumor shrinkage in order to increase resectability rate and improve survival. Several randomised studies and reviews show that a possible benefit, if any, of neoadjuvant chemotherapy is small and it is uncertain whether such a potential survival benefit outweighs the morbidity caused by such a treatment [43-51]. Several studies show that preoperative radiotherapy in combination with chemotherapy is recommended compared to surgery alone. Preoperative chemoradiotherapy might improve local control and therefore improve the prognosis of esophageal carcinomas. Currently, a randomized phase III trial is being conducted in The Netherlands.

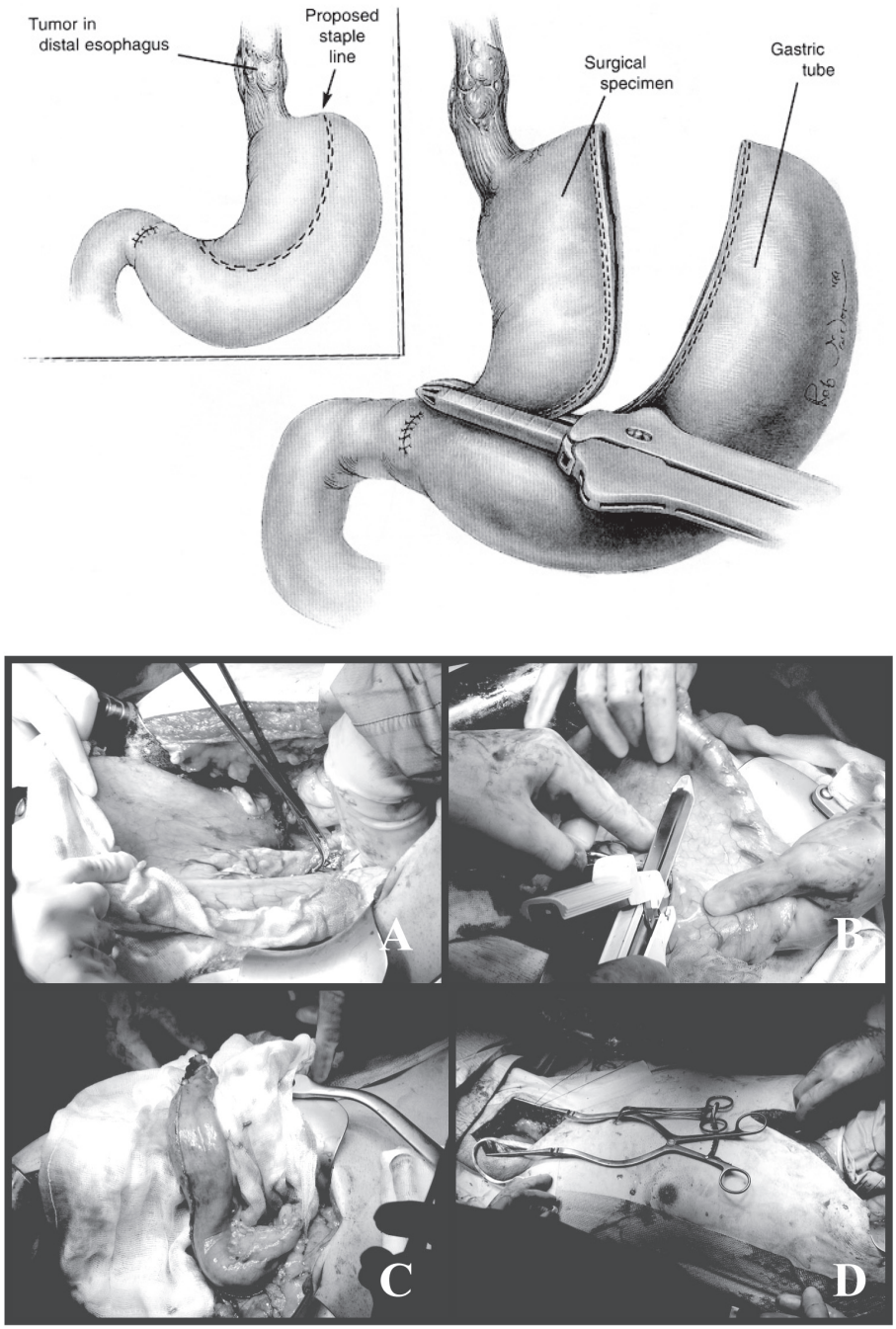


Figure 1.6 Schematic overview of surgical resection of GEJ adenocarcinoma. Photographs below the scheme show upper abdominal incision (A.), and creation of the gastric tube (A. B. C.) to restore continuation of the gastrointestinal tract after transhiatal en-bloc removal of esophagus and gastric cardia including tumor and adjacent lymph nodes (D.). Esophagogastrostomy will be performed in the neck (smaller incision left side of picture D.). *Also see color figures page 291.*

AIMS AND OUTLINE OF THIS THESIS

Aims

Esophageal adenocarcinoma is a highly aggressive disease from which more than 80% of patients die within 5 years after diagnosis. Patients with Barrett's esophagus have a 30 to 125 times higher risk of developing esophageal adenocarcinoma as compared to the general population. Presently, histological detection of dysplasia is the best available marker for cancer risk. Intensive endoscopic surveillance, including tissue biopsies, is aiming at early detection of high grade dysplasia and early adenocarcinoma. Patients with high grade dysplasia or early adenocarcinoma have a survival rate of 80% after surgery, which stress the importance of early detection. To date it is not possible to predict which patients with Barrett's esophagus will progress to invasive carcinoma. Stratification of Barrett's esophagus patients that are at risk of esophageal adenocarcinoma and those not at risk, is therefore urgently needed. This would allow more aggressive or preventative treatment of the people at risk, but also release the vast majority of people who are not at risk from unnecessary endoscopic surveillance.
The genetic alterations underlying esophageal carcinogenesis remain poorly understood. Knowledge of molecular biology could be of additional value to the histological diagnosis of dysplasia in predicting neoplastic progression. The aim of this thesis was, first, to better understand the molecular mechanisms in GEJ adenocarcinomas and its neoplastic progression and, second, to identify genetic markers that, in the end, might distinguish Barrett's epithelia that will progress to esophageal adenocarcinoma from Barrett's epithelia that will not progress to malignancy. Genetic alterations in Barrett's esophagus and GEJ adenocarcinoma were correlated to clinicopathological characteristics and patient survival. Moreover, aspects on epidemiology and treatment were investigated.

Outline

Chapter 2 provides a review of the literature on the molecular biology of esophageal adenocarcinoma. In general, genetic instability forms the hallmark of cancer development resulting in the activation of proto-oncogenes and inactivation of tumor suppressor genes. In this thesis, mutation analyses (Single Stranded Conformation Polymorphism (SSCP) studies) and Loss of Heterozygosity (LOH) studies aiming to identify alterations in (candidate) proto-oncogenes and tumor suppressor genes formed the main approach to study genetic alterations in GEJ adenocarcinomas. A schematic overview of the LOH analysis is provided in Figure 1.7.
Part II concerns molecular biological studies within GEJ adenocarcinomas. The contribution of the Wnt signaling pathway, an oncogenic pathway with great importance in colorectal carcinomas amongst others, was studied in **Chapter 3.** This chapter presents immunohistochemical data on diverse components of the Wnt signaling pathway in GEJ adenocarcinomas. Expression data were correlated to clinicopathological characteristics. In **Chapter 4** mutation and LOH analysis of the AXIN1 locus was performed in primary GEJ adenocarcinomas and in

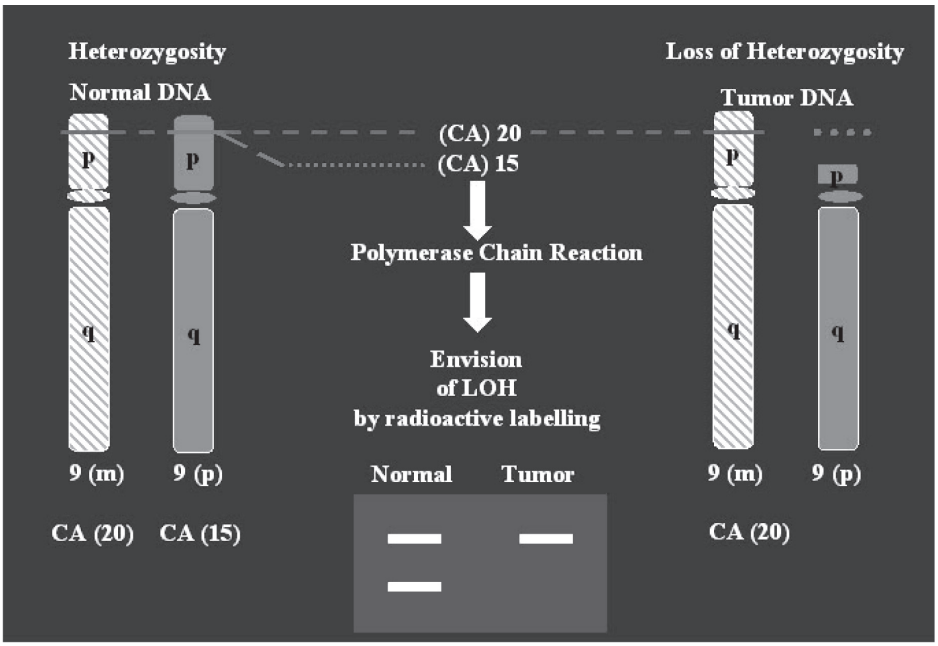


Figure 1.7 Schematic overview of Loss of Heterozygosity (LOH) analysis. Amplified fragments of maternal and paternal DNA of chromosome 9 are compared between normal and tumor DNA of a patient. Length of CA repeats within the fragments differs in the normal DNA, i.e. there is heterozygosity (maternal contains 20 CA repeats and paternal contains 15 CA repeats). In patients tumor DNA part of the short arm of paternal chromosome 9, containing the 15 CA repeat, is lost and therefore the heterozygosity is lost. This is visualized on a gel after radioactive labeling. Only the maternal 20 CA repeat is visualized in the tumor as compared to the normal DNA in which both fragments are shown and therefore this tumor shows LOH. Within LOH analysis, a separation of fragments takes place through difference in length, whereas Single Strand Conformation Polymorphism (SSCP) analysis separates fragments by a different conformation between normal and mutated DNA. *Also see color figures page 291.*

GEJ adenocarcinoma cell lines aiming to find an explanation for Wnt pathway activation in GEJ adenocarcinomas.

Chapter 5 describes the identification of an overlapping region of LOH at the long arm of chromosome 14, possibly harboring a GEJ adenocarcinoma suppressor gene. The 7.1 mega base pairs minimal deletion was located at 14q31.1-32.11 and identified using primary GEJ adenocarcinomas, GEJ adenocarcinoma xenografts as well as GEJ adenocarcinoma cell lines. In **Chapter 6** an immunohistochemical study of neuroendocrine cells in GEJ adenocarcinoma and adjacent Barrett's esophagus is described using chromogranin A antibody. Expression was correlated to TNM stage, tumor differentiation grade and survival.

Part III of the thesis focuses on epidemiological aspects of GEJ adenocarcinomas. Comorbidity patterns in esophageal adenocarcinomas were compared with patterns in cardia adenocarcinomas, esophageal squamous cell carcinomas and subcardia gastric carcinomas to gain more insight in variation of risk factor profiles in **Chapter 7**. Moreover, comorbidity patterns in these tumor types were, retrospectively, correlated to clinicopathological characteristics,

treatment choice (either curative or palliative) and survival in **Chapter 8**. This was done in order to determine a possible role for comorbidity in prognosis and treatment choice.

Part IV deals with aspects of surgery. Surgical resection of cancers of the esophagus and GEJ is associated with substantial morbidity and mortality. Local endoscopic techniques to detect and to treat high grade dysplasia and early carcinoma have become more and more available in recent years. A retrospective study described in **Chapter 9** evaluated the outcome of transhiatal esophagectomy in 120 patients with early adenocarcinoma of the esophagus or gastro-esophageal junction. The question was addressed whether surgical resection is necessary in patients with early malignancies or whether local endoscopic techniques might be suffcient. In order to precisely classify this series of early cancers, the mucosa and submucosa were subdivided into six successive layers. Additionally the depth of tumor infiltration was correlated to lymphatic dissemination and recurrence free period. **Chapter 10** describes the development and validation of a simple risk score combining clinical characteristics along with hospital volume to predict surgical mortality after esophagectomy which may be useful for risk adjustment in quality of care assessment.

Part V of this thesis aims to identify patients at high risk for developing GEJ adenocarcinoma. Patients with Barrett's esophagus receive endoscopic surveillance to detect dysplasia and to diagnose carcinoma at an early and possibly treatable stage. The identification of genes that confer susceptibility for adenocarcinoma formation in Barrett's esophagus would imply improved manageability of Barrett's patients. The CHEK2 gene, located on human chromosome 22q12, encodes a cell cycle checkpoint kinase that is implicated in DNA damage responses. CHEK2*1100delC is a truncating germline variant that abrogates kinase activity and confers low-penetrance susceptibility to breast cancer. As CHEK2 and p53, having an important role in the development of esophageal adenocarcinoma, are participants of the same biological pathway, we aimed to establish whether CHEK2*1100delC confers susceptibility to esophageal cancer in **Chapter 11**.
In recent literature, single nucleotide polymorphisms, defined as germline variant sequences of DNA in the population that are non-pathogenic, have been described to confer susceptibility to development of various cancers. **Chapter 12** describes the investigation of a possible prognostic role of different genetic polymorphisms in a large cohort of GEJ adenocarcinoma patients as compared to healthy persons. Moreover the distribution of polymorphism genotypes was investigated in a large cohort of esophageal squamous cell carcinoma patients.
Finally in **Chapter 13**, it was aimed to identify molecular markers that predict progression to malignancy in Barrett's patients. First, the question was addressed whether GEJ adenocarcinomas are monoclonal or polyclonal proliferations. To this end, multiple biopsies per carcinoma were selected from GEJ adenocarcinoma resection specimens. These were investigated for loss of heterozygosity (LOH) of chromosomal loci 17p (p53), 9p (p16), 5q (APC, MCC), 13q

(Rb) and 18q (DCC, DPC4/SMAD4), for p53 mutations (exon 5 to 8) and for mutations in the homopolymeric C-stretch (D310) within the mitochondrial DNA (mtDNA) displacement loop. Second, the usefulness of these aberrations as molecular progression markers was studied in the 'metaplasia-low grade dysplasia-high grade dysplasia-carcinoma sequence' in Barrett's esophagus adjacent to GEJ adenocarcinoma.

The studies described in this thesis are summarized in **Part VI**, **Chapter 14**. The Epilogue, **Chapter 15**, contains a general discussion. Moreover clinical applications as well as potential directions for future studies are addressed.

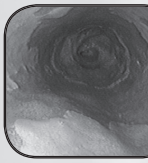
REFERENCES

1. Parkin DM, Bray FI, Devesa SS: Cancer burden in the year 2000. The global picture. Eur J Cancer 37 Suppl 8:S4-66, 2001
2. Daly JM, Fry WA, Little AG, Winchester DP, McKee RF, Stewart AK, Fremgen AM: Esophageal cancer: results of an American College of Surgeons Patient Care Evaluation Study. J Am Coll Surg 190:562-72; discussion 572-3, 2000
3. Devesa SS, Blot WJ, Fraumeni JF, Jr.: Changing patterns in the incidence of esophageal and gastric carcinoma in the United States. Cancer 83:2049-53, 1998
4. Powell J, McConkey CC: Increasing incidence of adenocarcinoma of the gastric cardia and adjacent sites. Br J Cancer 62:440-3, 1990
5. Wijnhoven BP, Louwman MW, Tilanus HW, Coebergh JW: Increased incidence of adenocarcinomas at the gastro-oesophageal junction in Dutch males since the 1990s. Eur J Gastroenterol Hepatol 14:115-22, 2002
6. Visser O, Siesling SS, Dijck van JAAM: Incidence of cancer in the Netherlands 1999/2000. Eindhoven Cancer Registry, 2003, pp 24-25
7. Ruol A, Parenti A, Zaninotto G, Merigliano S, Costantini M, Cagol M, Alfieri R, Bonavina L, Peracchia A, Ancona E: Intestinal metaplasia is the probable common precursor of adenocarcinoma in barrett esophagus and adenocarcinoma of the gastric cardia. Cancer 88:2520-8, 2000
8. Wijnhoven BP, Siersema PD, Hop WC, van Dekken H, Tilanus HW: Adenocarcinomas of the distal oesophagus and gastric cardia are one clinical entity. Rotterdam Oesophageal Tumor Study Group. Br J Surg 86:529-35, 1999
9. Heidl G, Langhans P, Mellin W, Bunte H, Grundmann E: Adenocarcinomas of esophagus and cardia in comparison with gastric carcinoma. J Cancer Res Clin Oncol 120:95-9, 1993
10. Gammon MD, Schoenberg JB, Ahsan H, Risch HA, Vaughan TL, Chow WH, Rotterdam H, West AB, Dubrow R, Stanford JL, Mayne ST, Farrow DC, Niwa S, Blot WJ, Fraumeni JF, Jr.: Tobacco, alcohol, and socioeconomic status and adenocarcinomas of the esophagus and gastric cardia. J Natl Cancer Inst 89:1277-84, 1997
11. Menke-Pluymers MB, Hop WC, Dees J, van Blankenstein M, Tilanus HW: Risk factors for the development of an adenocarcinoma in columnar-lined (Barrett) esophagus. The Rotterdam Esophageal Tumor Study Group. Cancer 72:1155-8, 1993
12. Vaughan TL, Davis S, Kristal A, Thomas DB: Obesity, alcohol, and tobacco as risk factors for cancers of the esophagus and gastric cardia: adenocarcinoma versus squamous cell carcinoma. Cancer Epidemiol Biomarkers Prev 4:85-92, 1995
13. Zhang ZF, Kurtz RC, Sun M, Karpeh M, Jr., Yu GP, Gargon N, Fein JS, Georgopoulos SK, Harlap S: Adenocarcinomas of the esophagus and gastric cardia: medical conditions, tobacco, alcohol, and socioeconomic factors. Cancer Epidemiol Biomarkers Prev 5:761-8, 1996
14. Brown LM, Hoover R, Silverman D, Baris D, Hayes R, Swanson GM, Schoenberg J, Greenberg R, Liff J, Schwartz A, Dosemeci M, Pottern L, Fraumeni JF, Jr.: Excess incidence of squamous cell esophageal cancer among US Black men: role of social class and other risk factors. Am J Epidemiol 153:114-22, 2001
15. Lagergren J, Bergstrom R, Lindgren A, Nyren O: Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma. N Engl J Med 340:825-31, 1999
16. Velanovich V, Hollingsworth J, Suresh P, Ben-Menachem T: Relationship of gastroesophageal reflux disease with adenocarcinoma of the distal esophagus and cardia. Dig Surg 19:349-53, 2002
17. Ye W, Chow WH, Lagergren J, Boffetta P, Boman G, Adami HO, Nyren O: Risk of adenocarcinomas of the oesophagus and gastric cardia in patients hospitalized for asthma. Br J Cancer 85:1317-21, 2001
18. Hage M, Siersema PD, van Dekken H, Steyerberg EW, Dees J, Kuipers EJ: Oesophageal cancer incidence and mortality in patients with long-segment Barrett's oesophagus after a mean follow-up of 12.7 years. Scand J Gastroenterol 39:1175-9, 2004
19. Lagergren J, Bergstrom R, Adami HO, Nyren O: Association between medications that relax the lower esophageal sphincter and risk for esophageal adenocarcinoma. Ann Intern Med 133:165-75, 2000

20. Vaughan TL, Farrow DC, Hansten PD, Chow WH, Gammon MD, Risch HA, Stanford JL, Schoenberg JB, Mayne ST, Rotterdam H, Dubrow R, Ahsan H, West AB, Blot WJ, Fraumeni JF, Jr.: Risk of esophageal and gastric adenocarcinomas in relation to use of calcium channel blockers, asthma drugs, and other medications that promote gastroesophageal reflux. Cancer Epidemiol Biomarkers Prev 7:749-56, 1998
21. Chow WH, Blaser MJ, Blot WJ, Gammon MD, Vaughan TL, Risch HA, Perez-Perez GI, Schoenberg JB, Stanford JL, Rotterdam H, West AB, Fraumeni JF, Jr.: An inverse relation between cagA+ strains of Helicobacter pylori infection and risk of esophageal and gastric cardia adenocarcinoma. Cancer Res 58:588-90, 1998
22. Vicari JJ, Peek RM, Falk GW, Goldblum JR, Easley KA, Schnell J, Perez-Perez GI, Halter SA, Rice TW, Blaser MJ, Richter JE: The seroprevalence of cagA-positive Helicobacter pylori strains in the spectrum of gastroesophageal reflux disease. Gastroenterology 115:50-7, 1998
23. Warburton-Timms VJ, Charlett A, Valori RM, Uff JS, Shepherd NA, Barr H, McNulty CA: The significance of cagA(+) Helicobacter pylori in reflux oesophagitis. Gut 49:341-6, 2001
24. Wu AH, Crabtree JE, Bernstein L, Hawtin P, Cockburn M, Tseng CC, Forman D: Role of Helicobacter pylori CagA+ strains and risk of adenocarcinoma of the stomach and esophagus. Int J Cancer 103:815-21, 2003
25. Nilsson IM, Blomback M, Ramgren O: Investigations on hemophilia A and B carriers. Bibl Haematol 26:26-9, 1966
26. Sampliner RE: Appearance and prognosis of dysplasia in Barrett's esophagus. Chest Surg Clin N Am 12:69-76, ix, 2002
27. Spechler SJ, Goyal RK: The columnar-lined esophagus, intestinal metaplasia, and Norman Barrett. Gastroenterology 110:614-21, 1996
28. Riegman PH, Vissers KJ, Alers JC, Geelen E, Hop WC, Tilanus HW, van Dekken H: Genomic alterations in malignant transformation of Barrett's esophagus. Cancer Res 61:3164-70, 2001
29. Reid BJ: p53 and neoplastic progression in Barrett's esophagus. Am J Gastroenterol 96:1321-3, 2001
30. Jankowski JA, Wright NA, Meltzer SJ, Triadafilopoulos G, Geboes K, Casson AG, Kerr D, Young LS: Molecular evolution of the metaplasia-dysplasia-adenocarcinoma sequence in the esophagus. Am J Pathol 154:965-73, 1999
31. Rosch T: Endosonographic staging of esophageal cancer: a review of literature results. Gastrointest Endosc Clin N Am 5:537-47, 1995
32. van Overhagen H, Berger MY, Meijers H, Tilanus HW, Kok TC, Stijnen T, Lameris JS: Influence of radiologically and cytologically assessed distant metastases on the survival of patients with esophageal and gastroesophageal junction carcinoma. The Rotterdam Esophageal Tumor Study Group. Cancer 72:25-31, 1993
33. Mannell A, Becker PJ: Evaluation of the results of oesophagectomy for oesophageal cancer. Br J Surg 78:36-40, 1991
34. Lambert R: Treatment of esophagogastric tumors. Endoscopy 32:322-30, 2000
35. Siersema PD, Hop WC, Dees J, Tilanus HW, van Blankenstein M: Coated self-expanding metal stents versus latex prostheses for esophagogastric cancer with special reference to prior radiation and chemotherapy: a controlled, prospective study. Gastrointest Endosc 47:113-20, 1998
36. Siersema PD, van Lanschot JJ, Fockens P, Tilanus HW: [Gastrointestinal surgery and gastroenterology. III. Diagnosis and treatment of esophageal carcinoma]. Ned Tijdschr Geneeskd 143:1947-52, 1999
37. Earlam R, Cunha-Melo JR: Oesophageal squamous cell carcinoma: I. A critical review of surgery. Br J Surg 67:381-90, 1980
38. Katariya K, Harvey JC, Pina E, Beattie EJ: Complications of transhiatal esophagectomy. J Surg Oncol 57:157-63, 1994
39. Tilanus HW, Hop WC, Langenhorst BL, van Lanschot JJ: Esophagectomy with or without thoracotomy. Is there any difference? J Thorac Cardiovasc Surg 105:898-903, 1993
40. DeMeester TR, Zaninotto G, Johansson KE: Selective therapeutic approach to cancer of the lower esophagus and cardia. J Thorac Cardiovasc Surg 95:42-54, 1988
41. Hulscher JB, van Sandick JW, de Boer AG, Wijnhoven BP, Tijssen JG, Fockens P, Stalmeier PF, ten Kate FJ, van Dekken H, Obertop H, Tilanus HW, van Lanschot JJ: Extended transthoracic resection

compared with limited transhiatal resection for adenocarcinoma of the esophagus. N Engl J Med 347:1662-9, 2002
42. van Lanschot JJ, Tilanus HW, Obertop H: [Transhiatal or transthoracic resection of esophageal carcinoma based on tumor location, positive high-thoracic lymph nodes and preoperative physical condition]. Ned Tijdschr Geneeskd 147:2097-100, 2003
43. Surgical resection with or without preoperative chemotherapy in oesophageal cancer: a randomised controlled trial. Lancet 359:1727-33, 2002
44. Kelsen DP, Ginsberg R, Pajak TF, Sheahan DG, Gunderson L, Mortimer J, Estes N, Haller DG, Ajani J, Kocha W, Minsky BD, Roth JA: Chemotherapy followed by surgery compared with surgery alone for localized esophageal cancer. N Engl J Med 339:1979-84, 1998
45. Ancona E, Ruol A, Santi S, Merigliano S, Sileni VC, Koussis H, Zaninotto G, Bonavina L, Peracchia A: Only pathologic complete response to neoadjuvant chemotherapy improves significantly the long term survival of patients with resectable esophageal squamous cell carcinoma: final report of a randomized, controlled trial of preoperative chemotherapy versus surgery alone. Cancer 91:2165-74, 2001
46. Kok TC, Van der Gaast A, Dees J, Eykenboom WM, Van Overhagen H, Stoter G, Tilanus HW, Splinter TA: Cisplatin and etoposide in oesophageal cancer: a phase II study. Rotterdam Oesophageal Tumor Study Group. Br J Cancer 74:980-4, 1996
47. Law S, Fok M, Chow S, Chu KM, Wong J: Preoperative chemotherapy versus surgical therapy alone for squamous cell carcinoma of the esophagus: a prospective randomized trial. J Thorac Cardiovasc Surg 114:210-7, 1997
48. Maipang T, Vasinanukorn P, Petpichetchian C, Chamroonkul S, Geater A, Chansawwaang S, Kuapanich R, Panjapiyakul C, Watanaarepornchai S, Punperk S: Induction chemotherapy in the treatment of patients with carcinoma of the esophagus. J Surg Oncol 56:191-7, 1994
49. Nygaard K, Hagen S, Hansen HS, Hatlevoll R, Hultborn R, Jakobsen A, Mantyla M, Modig H, Munck-Wikland E, Rosengren B, et al.: Pre-operative radiotherapy prolongs survival in operable esophageal carcinoma: a randomized, multicenter study of pre-operative radiotherapy and chemotherapy. The second Scandinavian trial in esophageal cancer. World J Surg 16:1104-9; discussion 1110, 1992
50. Roth JA, Pass HI, Flanagan MM, Graeber GM, Rosenberg JC, Steinberg S: Randomized clinical trial of preoperative and postoperative adjuvant chemotherapy with cisplatin, vindesine, and bleomycin for carcinoma of the esophagus. J Thorac Cardiovasc Surg 96:242-8, 1988
51. Schlag PM: Randomized trial of preoperative chemotherapy for squamous cell cancer of the esophagus. The Chirurgische Arbeitsgemeinschaft Fuer Onkologie der Deutschen Gesellschaft Fuer Chirurgie Study Group. Arch Surg 127:1446-50, 1992

Chapter 2



The molecular biology of esophageal adenocarcinoma

Partly adapted from Journal of Surgical Oncology, 2005 Dec 1;92(3):169-90

Linetta B Koppert
Bas PL Wijnhoven
Herman van Dekken
Hugo W Tilanus
Winand NM Dinjens

ABSTRACT

Barrett's esophagus is an acquired metaplastic change that occurs in the distal esophagus secondary to chronic gastroesophageal reflux. This premalignant condition forms the most important risk factor for developing esophageal adenocarcinoma, which is an extremely aggressive tumor with a 5-year survival rate of less than 25%. Carcinomas that arise in the setting of Barrett's esophagus are thought to develop as part of the metaplasia-dysplasia-carcinoma sequence. We aimed to review the current knowledge on the genomic alterations involved in the development of Barrett's esophagus and its progression to dysplasia and/or cancer. Several changes in gene structure, gene expression and protein structure are associated with the progression of Barrett's esophagus to adenocarcinoma. Accumulation of these changes seems to be essential, rather than the exact sequence of these changes. Multiple molecular pathways are involved and interact with each other. Alterations in tumor suppressor genes, amongst which p53 and p16, are early events in the metaplasia-dysplasia-adenocarcinoma sequence, followed by loss of cell cycle checkpoints. Ongoing genomic instability leads to cumulative genetic errors and thereby the generation of multiple clones of transformed cells. Within the multistep process of esophageal adenocarcinogenesis, to date no single molecular marker came forward able to predict who will and who will not develop cancer in the setting of Barrett's esophagus. Instead, panels of markers need to be developed in the future allowing to indicate disease progression. Identification of crucial molecular pathways involved in esophageal adenocarcinogenesis would ultimately improve therapy and facilitate development of new treatment strategies.

INTRODUCTION

Cancer of the esophagus represents the 9th leading cancer in the world and is associated with a five-year survival rate under 25%. Understanding of the molecular biology is a prerequisite to predict prognosis and to tailor treatment. The development of esophageal cancer is a multistep phenomenon involving genetic events that result in key abnormalities of cell cycle regulation, growth factor activity and intercellular adhesion mechanisms [1]. This review focusses on esophageal adenocarcinoma, which is associated with an increasing incidence in the Western world as compared to squamous cell carcinomas.

It is now generally accepted that esophageal adenocarcinomas develop from a premalignant lesion of the esophagus, referred to as Barrett's esophagus. Barrett's esophagus is an acquired condition in which the normal squamous cell epithelium of the esophagus is replaced by metaplastic columnar epithelium due to chronic gastroesophageal reflux disease. Barrett's esophagus is associated with a predisposition to adenocarcinoma of the distal esophagus or the gastric cardia, i.e. an about 30-125 fold higher risk compared to the general population, with an estimated incidence of 1 in 180 patient-years [2-5]. Although high grade dysplasia of Barrett's esophagus is generally considered a precursor to invasive carcinoma, the endoscopic as well as histopathologic recognition of this lesion can be rather difficult. Problems concern amongst others, sampling errors, intra- and interobserver variation in diagnosis and grading of dysplasia, differentiation of high grade dysplasia and cancer, indetermination for dysplasia due to reactive or regenerative changes. Efforts are required to better understand the pathophysiology of Barrett's esophagus and its progression to cancer. Molecular genetic analysis and development of novel biomarkers might help to identify those patients at increased risk for malignant transformation. Biopsy samples of Barrett's esophagus that histologically appear as low risk for neoplastic progression might in fact harbor genetic alterations that are indicative for a high risk profile. Molecular genetic knowledge might ultimately lead to more adequate cancer prevention and therapeutic interventions. In recent years a considerable amount of data has been accumulated regarding the molecular biological characterization of esophageal adenocarcinoma.

Genetic alterations in human cancers

Cancer is a disease of the genome with accumulation of sequence alterations (mutations) in premalignant tissue, leading to evolution of cell clones with increasing genomic instability and finally to cells with invasive and metastatic capabilities [6]. Most mutations found in human malignancy are somatic and found only in the patient's tumor tissue. It is generally accepted that some of these alterations are causally involved in the transition of a normal cell into a tumor cell [7]. Additionally, epigenetic alterations like methylation of DNA sequences resulting in gene silencing can play a role in the cellular transformation, which is a multistep process of genetic and epigenetic alterations [8]. These alterations render the cell independent

of regulated proliferative and cell death pathways and deliver the cells with proliferative, invasive and metastasizing capacities. Evidence has been presented that at least 5 to 10 specific genetic alterations are necessary to generate the malignant phenotype and most tumors are characterized by genomic instability facilitating the accumulation of mutations [9]. As a consequence, a malignant tumor is generated composed of cells with an increased proliferative activity, a prolonged lifespan and with metastasizing capacity. The genomic instability occurs in two different forms: one characterized by chromosomal instability (aneuploidy) and the other characterized by microsatellite instability [10]. The targets of the genomic instability, at the nucleotide or at the chromosomal level, comprise 4 classes of genes:

1. Proto-oncogenes. These are regulatory genes found in normal cells that perform a regulated role in activation of cell proliferation or inhibition of apoptosis (regulated cell death). Upon activation (through point mutation, gene amplification, chromosomal translocation, insertion etcetera) these dominant genes turn into oncogenes with continuous stimulation of cell proliferation or continuous inhibition of apoptosis.
2. Tumor suppressor genes. Tumor suppressor genes are recessive genes, with in normal cells a role in inhibition of cell proliferation or stimulation of apoptosis. Both gene copies need to be inactivated for their tumor suppressive effect to be lost which can be caused by both genetic and epigenetic phenomena: mutation, deletion of (part of) the gene and epigenetic silencing through promoter methylation.
3. Mismatch repair genes. These genes are in normal cells involved in repair of DNA sequence mistakes occurring primarily during DNA replication. Inactivation of both copies of these recessive genes results in defective DNA repair and as a consequence in a genome wide accumulation of mutations, also in proto-oncogenes and tumor suppressor genes. Because tandem repeat DNA sequences (microsatellites) are especially vulnerable for DNA replication mistakes, microsatellite instability is a hallmark of a defect in DNA repair [11]. The genes PMS1, PMS2, MLH1, MSH2, MSH6 and the recently discovered MBD4 (MED1) are all associated with microsatellite instability [12,13].
4. Mitotic checkpoint genes. These genes are involved in control mechanisms ensuring the proper separation of chromosomes during cell division. When the mitotic checkpoint is defective, improper allocation of the chromosomes to the daughter cells takes place during mitosis. This results in chromosomal instability and an abnormal number of chromosomes per cell (aneuploidy) [14]. To date nine human genes with a role in mitotic checkpoint control have been discovered[14]. Inactivation of mitotic checkpoint genes results in chromosomal instability and an abnormal chromosome number (aneuploidy) [14]. Mutation analysis of the human mitotic checkpoint genes in aneuploid cancers revealed only few alterations and probably genes yet to be discovered are responsible for most of the checkpoint defects found in aneuploid cancers.

The activation of proto-oncogenes and inactivation of tumor suppressor genes as the result of genetic instability form the key genetic foci contributing to tumor development. To date there are no proto-oncogenes nor tumor suppressor genes that are activated or inactivated in all cancers. Even comparable cancers from the same organ and cell type never share alterations in the same genes completely. However, comparable tumors might probably have alterations in the same molecular pathways but with different components affected [15].
To date, evidence is accumulating that cancer cells originate from transformation of tissue stem cells. These transformed tissue stem cells are thought to function as cancer stem cells that maintain the generation of neoplastic clones in a tumor. The existence of cancer stem cells is established in leukemia more than a decade ago, and recently in breast and brain tumors [16-18]. In all these tumors it is thought that the cancer is a reflection of the corresponding normal tissue stem cells. However, Houghton et al. recently proposed a new concept in gastric carcinogenesis, i.e. that bone marrow-derived cells might represent a potential source of epithelial cancers [19]. Working with mice infected by a Helicobacter strain, they found that these bone marrow-derived cells were able to home, repopulate the chronically inflamed gastric mucosa and contribute over time to metaplasia, dysplasia and cancer [19,20]. This results supports the idea that chronic inflammation fosters cancer development. Even more remarkable, the results of Houghton et al. provide evidence that epithelial cancers can originate from bone marrow derived stem cells. A similar phenomenon could perhaps occur in different types of inflammation-linked cancers as well. To date there are no literature data on esophageal adenocarcinogenesis concerning involvement of bone marrow-derived stem cells. The value of these new findings and concepts in carcinogenesis awaits future experimental confirmation.

This review aims to summarize current knowledge on genomic aberrations in esophageal adenocarcinomas as from recent years. Described genomic abberations are categorized as follows: Proto-oncogenes, Tumor suppressor genes, Mismatch repair genes (microsatellite instability), Mitotic checkpoint genes (DNA content and chromosomal abnormalities), Cell proliferation and Apoptosis, Genes involved in controlling the cell cycle, and Cell-cell adhesion genes. For comprehensive overviews published before, the authors refer to reviews by Jenkins et al. and Wijnhoven et al. [21,22].

PROTO-ONCOGENES

Proto-oncogenes are cellular genes, which can be converted into oncogenes by activating mutations. These mutations are somatic events.

Growth factors and their receptors EGF, TGF-α and EGFR

The Epidermal Growth Factor (EGF) has a stimulatory effect on epithelial cell proliferation in the gastrointestinal tract. EGF and Transforming Growth Factor-α (TGF-α) belong to the family of growth factors that binds to the epidermal growth factor receptor (EGFR or c-erbB1). EGFR directed therapies are already available for treatment of human tumors with epithelial origin. EGFR is located at chromosome 7p12-13, a frequently amplified region in esophageal adenocarcinomas which has been correlated with the occurrence of lymph node metastasis [23,24]. EGFR overexpression correlated with decreased postoperative survival of patients with locally advanced esophageal adenocarcinoma treated with preoperative chemoradiotherapy [25]. EGFR directed therapies such as the EGFR kinase inhibitor Gefitinib (Iressa) are already available for treatment of human tumors with epithelial origin with clinical responses in EFGR mutated non-small cell lung cancers [26-28]. Barber et al. recently published about mutations in colorectal carcinomas [29].

TGF-α binds to EGFR and stimulates cell division. Amplification of the TGF-α gene at chromosome 2p13 has been found in the progression of esophageal adenocarcinoma [30]. TGF-α protein expression was significantly associated with tumor progression and lymph node metastasis [31]. In Barrett's tissue, TGF-α has been found to activate Vascular Endothelial Growth Factor (VEGF) which plays a role in the vascularization of adenocarcinomas [32]. Expression of VEGF was indeed increased in Barrett's adenocarcinomas [33].

c-erbB2

The c-erbB2 proto-oncogene (HER2/neu) is localized at chromosome 17q21 and shares significant homology with EGFR. As a result of its potential role in the selection of therapy, HER2/neu has reached a near-standard-of-practice status in breast cancer. The humanized monoclonal antibody Trastuzumab (Herceptin, Hoffmann-La Roche, Grenzach-Wyhlen, Germany) that inhibits the proliferation of c-erbB2 overexpressing tumor cells has become available commercially for the treatment of patients with breast cancer. C-erbB2 protein overexpression and/or amplification of the c-erbB2 gene occurred in approximately 10-70% of esophageal adenocarcinomas [30,34-41]. Overexpression of c-erbB2 was not demonstrated in metaplastic or low grade dysplastic Barrett's epithelium suggesting that it is a late event in the dysplasia-carcinoma sequence [35,42]. Walch et al. showed that both c-erbB2 and Growth Factor Receptor-bound protein 7 (GRB7) alterations displayed concomitant elevated expression levels and increased copy numbers in high grade dysplasia and carcinoma [42]. C-erbB2 overexpression in adenocarcinomas correlated significantly with tumor invasion, lymph node involvement, distant metastasis and status of residual tumor after resection [38,39]. Furthermore, c-erbB2 amplification correlated with shortened patient survival and independently predicted poor outcome in patients with Barrett related adenocarcinoma [43]. We found c-erbB2 amplification to be present in 5 out of 13 esophageal adenocarcinoma cell lines and in 18 out of 49 primary esophageal or gastric cardia adenocarcinomas (Dinjens et al., Van Dekken et al., unpublished results).

FGF

The Fibroblast Growth Factors (FGFs) form an important proangiogenic factor with the capability to regulate growth and differentiation of various cell types. The expression of acidic and basic FGF (aFGF, bFGF) has also been studied in esophageal adenocarcinoma and Barrett's metaplasia. bFGF expression levels were significantly increased in Barrett's adenocarcinomas and dysplastic tissues as compared to normal esophageal mucosa and metaplasia [33].

TGF-β

Transforming Growth Factor (TGF-β), a potent mediator of cell growth, plays various roles in the process of malignant progression. TGF-β is a potent inhibitor of cell proliferation, an inducer of differentiation in epithelial cells of the intestine in vitro, and a suppressor of genomic instability [44]. Cancer cells can become resistant to TGF-β cell growth inhibition. TGF-β induces diverse cellular changes trough heteromeric complexes of TGF-β receptor type I and II such that the loss of either receptor dramatically alters the cellular response [45]. Loss of TGF-β receptor type II expression appeared to be associated with Barrett's esophagus and esophageal adenocarcinomas [46,47]. Pathways of TGF-β signaling involve SMAD proteins, of which the SMAD2 and SMAD4 protein were originally isolated as the MADR2 and DPC4 tumor suppressor gene respectively, both located at chromosome 18q, a locus which frequently shows loss in esophageal adenocarcinomas. Mutational inactivation of TGF-β signaling pathways was investigated by Tanaka et al. and was found to be rare [48]. The Runt domain transcription factor 3 (RUNX 3) tumor suppressor gene was shown to inhibit cell proliferation and induce apoptosis by reinstating TGF-β responsiveness in an esophageal adenocarcinoma cell line [49].

Ras-family

Activation of the Ras oncogene is commonly found in gastrointestinal tract cancers. The 3 Ras isoforms comprise H-Ras, K-Ras and N-Ras and are essential components in normal cell division and differentiation. Reports at point mutations in K-Ras in Barrett's esophagus and in esophageal adenocarcinomas showed them to be rare [50-52]. Another report described K-Ras mutations in 0% of Barrett's metaplasias, 0% of low grade dysplasias, 30% of high grade dysplasias and 40% of adenocarcinomas [53]. Activation of the Ras proto-oncogenes seems to be of little importance in Barrett's adenocarcinomas.

c-myc

The c-myc gene forms a transcription factor with a role in regulation of proliferation-associated genes crucial for cell cycle control. C-myc is located on chromosome 8q24 and activation may contribute to tumor progression by preventing cells from entering the G0-resting phase. Amplification was reported in none of metaplasias and low grade dysplasias, but in 25% of high grade dysplasias and 44% of adenocarcinomas [54]. Studies suggested that c-myc is the target gene of the chromosome 8q high level amplifications found in esophageal adeno-

carcinomas [55-58] (Figure 2.1). Tselepis et al. showed upregulation of c-myc protein in 50% of metaplasias and 90% of adenocarcinomas with a role for bile acids in an acid environment as potent inducers of this oncogene in vitro [59].

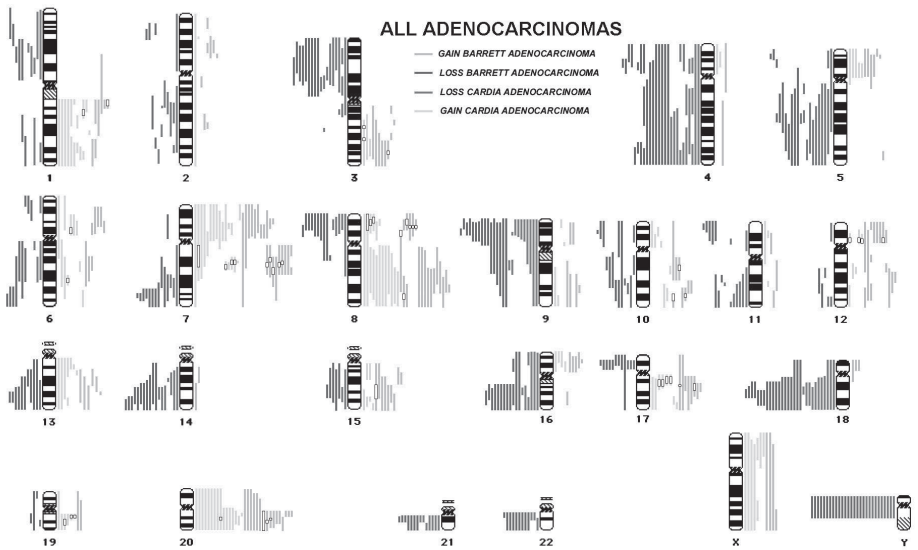


Figure 2.1 Combined CGH data from Van Dekken et al. (20 gastric cardia adenocarcinomas) and from Riegman et al. (30 Barrett adenocarcinomas) [167,171]. Boxes indicate regions of high-level amplification. *Also see color figures page 291.*

src

The cellular oncogene c-src and its viral homologue v-src are cellular oncogenes, which encode a nonreceptor tyrosine kinase involved in a number of signal transduction pathways. Src may deregulate cell-adhesion by anchorage-dependent growth control, thereby maintaining cells in the proliferative state [60]. Increased src activity was found in Barrett's epithelium with a 6 fold increase in esophageal adenocarcinomas [61]. Moreover, Jankowski et al. found that 20% of a series of esophageal adenocarcinomas and Barrett's esophagus expressed src [62].

Prostaglandins

The rate-limiting step in the synthesis of prostaglandins is the conversion of arachidonic acid to prostaglandin. The enzyme that controls this crucial step is cyclooxygenase (COX), of which there are 2 isoforms, COX-1 and COX-2. COX-1 expression is predominantly constitutive whereas COX-2 expression is inducible by mediators of inflammation. The mechanisms by which COX-2 is thought to be involved in carcinogenesis include resisting apoptosis, increasing cell proliferation, stimulating angiogenesis and modulating the invasive properties of cancer cells [63]. COX-2 was shown to be functionally active in Barrett's esophagus since treat-

ment with COX-2 inhibitors hindered proliferation of Barrett cells in culture as well as adenocarcinoma cells [64,65]. Proliferation was restored by prostaglandin treatment [64]. Enhanced COX-2 expression was detected in high amount in metaplasia (75-91%), low grade (83%) and high grade dysplasia (100%) and adenocarcinoma (97-100%) [66,67]. Elevated COX-2 protein expression was significantly associated with reduced survival after esophagectomy for adenocarcinoma [68]. Moreover, COX-2 inhibition with the selective inhibitor rofecoxib showed a reduction of COX-2 expression, prostaglandin release and cell proliferation in Barrett's patients, thereby forming a promising chemoprevention agent against dysplasia and carcinoma [69]. Results of clinical studies to investigate the effect of COX-2 inhibitors as a novel treatment modality for patients with Barrett's esophagus and adenocarcinoma have to be awaited. Since complex interactions seemed to exist between COX-2 and autocrine production of gastrin on Barrett's adenocarcinogenesis, this needs to be considered when evaluating chemopreventive strategies in Barrett's adenocarcinoma [70].

BRAF

The Ras oncogene transmits extracellular growth signals trough the MAP kinase pathway, resulting in the activation of RAF kinase. The 3 isoforms of RAF comprise ARAF, BRAF and CRAF. Activating BRAF mutations were discovered in a variety of malignancies [71]. BRAF mutations are generally nonoverlapping with K-Ras, suggesting that only one mutational event in the pathway, comprising RAS, MAP kinase and BRAF, may be needed for tumorigenesis [72-74]. Sommerer et al. detected activating BRAF mutations in 0% of low grade dysplasias, in 4% of high grade dysplasias and in 11% of adenocarcinomas [75]. Interestingly, K-Ras mutations were detected in 11% and 21% of high grade dysplasias and adenocarcinomas respectively, all lesions with K-Ras mutations having an intact BRAF gene [75]. The inhibition of the RAS-MAP-RAF kinase pathway might be a new diagnostic or even therapeutic strategy in Barrett's adenocarcinogenesis [76].

PIK3CA

Systematic cancer genome analysis has recently led to the discovery of somatic mutations in the PIK3CA gene, a member of the phosphatidylinositol 3-kinases (PI3Ks) [76]. PIK3CA is a lipid kinase located at chromosome 3q26 that regulates signaling pathways important in cell proliferation, adhesion, cell survival and motility. Somatic PIK3CA mutations were detected in colon cancers, brain cancers, gastric, breast and lung cancers [77]. Mutant PIK3CA is likely to function as an oncogene and therefore may prove useful for diagnostic and therapeutic purposes [77]. No data exist on PIK3CA gene mutation analysis in esophageal adenocarcinomas, although amplification of PIK3CA was detected in 5.7% of 87 Barrett's adenocarcinomas [78]. Interestingly PIK3CA amplification showed significant correlations with low tumor stage, absence of nodal involvement, small tumor size and lower T-status [78].

HNF3α

The Hepatocyte Nuclear Factor 3α (HNF3α) gene is located at 14q13 and forms a member of the forkhead gene family. HNF3α is involved in liver differentiation and regeneration, amongst others. Amplification of chromosome 14q13 with overexpression of the HNF3α in esophageal adenocarcinomas suggested a potential oncogenic role for this gene [79].

TUMOR SUPPRESSOR GENES

In tumor suppressor genes both gene copies need to be inactivated for the tumor suppressive effect to be lost. One allele of these genes is frequently inactivated by Loss of Heterozygosity (LOH). The remaining copy is often inactivated by mutation or promotor methylation. To define chromosomal regions of deletion LOH-analysis is used, also referred to as Microsatellite Allelotyping or Allelic Imbalance analysis. Using the Comparative Genomic Hybridization (CGH) technique, genome wide detection of amplifications and losses can be performed. Frequent loss of one allele involving a chromosomal arm or locus suggests the presence, at or near that locus, of a tumor suppressor gene. Several groups have evaluated chromosomal regions for LOH in Barrett's metaplasia, dysplasia and adenocarcinomas of the gastro-esophageal junction (Table 2.1B). Most common areas of chromosomal loss with their target genes will be discussed.

Chromosome 3p: FHIT, VHL and PPARγ

Fragile sites are genomic regions that predispose for structural chromosome aberrations such as translocations or deletions. The Fragile Histidine Triad (FHIT) gene is localized on chromosome 3p14 and spans the common fragile site FRA3B. Even though its role in carcinogenesis is still unclear, this gene is frequently inactivated by carcinogen-induced intragenic deletions in many types of cancers. Rare silent point mutations were found in esophageal adenocarcinomas [80]. A relationship between genomic deletions and the presence of abnormal FHIT transcripts (observed in 86% of Barrett's esophagus and 93% of associated carcinomas) was found by Micheal et al. [81]. Esophageal adenocarcinoma cell line experiments provided evidence for selection of loss of a functional FHIT gene by translocation events at the fragile site harboring FHIT [82]. Abnormal FHIT transcript were observed in 44% of 18 esophageal adenocarcinomas, genomic deletions were found in 44% also with overlap of both types of alterations in only 2 out of 18 patients [83]. Since genomic deletions were observed in 80% of squamous cell carcinomas of the esophagus, the authors propose the FHIT gene to be a common target for carcinogens such as smoking and alcohol, known risk factors for the development of particularly esophageal squamous cell cancer [83]. Array-based CGH showed DNA sequence copy number loss of FHIT in 28% of 18 Barrett's carcinoma samples as part of approximately 50 genes identified [84]. However, aberrant FHIT transcripts were also detected in normal, non-cancerous tissues of the gastrointestinal tract, questioning the role of FHIT as

Table 2.1 Summary of chromosomal losses in Barrett's esophagus and adenocarcinomas described in the literature, CGH studies (A) and LOH studies (B)

Table 2.1A	**CGH studies**									
	Walch et al.[166]				*Riegman et al.*[167]				*Van Dekken et al.*[57]	*Varis et al.*[174]
chromosome	MET	LGD	HGD	BA	MET	LGD	HGD	BA	BA/CA	BA
3p	n.d.	n.d.	n.d.	n.d.	-	-	-	30%	n.d.	n.d.
4p	n.d.	n.d.	n.d.	n.d.	-	-	-	47%	54%	-
4q	-	-	36%	50%	-	-	-	40%	54%	22%
5q	-	-	54%	43%	-	22%	60%	27%	36%	22%
7q	-	50%	-	33%	-	-	60%	37%	n.d.	-
8p	-	-	27%	-	-	-	-	27%	n.d.	-
9p	33%	-	45%	43%	-	33%	40%	53%	29%	17%
9q	-	50%	-	-	-	-	-	-	n.d.	n.d.
12p	n.d.	n.d.	n.d.	n.d.	-	-	-	-	n.d.	n.d.
12q	67%	-	-	-	-	-	30%	-	n.d.	n.d.
13q	83%	28%	45%	-	-	-	-	33%	n.d.	n.d
14q	-	-	36%	30%	-	-	-	37%	46%	-
16q	n.d.	n.d.	n.d.	n.d.	-	-	-	40%	36%	n.d.
17p	n.d.	n.d.	n.d.	n.d.	-	22%	40%	30%	29%	n.d.
18q	-	-	36%	40%	-	-	70%	63%	43%	-
Y	50%	100%	91%	76%	-	22%	40%	60%	64%	n.d.

Table 2.1B	LOH studies												
	Wu et al. [125]				*Gleeson et al.* [102]		*Barrett et al.*[105]		*Nobukawa et al.*[104]		*Dolan et al.*[89]	*Sanz-Ortega et al.*[90]	*Raja et al.*[91]
chromosome	MET	LGD	HGD	BA	MET	BA	PM	CA	HGD	CA	CA	BA	BA
3p									19%	26%	64%	40%	34%
4q					71%	29%							
5q	10%	21%	27%	41%	59%		54%	66%	43%	35%	45%	33%	64%
9p					50%	43%	91%	75%	40%	35%	52%	50%	57%
9q					47%	43%							
10q													22%
11p											61%		
12p						47%							
12q					71%	65%							
13q											50%		
17p	14%	42%	79%	75%	57%	76%	100%	100%	48%	57%	96%	54%	46%
17q											55%		
18q	32%	42%	73%	69%	29%	75%	41%	57%			70%		63%

CGH, Comparative Genomic Hybridization; LOH, Loss of Heterozygosity; MET, metaplasia; LGD, low grade dysplasia; HGD, high grade dysplasia; BA, Barrett adenocarcinoma; CA, carcinoma; PM, premalignant stage; n.d., no data; -, <25%

a tumor suppressor [85]. Its apparent involvement might simply reflect its location within an unstable region of the genome.

Frequent loss is described at chromosome 3p25-26 encompassing two candidate tumor suppressor genes: the Von Hippel-Lindau (VHL) gene and the peroxisome proliferator activated receptor-gamma (PPARγ) gene. Both genes are mutated in VHL disease and colon carcinomas, respectively [86,87]. LOH analysis revealed 3p25-26 loss in 67% of 36 distal esophageal and gastric cardia carcinomas whereas no mutations were detected [88]. A high percentage of LOH at the 3p VHL locus was also shown by Dolan et al., Sanz-Ortega et al. and Raja et al. suggesting that other candidate tumor suppressor genes located at 3p might be involved in esophageal adenocarcinogenesis [89-91]. Moreover, LOH at the VHL gene locus (and also at the APC, p16, DCC and MSH3 mismatch repair gene locus) could be detected in histologically normal tissue and in adjacent adenocarcinoma, being potential markers of early neoplastic progression [92].

Chromosome 5q: MCC and APC

The Mutated in Colorectal Cancer (MCC) tumor suppressor gene is closely linked to the Adenomatosis Polyposis Coli (APC) tumor suppressor gene at chromosome 5q21. Bektas et al. performed LOH analysis in 36 patients with Barrett's metaplasia and/or low grade dysplasia and/or high grade dysplasia and adenocarcinoma [93]. MCC allelic loss percentages were 6%, 0%, 0% and 25% respectively [93]. So far, no reports have been published on MCC mutation analysis in esophageal adenocarcinomas. Also in colorectal and gastric cancers, with high MCC LOH, mutation of the retained MCC allele is uncommon, suggesting that MCC does not function as a tumor suppressor gene in gastrointestinal malignancies.

Performing LOH analysis we observed APC allelic loss in 16% of Barrett's metaplasias, 23% of low grade dysplasias, 48% of high grade dysplasias and 50% of adenocarcinomas (Chapter 13). Analogous to Zhuang et al. patterns of allelic loss of the APC gene in premalignant tissue and in carcinomas were identical in all stages of neoplastic progression, suggesting the emergence of a clonal population of cells [94]. A very low rate of APC mutations was described in esophageal cancers [95-97]. Another putative tumor suppressor gene might therefore be the target of the frequent LOH on 5q and deletion of the APC locus may just be the result of large deletions on 5q and may therefore not be important in esophageal carcinogenesis [96]. On the other hand, an alternative mechanism could be responsible for inactivation of the APC gene. In gastric cancer, methylation of the promotor region of the APC gene was strongly associated with silencing of its expression [98]. Kawakami et al. observed hypermethylation of the promotor region of the APC gene in 92% of 52 Barrett's adenocarcinomas and in 40% of 43 patients with Barrett's metaplasia [99]. Interestingly, hypermethylated APC DNA was observed in the plasma in part of the patients and high levels significantly correlated with reduced patient survival [99]. Monoallelic APC promotor methylation was found to be frequently altered in histologically normal-appearing gastric mucosa from patients with gastric or esophageal adenocarcinoma [100].

Chromosome 9p: p16

Inactivation of the p16 tumor suppressor gene (also MTS1 or CDKN2A gene) at 9p21 is one of the most common genetic abnormalities in human cancers. P16 encodes a cell cycle regulatory protein that inhibits cyclin-dependent kinases 4 and 6, preventing the phosphorylation of the Rb protein and the release of a transcription factor. This blocks cell cycle progression in the G1-S phase. P16 becomes inactivated by a two-hit mechanism that can involve 9p21 LOH, mutation, homozygous deletion or CpG island methylation (i.e. an epigenetic event). In esophageal adenocarcinomas frequent allelic loss at 9p21 has been described [89-91,95,101-105]. Point mutations in exons 1 and 2 of the p16 gene were found to be rare in esophageal adenocarcinomas (ca. 5%) in contrast to squamous cell carcinomas [80,106-108]. Barrett et al. reported a higher prevalence (23%) of p16 gene mutations in adenocarcinomas with LOH of 9p21 [109]. However, in this study only aneuploid cell populations were investigated, which might not be representative for esophageal carcinomas in general and thus might explain the higher prevalence of p16 gene mutations. Gonzalez et al. reported homozygous deletions of the p16 gene in 3 of 12 (25%) esophageal adenocarcinomas [95]. P16 promoter methylation (with or without p16 LOH) is a common mechanism of p16 inactivation during neoplastic progression in Barrett's esophagus, and is already present in non-dysplastic premalignant Barrett's epithelium [110-114]. Wong et al. stated that p16 lesions are the earliest known somatic genetic/epigenetic abnormalities in Barrett's esophagus occurring in more than 85% of cases at all histological grades of dysplasia [112]. Cells with p16 aberrations, either hemizygous or nullizygous clones, undergo clonal expansion to involve large regions of the esophagus, creating a field in which other premalignant lesions can arise that can result in the formation of esophageal adenocarcinoma [112]. P16 inactivation may therefore be a useful biomarker to stratify patients' risk of progression of Barrett's metaplasia to esophageal cancer [115,116]. Suspiro et al. reported 9p LOH in 35% of 18 Barrett's metaplasia patients without dysplasia or cancer (as well as 39% 17p LOH) regarding 9p and 17p LOH as useful markers for risk stratification within endoscopic surveillance programs [117]. Other tumor suppressor genes on 9p being p15 and $p14^{ARF}$ were rarely altered in esophageal adenocarcinomas [106,109,114].

Chromosome 13q: retinoblastoma

The Retinoblastoma (Rb) gene encodes a cell cycle-associated protein important in regulating the G1-S checkpoint of the cell cycle. Functional alterations play a key role in carcinogenesis and cell proliferation. Rb allelic loss was found in none of the Barrett's metaplasias, 7% of the low grade dysplasias, 8.3% of the high grade dysplasias and 18.5% of the carcinomas by Sarbia et al [118]. There are no reports on mutation analysis of the Rb gene in esophageal adenocarcinomas.

Table 2.2 Summary of gains and high level amplifications in Barrett's esophagus and adenocarcinomas described in the literature

Chromosome or chromosomal region	Histological stage	Method	Publication
8q24, 20q, 17q21, 7p11-15	Gastro-esophageal junction adenocarcinoma	CGH	*Moskaluk et al.*[55]
20pq, 8q, 7p, 13q, 12q, 15q, 1q, 3q, 5p, 6p, 19q, Xpq, 17q, 18p	Gastro-esophageal junction adenocarcinoma	CGH	*Van Dekken et al.*[57]
5q, 7q, 13q, 17q, X	High grade dysplasia	CGH	*Van Dekken et al.*[249]
7, 8, 11, 17	Adenocarcinoma	ISH	*Beuzen et al.* [175]
20q12-q13.1, 17q12-21, 8, 10q, 13	Barrett's adenocarcinoma	CGH	*Varis et al.*[174]
8q23-24, 6p 8q23-24, 2p23-24 8q23-24, 20q, 2p23-24, 4q, 21q 8q23-24, 20q 8q23-24, 20q, 2p23-24, 6p, 10q, 17q11-22	Metaplasia Low grade dysplasia High grade dysplasia Adenocarcinoma Lymph node metastases	CGH	*Walch et al.*[166]
3q, 7p, 17q, 22q	Barrett's adenocarcinoma and related lymph node metastases	CGH	*Walch et al.*[176]
7p, 7q, 13q, 17q	High grade dysplasia	CGH	*Riegman et al.*[167]
- 4q, 21q, 1q, 16p, 19q, 20q, 22q 8q, 4q, 19q, 1q, 20q, 21q, 22q, 18p, 3q, 16p, 20p, 21p, 14q	Low grade dysplasia High grade dysplasia Adenocarcinoma	CGH	*Croft et al.*[165]
4, 8	Metaplasia, low grade dysplasia, high grade dysplasia, adenocarcinoma	FISH	*Doak et al.* [164]
15q21, 8q24.12-24.1, 2p12-11, 17q21.3, 4p15.3, 7q21.1, 11q22.3, 18q11.2, 20q13.1, 20q13.2-13.3, 20q13, 1p13.2, 2p22.3-22.1, 3q26, 5p15.2, 7q31, 9p11.2, 15q25-26, 17q11, 20q13.1-13.2, 1p36, 1p34.3, 2q33.3-34, 5p15.2, 5q31.1, 7q21-22, 7q21.3-22, 8p22, 8p22-21.3, 8p11.2-11.1, 9q34, 9q34.1, 10p13, 10p11-q11, 11q13, 11q13-14, 12p13.1-12, 12q13.2-13.3, 13q14.3, 15q11-13, 15q26.1, 16q22.1, 16q24.3, 17q11.2, 17q23, 17q23.2-25.3, 18p11.31-11.2, 22q11.21	Barrett's adenocarcinoma	Array CGH	*Albrecht et al.* [84]

CGH, Comparative Genomic Hybridisation; (F)ISH, (Fluorescence) In Situ Hybridization

Chromosome 17p: p53

Loss or mutation of tumor suppressor gene p53 is probably the most common single genetic change in cancer. This reflects the central importance of p53, which has several functions in the cell. One is as a transcription factor. Tetramers of p53 bind DNA and can activate transcription of reporter genes situated downstream of a p53 binding site. However, p53 is believed to have a much broader role in the cell, which has been summarized as 'the guardian of the genome'. Normal cells with damaged DNA arrest at the G1-S cell cycle checkpoint until damage is repaired, cells that lack p53 or have a mutant form do not arrest and replication of damaged DNA presumably leads to random genetic changes, some of which are oncogenic, similar to cells with a defective mismatch repair system. In addition, cells that lack p53 or contain mutant p53 escape apoptosis. Molecular techniques can detect p53 gene alterations, such as single strand conformation polymorphism (SSCP) analysis, sequencing and LOH analysis. The prolonged half-life of the mutant p53 protein and the concomitant increased cellular p53 concentration makes visualization by immunohistochemistry possible. Deletion of one allele of p53 in combination with a functionally inactivating mutation of the other p53 allele is among the most common combinations of genetic alterations documented in human cancer. P53 was the first such gene to be investigated in esophageal adenocarcinomas [119-121]. P53 LOH and mutations seem to be relatively early events in neoplastic progression in Barrett's esophagus because it develops in diploid cells before aneuploidy and other LOH events involving 5p, 13p and 18q [105,115,122,123]. LOH of the p53 locus has been found in 75-80% of esophageal adenocarcinomas as well as in 79% of high grade dysplasias, 42% of low grade dysplasias and 14% of Barrett's metaplasias [102,124,125]. P53 mutations were found in 29-66% of Barrett's metaplasias/low grade dysplasias and in 40-88% of high grade dysplasias/adenocarcinomas [126-130]. In contrast, nuclear p53 protein overexpression was found often in absence of p53 mutations, therefore immunohistochemistry seems to be a poor indicator of p53 gene mutations and probably an alternative mechanism is responsible for p53 protein expression [126-131].

17p LOH analysis performed on endoscopic biopsies identified patients with Barrett's esophagus at risk of neoplastic progression within surveillance programs, therefore it could supplement histology in determining the frequency of endoscopy during surveillance [117,132]. Moreover, since p53 mutations could be detected before development of high grade dysplasia or adenocarcinoma in Barrett's esophagus patients in surveillance programs, it may also be useful in stratifying the risk for development of adenocarcinoma within Barrett's esophagus [133]. Reid et al. found patients with p53 LOH in the flow cytometric-purified biopsy samples to be at increased risk for progression to adenocarcinoma, high grade dysplasia, increased 4N and aneuploidy [134]. LOH at 17p13 (but also LOH at 3p21, 5q21 and 9p21) might have a role as potential biomarker for high chance of developing adenocarcinoma in patients with Barrett's esophagus [90]. Several groups have investigated clinical significance of p53 mutations. Patients

with p53 mutations and/or p53 protein overexpression in the tumor after surgical resection had significant reduced 5-year survival relative to patients with wild-type p53 [135-137].
In conclusion, there is overwhelming evidence that p53 gene alterations are early and frequent events in esophageal adenocarcinomas and that this gene is associated with malignant transformation of Barrett's esophagus. Moreover, p53 alterations could be of value in the prevention as well as planning of treatment strategies in future studies.

Chromosome 18q: SMAD4 (DPC4) and DCC

LOH analysis revealed allelic loss of 18q in 63-70% of esophageal adenocarcinomas [89,91]. Putative tumor suppressor genes in this region comprise the Deleted in Colorectal Cancer (DCC) and SMAD4 (Deleted in Pancreatic Cancer: DPC4) genes. Allelic loss of SMAD4 was described in 46% of 35 Barrett's esophagus patients with premalignant epithelium and/or adenocarcinoma [138]. Mutational analysis of SMAD4 did not reveal inactivating alterations indicating that the targeted gene remains to be identified [138].

MISCELLANEOUS TUMOR SUPPRESSOR GENES AND INACTIVATION

Chromosome 7q

Frequent LOH has been described at 7q31 in numerous malignancies. Suppressor of Tumorigenicity 7 (ST7) has been identified as a candidate tumor suppressor gene at this region. 7q LOH was detected in 32% of 25 esophageal adenocarcinomas, but no mutations were detected in this region [139]. Therefore ST7 is not the target gene at 7q in esophageal adenocarcinomas [139].

Chromosome 14q

LOH at 14q31-32.1 (harboring candidate gene Thyroid Stimulating Hormone Receptor TSHR) occurred in 46% of Barrett's adenocarcinomas and gastric cardia adenocarcinomas [57]. To date there is no clear indication as to what 14q tumor suppressor gene is the target for 14q loss.

Methylation

The bi-allelic inactivation of tumor suppressor genes cannot always exclusively be explained by genetic alterations. LOH at for example the APC locus has been detected frequently without mutations in the remaining allele in esophageal adenocarcinoma [94,105]. Therefore other non-genetic events could contribute to gene inactivation in esophagal adenocarcinoma. Hypermethylation of CpG islands in the promotor region is such a non-genetic inactivation mechanism. Methylation of p16 is described to be the predominant mechanism for p16 inactivation in esophageal adenocarcinogenesis as early as metaplasia [112-114,140,141]. Methylation of APC is described in esophageal adenocarcinoma as well as Barrett's metaplasia [99,141]. Patients

whose tumors had more than 50% of a 7 gene-profile methylated had significantly poorer survival and earlier tumor recurrence [142]. Moreover aberrant hypermethylation at many different loci within a panel of 20 genes suggested an overall deregulation of methylation control in Barrett adenocarcinogenesis [143]. Interestingly, normal and metaplastic tissues from patients with evidence of associated dysplasia or cancer had a significantly higher incidence of hypermethylation than similar tissues from patients with no further progression of metaplasia [143].

MISMATCH REPAIR GENES:

Microsatellite instability

The Mismatch Repair (MMR) system is one of the mechanisms by which rapid repair of damaged DNA can be achieved. This system primarily removes nucleotides mispaired by DNA polymerases, and insertion or deletion loops resulting from slippage during replication or recombination. MMR failure results in the accumulation of single nucleotide mutations and alterations in length of simple, repetitive microsatellite sequences throughout the genome. This is known as Microsatellite Instability (MSI). MMR deficiency has been shown to be an important defect in about 15% of sporadic and hereditary colorectal cancers, gastric, ovarian and endometrial cancers. In Hereditary Non-Polyposis Coli Colorectal Cancer (HNPCC) syndrome germline mutations in MMR genes (most frequently MLH1 and MSH2) are commonly found. Concerning colorectal cancer a panel of 5 microsatellites has been validated and is recommended to characterize cancers as 'MSI-high' (2-5 markers out of 5 markers show instability), 'MSI-low' (1 marker) or 'MSS (stable)'. Such criteria lack for other human solid tumors. Several groups published on MSI and MMR in esophageal carcinomas. These studies are summarized in Table 2.3.

Meltzer et al. suggested that the instability may develop as an early event in Barrett's associated neoplastic progression since among 25 flow cytometry sorted adenocarcinomas instability occurred in 8 (32%) [144]. Moreover in 4 of these 8 positive cases, the diploid component of the tumor showed instability [144]. Several studies have confirmed the low prevalence of MSI, between 5-10%, in esophageal adenocarcinomas [89,103,107,125,145]. Interestingly, Wu et al. found a trend towards an improved survival for esophageal adenocarcinomas demonstrating MSI, whereas others could not establish an association with survival or clinicopathologic features such as tumor grade, pathologic stage, perineural or vascular invasion, and also not with p53 alterations or mutations in the recently reported DNA repair gene MBD4 [125,146,147]. It is not yet known which mismatch repair genes are responsible for the MSI observed in Barrett's adenocarcinoma.

Table 2.3 Microsatellite Instability in GEJ adenocarcinomas, a summary of the literature

Publication	Number of markers used	Number of carcinomas investigated	Results MSI			Mismatch repair genes investigated
Meltzer et al.[144]	5	(28 Barrett's metaplasias) 36 esophageal adenocarcinomas	(2/28) 8/36	(7%) 22%	(1 or more markers) 1 or more markers	No data
Gleeson et al. [250]	139	17 esophageal adenocarcinomas	1/17 16/17	6% 94%	58/128 markers 1/128 to 10/123 markers	No data
Keller et al. [145]	8	15 Barrett's adenocarcinomas	2/15	13%	At least 1 marker	No data
Muzeau et al. [251]	39	26 Barrett's adenocarcinomas	6/26 3/26	23% 12%	1 marker 2 markers	No data
Wu et al. [125]	14	92 esophageal or GEJ adenocarcinomas (56 with Barrett)	5/92	5%	4-13 markers	No data
Kulke et al. [146]	5	80 Barrett's adenocarcinomas	13/80	16%	1-2 markers	No cases with loss of MLH1 or MSH2 staining
Wijnhoven et al. [88]	2	43 GEJ adenocarcinomas	1/43	2%	1-2 markers	No data
Evans et al. [147]	15	27 esophageal adenocarcinomas	18/27	65%	1-5 markers	4/18 with loss of MLH1 and/or MSH2 staining
Falkenback et al. [252]	7	59 Barrett's adenocarcinomas	No data			2/59 with loss of MLH1 or MSH2 staining; both MSI-high (5 loci)
		395 adenocarcinomas	73/395	18%		

MITOTIC CHECKPOINT GENES:

DNA content and chromosomal abnormalities

DNA content/aneuploidy

Due to defects in mitotic checkpoint genes cells can obtain an abnormal number of chromosomes, defined as aneuploidy [148]. To date nine human genes with a clear role in mitotic checkpoint control and prevention of aneuploidy have been discovered [14,149]. Despite the fact that 95% of esophageal adenocarcinomas is aneuploid, the gene(s) involved in aneuploidy in these tumors are still unknown. Gene expression levels of the MAD2 and BUB1 mitotic spindle checkpoint genes were assessed in 37 Barrett's patients by real time PCR, although no correlation with aneuploidy could be established [150]. The APC tumor suppressor gene plays a role in chromosome segregation by binding to the microtubules that form the spindle apparatus [15,149,151]. In Barrett's epithelium and related adenocarcinomas frequent loss of the APC gene (5q21) as well as gene inactivation by hypermethylation has been described but few gene mutations (see Tumor Suppressor Genes paragraph). Whether loss of APC function plays a role in aneuploidy observed in Barrett and carcinoma remains to be demonstrated. Aneuploidy can be detected by measuring the DNA content of individual cells. With flow cytometry normal cells have a DNA content ranging from 2N (in G1 phase of cell cycle) to 4N (in G2/M phase of cell cycle). By using flow cytometry it has been shown that the evolution from normal esophagus to premalignant Barrett's metaplasia was frequently associated with aneuploidy and increased G2/M fraction (4N) of the metaplastic cells [152-154]. It has been suggested that nuclear DNA ploidy analysis on flow cytometry might provide a more objective and reliable biomarker associated with progression from metaplasia to carcinoma than the histological diagnosis of high grade dysplasia [155,156]. Indeed, prospective studies indicate that both aneuploidy and dysplasia may be prognostic factors for malignant transformation in Barrett's epithelium [153,157,158]: 70% of the patients with aneuploidy or increased G2/tetraploid fractions in biopsy specimens obtained during initial endoscopic evaluation developed high grade dysplasia or cancer, whereas none of the patients without flow cytometric abnormalities on initial evaluation showed progression to invasive carcinoma or high-grade dysplasia [153]. A combination of histological dysplasia any grade and the measurement of DNA abnormalities might therefore help identify Barrett's esophagus patients at risk for adenocarcinoma [155,156]. Rabinovitch et al. proposed that patients with negative, indefinite or low-grade dysplasia biopsy results in a surveillance program without increased 4N or aneuploidy may have subsequent surveillance deferred for up to 5 years [159].

Specific Chromosomal abnormalities

Detection of aneuploidy is based on the nuclear DNA content. No information is obtained about detailed chromosomal abnormalities in these cells. Techniques to investigate these chromosomal abnormalities comprise karyotyping, for which cells have to be cultured, In

Situ Hybridization (ISH), by which numerical aberrations can be detected as well as amplification or loss of specific DNA loci, and LOH analysis, which investigates specific chromosomal regions for amplification or loss. The CGH technique allows genome wide screening for chromosomal imbalances.

The most consistent numerical chromosomal abnormalities found in cytogenetic studies of dysplastic Barrett's mucosa and adenocarcinoma comprised loss of the Y-chromosome, namely in 31-93% of tumors, correlating with the development of carcinoma [160-162]. Karyotyping revealed frequent structural rearrangements in esophageal adenocarcinomas in the 1p, 3q, 11p-13, and 22p regions [160,163]. Using Fluorescence In Situ Hybridization (FISH), Doak et al. showed chromosome 4 and 8 hyperploidy to represent the earliest and most common alterations identified using tissue from endoscopic cytology brushings (metaplasia 89% and 71%, low grade dysplasia 90% and 75%, high grade dysplasia 88% and 100%, carcinoma 100% and 100% respectively)[164]. CGH showed significant chromosome changes to be absent in low grade dysplastic lesions whereas a large amount of widespread instability was present in high dysplastic lesions (most often chromosome 4 amplification) and adenocarcinomas (with chromosome 8 amplified most frequently) [165]. Furthermore, trisomies for chromosomes 5 and 7 and translocations involving chromosome 3 and 6 in Barrett's esophagus were described [162]. LOH studies and CGH studies reported in the literature showed frequent chromosomal losses in the metaplasia-dysplasia-adenocarcinoma sequence concerning amongst others chromosomes 4q, 5q, 7q, 9p, 13q, 17p and 18q and revealed over representation of chromosomes 6p, 7, 8q, 11, 12q, 14 and 20q [55,57,58,84,89-91,102,104,105,109,112,125,160,161,164,166-176], with a detailed summary of part of these studies in Tables 2.1A, 2.1B (losses) and Table 2.2 (gains and high level amplifications). Figure 2.1 shows combined CGH data from Van Dekken et al. (20 gastric cardia adenocarcinomas) and from Riegman et al. (30 Barrett adenocarcinomas) [167,171]. It remains to be determined whether any of these abnormalities are predictive markers of progression to malignancy. However, loss of 7q33-q35 was found to represent a significant distinction between low grade and high grade dysplasia whereas loss of 16q21-q22 and gain of 20q11.2-q13.1 were disclosed to significantly discriminate between high grade dysplasia and adenocarcinoma [167]. Detection of DNA sequence copy number changes using array CGH revealed changes, mainly gains, of approximately 50 genes in Barrett's adenocarcinoma, providing new candidate biomarkers in esophageal adenocarcinogenesis [84].

CELL PROLIFERATION AND APOPTOSIS

In the light of molecular concepts about carcinogenesis it is important to realize that the accumulation of genetic alterations is only effective in combination with cell division. Dividing normal cell populations maintain the balance between cell proliferation and cell loss. If there

is increased proliferation, decreased apoptosis or both, uncontrolled growth occurs and this may result in tumor formation [177].

Cell proliferation

In order to assess the amount and distribution of cell proliferation in paraffin-embedded tissues monoclonal antibodies have been developed to detect cell cycle modulators. Several studies used a monoclonal Ki-67 (MIB-1) and PCNA antibody to study the proliferative properties in Barrett's esophagus and adenocarcinomas. An increased number of proliferating cells and an expansion of the proliferative compartment was demonstrated in Barrett's esophagus and adenocarcinoma [178-181]. PCNA immunostaining was mainly seen in the basal cells of the neck/foveolar epithelial compartment of the glands in Barrett's esophagus. However, in mucosa with high-grade dysplasia, the proliferative compartment extended upwards into the superficial layers of the glands [182-184]. Ki-67 staining pattern also correlated with the histologic findings in Barrett's esophagus: the number and localization of Ki-67 positive nuclei was significantly different between non, low and high grade dysplastic Barrett's and adenocarcinoma [182,185-190]. Interestingly, effective intra-esophageal acid suppression decreased cell proliferation and favored differentiation in Barrett's epithelium, but had no effect on the grade of dysplasia [191].

Apoptosis

Apoptosis, or programmed cell death, is one of the mechanisms responsible for cell loss. The net effect of cell division and apoptosis determines tissue growth. Apoptosis provides a protective mechanism by removing senescent, DNA-damaged, or diseased cells that could either interfere with normal function or lead to neoplastic proliferation. Apoptosis itself can be detected by use of immunohistochemical detection of DNA fragmentation as markers for apoptosis. An increase in apoptotic rate with increasing histologic severity in intestinal metaplasia/dysplasia and carcinoma was noted [178], whereas others found few apoptotic cells in Barrett's high-grade dysplasia and adenocarcinoma [192,193].

Fas and Fas Ligand

The Fas/APO-1 (CD95) gene encodes a transmembrane protein that is involved in apoptosis and is a member of the tumor necrosis factor receptor superfamily. Loss of its expression during carcinogenesis can result in the interruption of the apoptotic pathway. Hughes et al. found that expression of Fas on the cell surface by esophageal adenocarcinomas is reduced or absent whereas high levels of Fas mRNA were detected in these tumors [194]. Furthermore, they demonstrated in an esophageal adenocarcinoma cancer cell line that wild-type Fas protein is retained in the cytoplasm. Apparently, retention of wild-type Fas protein within the cytoplasm may represent the mechanism by which malignant cells evade Fas-mediated apoptosis [194]. Fas ligand binds to Fas, activating an apoptotic pathway. Younes et al. found that

Fas ligand was overexpressed in cases of Barrett's metaplasia with dysplasia and esophageal adenocarcinomas but that Fas was usually undetectable or expressed at low levels [195,196]. They propose that decreased Fas expression might protect Barrett's metaplasia with dysplasia and carcinoma from self-destruction while allowing them to evade immune surveillance [195,196].

Bcl-2 family (Bcl-2, Bcl-xl, Bax)

The Bcl-2 proto-oncogene at 18q21 encodes a protein that blocks apoptosis [197]. Divergent results have been published in the literature concerning Bcl-2 expression. It was found to be increased in reflux esophagitis, non dysplastic Barrett's and low grade dysplastic Barrett's epithelium, but low or virtually absent in high grade dysplasia and carcinomas [182,187,193,198-200]. Inhibition of apoptosis by overexpression of Bcl-2 protein occurs mainly early in the neoplastic progression. As malignancy appears, cells acquire other ways of avoiding apoptosis for instance by mutations in the p53 gene, since p53 dysfunction plays a major role in the progression from dysplasia to carcinoma in Barrett's esophagus and appears unrelated to Bcl-2 expression [201]. Van der Woude et al. proposed that the apoptotic balance in the transformation of metaplasia to adenocarcinoma switches from a pro- to a antiapoptotic phenotype due to increased Bcl-xl and decreased Bax expression [200]. Bcl-xl expression was shown to be elevated in the metaplasia-low grade-high grade dysplasia-adenocarcinoma sequence (27%, 60%, 71% and 59% respectively) [202]. Loss of Bcl-2 expression in Barrett's dysplasia and adenocarcinoma was associated with tumor progression as well as worse survival but not with response to neoadjuvant chemoradiation [203].

Mdm2

The p53 gene is an important tumor suppressor gene functioning both in cell cycle arrest and apoptosis. The human homologue of the murine double minute-2 gene (mdm2) at 12q15 is a known regulator of p53 activity. Its expression results in stabilization of the wild-type p53 protein and loss of its tumor suppressor function. Soslow et al. showed mdm2 to be overexpressed in esophageal adenocarcinomas [128]. Recently, a mdm2 promotor polymorphism was described that decreased p53 function in Li Fraumeni patients without a p53 mutation [204].

Survivin and other IAPs

Survivin forms an inhibitor of apoptosis protein (IAP) [205]. It is expressed in the G2/M phase of the cell cycle and its interaction with the mitotic spindle apparatus has been reported to be essential for anti-apoptotic function [205]. The expression of survivin has been shown to predict a poor prognosis in terms of survival and disease recurrence for patients with colorectal cancer, neuroblastoma and bladder cancer [206-209]. Beardsmore et al. found survivin immunostaining in 95% of esophageal carcinomas (both squamous cell and adenocarcinomas) with a strong positive correlation between levels of survivin expression and the number of proliferating cells in the malignant tissue [199]. Although a predictive role for surviving in the response

to neoadjuvant chemoradiotherapy has been suggested in the literature, Beardsmore et al. were not able to show such a correlation in esophageal cancers [199]. Expression of survivin and other IAPs (cIAP1, cIAP2, NAIP, and XIAP) was elevated in a series of esophageal squamous cell carcinomas [210].

Telomerase

The limited life span of normal somatic cells is characterized by telomere shortening. Cells that are not subject to replicative senescence such as germline cells, stem cells and cancer cells are able to maintain telomere length. Telomerase is a ribonucleoprotein enzyme complex that is capable of restoring and maintaining telomere length by providing its own RNA template for the addition of telomeric sequences to chromosome ends. Telomerase activation is associated with increased expression of the telomere reverse transcriptase catalytic subunit (hTERT). Lord et al. measured mRNA expression levels of hTERT in Barrett's metaplasia, dysplasia and adenocarcinoma and found a significant increase in upregulation in the metaplasia-dysplasia-adenocarcinoma sequence as compared to patients normal tissue [211]. Moreover, a significant difference was found in hTERT activation when normal tissues of cancer patients were compared to squamous cell epithelia of non-cancer patients[211]. Another study demonstrated that the vast majority of esophageal adenocarcinomas and high grade dysplasias contained high levels of telomerase RNA and that the greatest increase occurred during the transition from low to high grade dysplasia [212].

Because of the tight link between cell proliferation and apoptosis in tumor growth the assessment of both parameters in a neoplasm is suggested to be a sensitive marker for neoplastic progression [179].

Genes involved in controlling the cell cycle

Proliferation is crucial for tumor development. Cell cycle progression is the result of proliferation promoting factors: the Cyclin Dependent Kinases (CDKs) and the Proliferation Inhibiting components (CDK-inhibitors, CDKIs). Cyclins form a family of proteins that complex with CDKs. The CDKIs can be categorized in the Kip/Cip proteins (p21, p27, p57) and the INK4 proteins (p15, p16, p18, p19). The development of dysplasia is characterized by a disregulation of cell cycling. In Barrett's metaplasia progression of an increased fraction of S-phase and G2/M phase cells is described, suggesting that cells escape G1-G0 phase checkpoints [213]. The Rb gene (mediating cell cycle progression), p16 gene (a CDKI) and p53 gene (inhibits cell cycle when DNA is damaged) have been described in the section 'Tumor suppressor genes'.

CYCLINS

Cyclin D1 (11q13)

Disregulation of the cell cycle in the development of dysplasia is in part due to increased cyclin D1 expression and hypermethylation or mutation of p16 with mobilization of cells from G0 to G1 with subsequent accumulation in the G2 phase [1]. Amplification of the cyclin D1 gene was observed in 16-26% of the esophageal adenocarcinomas, whereas increased nuclear expression of cyclin D1 was observed in 22-64% of the esophageal adenocarcinomas [214-219]. Within a prospective cohort of Barrett's patients a statistically significantly increased risk of progression to adenocarcinoma was shown in patients with cyclin D1 positive biopsy specimens [220]. The Rb protein is phosphorylated by cyclin D1 and thereby inactivated. The inactivated Rb facilitates transition of the cell from G1 into S-phase. Another group of CDKIs is the Cip/Kip family and includes p21, p27 and p57.

Cyclin B1 (5q12)

Cyclin B1 is synthesized in the early G2-phase and is believed to be involved in the control of G2-M-phase transition by promoting chromosome condensation, destruction of the nuclear membrane and assembly of the mitotic spindle. Overexpression of cyclin B1 is a frequent and early finding in the metaplasia - dysplasia - carcinoma sequence [221].

Cyclin E (19q12)

Cyclin E is involved in the process of cell entry into the S-phase of the cell cycle. Expression of the cyclin E protein was reported in dysplastic Barrett's mucosa as well as in adenocarcinomas, and increased nuclear staining was associated with regeneration and proliferation of adjacent metaplastic epithelium of erosions or ulcerations in Barrett's esophagus [222]. Amplification of 19q12 with cyclin E as the best candidate gene was observed in 13.8% of esophageal adenocarcinomas [223].

P21$^{WAF1/CIP1}$ (6p21)

The wild type p53 tumor suppressor gene can induce the CDKI p21. Through inhibition of CDKs the G1-S phase of the cell cycle can be downregulated. P21 expression was elevated in low grade dysplasia, high grade dysplasia and in Barrett's adenocarcinoma, but not in Barrett's metaplasia [224]. Interestingly no correlation between p21 and p53 staining in esophageal adenocarcinomas was found, indicating a p53 independent mechanism for upregulation of p21 [224,225]. Elevated p21 expression was significantly associated with better prognosis [217].

P27^{Kip-1} (12p13)

P27 induces a block during G1 in the cell cycle. Loss of p27 expression is thought to be tumorigenic and may lead to tumor progression. Most Barrett related adenocarcinomas are associ-

ated with loss of p27 as well as altered cellular localization (cytoplasmic instead of nuclear) [226]. Loss of nuclear and/or cytoplasmic staining for p27 correlated with higher histological grade, depth of invasion, presence of lymph node metastasis and shorter survival [226].

Cell-cell adhesion genes

Reduced cell-cell adhesion promotes growth of epithelial cells because contact inhibition of proliferation is lost. Moreover cell adhesion molecules are involved in the process of invasion and metastasis of cancers.

E-cadherin-catenin complex

E-cadherin belongs to the family of calcium-dependent cell adhesion molecules and forms part of the adherens junction complex promoting tight adhesion of epithelial cells. Loss of E-cadherin occurs in several cancers and is associated with the development of invasive properties. E-cadherin expression was shown to be reduced in both Barrett's esophagus and esophageal adenocarcinoma and in the latter this was correlated with a greater frequency of lymph node metastasis and decreased patient survival [227,228]. Mutations of the E-cadherin gene were rare in adenocarcinomas [229]. However, epigenetic silencing via aberrant methylation of the E-cadherin promotor seemed to be a common cause of inactivation in adenocarcinomas [230].

β-Catenin is involved in cell-cell adhesion by its interaction with E-cadherin and with the cytoskeleton. A second function of β-catenin concerns cell-signaling. The β-catenin protein can translocate to the nucleus where it complexes with the transcription regulator proteins T-Cell factor 4/Lymphoid Enhancer-binding Factor 1 (TCF4/LEF1) to activate transcription of oncogenes including i.e. c-myc and cyclin D1. In general the APC gene product forms a complex with other proteins and targets β-catenin for degradation preventing β-catenin dependent transcription of oncogenes. However, activated Wnt signaling protects β-catenin from APC-mediated degradation and increases β-catenin-dependent transcription [231,232]. Nuclear and cytoplasmic instead of membranous β-catenin localization has been described to occur frequently in esophageal adenocarcinomas, however mutations in β-catenin or APC appeared to be very rare [233-236].

CD44 protein family

The CD44 gene produces a variety of glycosylated cell surface proteins by alternative splicing of its at least 20 exons. CD44 is involved in cell-cell adhesion and cell-matrix interactions. Expression of CD44 in APC and TCF mutant mice was described to implicate its regulation by the Wnt pathway [237]. Several reports focused on the expression of certain splice variants in esophageal adenocarcinoma. CD44V6 was detected by immunohistochemistry in 55% of Barrett's metaplasias and 63% of adenocarcinomas [238]. CD44V6 expression was related to an aggressive pathological feature in adenocarcinomas [239]. With RT-PCR and southern blot

analysis CD44V5 en CD44V6 were found to be frequently and similarly expressed in both Barrett's metaplasia and adenocarcinoma, thus not closely associated with development and progression of esophageal adenocarcinomas according to Menges et al. [240].

Cathepsin B

The cysteine protease cathepsin B (CTSB) gene is localized at chromosome 8p22 and codes for a lysosomal enzyme that has been shown to be overexpressed or exhibit altered localization in cancers [241]. Overexpression or altered localization of CTSB is thought to result in degradation of the basement membrane facilitating tumor invasion and metastasis. Characterization of an amplicon at chromosome 8p22-23 revealed that CTSB was both amplified and overexpressed in esophageal adenocarcinomas. Gene amplification was observed in 13% of tumors, mRNA overexpression was observed in 25% of tumors and protein staining was detected even in 75% of tumors, suggesting that CTSB overexpression could result from other mechanisms in addition to gene amplification [223]. These data support an important role for CTSB gene amplification and CTSB protein overexpression in esophageal adenocarcinomas.

SUMMARY AND FUTURE PERSPECTIVES

Concerning Barrett's metaplasia and its malignant potential there is still need for improved understanding of the molecular biology in order to develop a more scientific approach to cancer prevention in these patients. Since the annual incidence rate for developing esophageal adenocarcinoma in Barrett's esophagus is as low as 0.5-1%, a cancer preventive strategy needs to be cost effective and acceptable to a large number of patients at relatively low risk [76]. Despite ongoing efforts to characterize the molecular changes leading to esophageal adenocarcinoma, no clinically applicable biomarkers for prediction of prognosis and/or response to therapy came forward to date. Moreover, the proposed existence of intratumoral heterogeneity of genetic alterations has important implications for the development of such biomarkers. Using FISH analysis Walch et al. demonstrated intratumoral heterogeneity which they ascribe to a probable existence of many divergent neoplastic subpopulations [166]. This highlights one of the main problems associated with surveillance of Barrett's patients namely sampling error. LOH and gene amplification analysis at several gene loci of multiple tumor samples per patient elucidated marked intratumoral heterogeneity in Barrett's adenocarcinomas [242]. In studies of Galipeau et al., Prevo et al. and Barrett et al. the subject of clonal expansion of cells with certain alterations have been extensively studied [105,115,243]. It seems likely that the acquisition of 9p LOH and 17p LOH predisposes to the evolution of aneuploid cell populations and other genetic abnormalities that culminate in the development of cancer [105,115,243]. Maley et al. addressed the role of genetic instability and clonal expansion in Barrett's metaplasia using prospective data [244]. They measured the size of cell clones with p53

alterations, p16 alterations, aneuploidy and tetraploidy in serial endoscopy biopsy samples with a follow-up of 8 years [244]. They demonstrated that not the single presence of p53 LOH, aneuploidy or tetraploidy predict progression to adenocarcinoma, but the size of clones with these lesions [244]. Thus the combination of both genetic instability and clone size predicted progression to cancer in their prospective cohort study [244].

In conclusion, the search for molecular markers to predict which Barrett's patients are at high risk for neoplastic progression should be focused on early genetic events. Such early events concern loss of p16 and p53 with aneuploidy as a probable driving force. The value of various genetic alterations, amongst which p16 and p53, as molecular markers in GEJ adenocarcinogenesis was investigated in chapter 13 of this thesis.

REFERENCES

1. Tselepis C, Perry I, Jankowski J: Barrett's esophagus: disregulation of cell cycling and intercellular adhesion in the metaplasia-dysplasia-carcinoma sequence. Digestion 61:1-5, 2000
2. Cameron AJ, Ott BJ, Payne WS: The incidence of adenocarcinoma in columnar-lined (Barrett's) esophagus. N Engl J Med 313:857-9, 1985
3. Hameeteman W, Tytgat GN, Houthoff HJ, van den Tweel JG: Barrett's esophagus: development of dysplasia and adenocarcinoma. Gastroenterology 96:1249-56, 1989
4. Menke-Pluymers MB, Hop WC, Dees J, van Blankenstein M, Tilanus HW: Risk factors for the development of an adenocarcinoma in columnar-lined (Barrett) esophagus. The Rotterdam Esophageal Tumor Study Group. Cancer 72:1155-8, 1993
5. Kim R, Weissfeld JL, Reynolds JC, Kuller LH: Etiology of Barrett's metaplasia and esophageal adenocarcinoma. Cancer Epidemiol Biomarkers Prev 6:369-77, 1997
6. Nowell PC: The clonal evolution of tumor cell populations. Science 194:23-8, 1976
7. Hanahan D, Weinberg RA: The hallmarks of cancer. Cell 100:57-70, 2000
8. Muyrers-Chen I, Paro R: Epigenetics: unforeseen regulators in cancer. Biochim Biophys Acta 1552:15-26, 2001
9. Renan MJ: How many mutations are required for tumorigenesis? Implications from human cancer data. Mol Carcinog 7:139-46, 1993
10. Lengauer C, Kinzler KW, Vogelstein B: Genetic instabilities in human cancers. Nature 396:643-9, 1998
11. Modrich P, Lahue R: Mismatch repair in replication fidelity, genetic recombination, and cancer biology. Annu Rev Biochem 65:101-33, 1996
12. Riccio A, Aaltonen LA, Godwin AK, Loukola A, Percesepe A, Salovaara R, Masciullo V, Genuardi M, Paravatou-Petsotas M, Bassi DE, Ruggeri BA, Klein-Szanto AJ, Testa JR, Neri G, Bellacosa A: The DNA repair gene MBD4 (MED1) is mutated in human carcinomas with microsatellite instability. Nat Genet 23:266-8, 1999
13. Lynch HT, de la Chapelle A: Genetic susceptibility to non-polyposis colorectal cancer. J Med Genet 36:801-18, 1999
14. Cahill DP, Lengauer C, Yu J, Riggins GJ, Willson JK, Markowitz SD, Kinzler KW, Vogelstein B: Mutations of mitotic checkpoint genes in human cancers. Nature 392:300-3, 1998
15. Fodde R, Smits R, Clevers H: APC, signal transduction and genetic instability in colorectal cancer. Nat Rev Cancer 1:55-67, 2001
16. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, Minden M, Paterson B, Caligiuri MA, Dick JE: A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature 367:645-8, 1994
17. Clarke MF: Neurobiology: at the root of brain cancer. Nature 432:281-2, 2004
18. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF: Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 100:3983-8, 2003
19. Houghton J, Stoicov C, Nomura S, Rogers AB, Carlson J, Li H, Cai X, Fox JG, Goldenring JR, Wang TC: Gastric cancer originating from bone marrow-derived cells. Science 306:1568-71, 2004
20. Marx J: Medicine. Bone marrow cells: the source of gastric cancer? Science 306:1455-7, 2004
21. Jenkins GJ, Doak SH, Parry JM, D'Souza FR, Griffiths AP, Baxter JN: Genetic pathways involved in the progression of Barrett's metaplasia to adenocarcinoma. Br J Surg 89:824-37, 2002
22. Wijnhoven BP, Tilanus HW, Dinjens WN: Molecular biology of Barrett's adenocarcinoma. Ann Surg 233:322-37, 2001
23. Vissers KJ, Riegman PH, Alers JC, Tilanus HW, van Dekken H: Involvement of cancer-activating genes on chromosomes 7 and 8 in esophageal (Barrett's) and gastric cardia adenocarcinoma. Anticancer Res 21:3813-20, 2001
24. Fitzgerald RC, Triadafilopoulos G: Recent developments in the molecular characterization of Barrett's esophagus. Dig Dis 16:63-80, 1998
25. Gibson MK, Abraham SC, Wu TT, Burtness B, Heitmiller RF, Heath E, Forastiere A: Epidermal growth factor receptor, p53 mutation, and pathological response predict survival in patients with locally advanced esophageal cancer treated with preoperative chemoradiotherapy. Clin Cancer Res 9:6461-8, 2003

26. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M: EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 304:1497-500, 2004
27. Dancey JE: Predictive factors for epidermal growth factor receptor inhibitors--the bull's-eye hits the arrow. Cancer Cell 5:411-5, 2004
28. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 350:2129-39, 2004
29. Barber TD, Vogelstein B, Kinzler KW, Velculescu VE: Somatic mutations of EGFR in colorectal cancers and glioblastomas. N Engl J Med 351:2883, 2004
30. Jankowski J, Hopwood D, Wormsley KG: Expression of epidermal growth factor, transforming growth factor alpha and their receptor in gastro-oesophageal diseases. Dig Dis 11:1-11, 1993
31. D'Errico A, Barozzi C, Fiorentino M, Carella R, Di Simone M, Ferruzzi L, Mattioli S, Grigioni WF: Role and new perspectives of transforming growth factor-alpha (TGF-alpha) in adenocarcinoma of the gastro-oesophageal junction. Br J Cancer 82:865-70, 2000
32. Couvelard A, Paraf F, Gratio V, Scoazec JY, Henin D, Degott C, Flejou JF: Angiogenesis in the neoplastic sequence of Barrett's oesophagus. Correlation with VEGF expression. J Pathol 192:14-8, 2000
33. Lord RV, Park JM, Wickramasinghe K, DeMeester SR, Oberg S, Salonga D, Singer J, Peters JH, Danenberg KD, Demeester TR, Danenberg PV: Vascular endothelial growth factor and basic fibroblast growth factor expression in esophageal adenocarcinoma and Barrett esophagus. J Thorac Cardiovasc Surg 125:246-53, 2003
34. Hardwick RH, Barham CP, Ozua P, Newcomb PV, Savage P, Powell R, Rahamin J, Alderson D: Immunohistochemical detection of p53 and c-erbB-2 in oesophageal carcinoma; no correlation with prognosis. Eur J Surg Oncol 23:30-5, 1997
35. Hardwick RH, Shepherd NA, Moorghen M, Newcomb PV, Alderson D: c-erbB-2 overexpression in the dysplasia/carcinoma sequence of Barrett's oesophagus. J Clin Pathol 48:129-32, 1995
36. Duhaylongsod FG, Gottfried MR, Iglehart JD, Vaughn AL, Wolfe WG: The significance of c-erb B-2 and p53 immunoreactivity in patients with adenocarcinoma of the esophagus. Ann Surg 221:677-83; discussion 683-4, 1995
37. Flejou JF, Paraf F, Muzeau F, Fekete F, Henin D, Jothy S, Potet F: Expression of c-erbB-2 oncogene product in Barrett's adenocarcinoma: pathological and prognostic correlations. J Clin Pathol 47:23-6, 1994
38. Polkowski W, van Sandick JW, Offerhaus GJ, ten Kate FJ, Mulder J, Obertop H, van Lanschot JJ: Prognostic value of Lauren classification and c-erbB-2 oncogene overexpression in adenocarcinoma of the esophagus and gastroesophageal junction. Ann Surg Oncol 6:290-7, 1999
39. Nakamura T, Nekarda H, Hoelscher AH, Bollschweiler E, Harbeck N, Becker K, Siewert JR, Harbec N: Prognostic value of DNA ploidy and c-erbB-2 oncoprotein overexpression in adenocarcinoma of Barrett's esophagus. Cancer 73:1785-94, 1994
40. Friess H, Fukuda A, Tang WH, Eichenberger A, Furlan N, Zimmermann A, Korc M, Buchler MW: Concomitant analysis of the epidermal growth factor receptor family in esophageal cancer: overexpression of epidermal growth factor receptor mRNA but not of c-erbB-2 and c-erbB-3. World J Surg 23:1010-8, 1999
41. Geddert H, Zeriouh M, Wolter M, Heise JW, Gabbert HE, Sarbia M: Gene amplification and protein overexpression of c-erb-b2 in Barrett carcinoma and its precursor lesions. Am J Clin Pathol 118:60-6, 2002
42. Walch A, Specht K, Braselmann H, Stein H, Siewert JR, Hopt U, Hofler H, Werner M: Coamplification and coexpression of GRB7 and ERBB2 is found in high grade intraepithelial neoplasia and in invasive Barrett's carcinoma. Int J Cancer 112:747-53, 2004
43. Brien TP, Odze RD, Sheehan CE, McKenna BJ, Ross JS: HER-2/neu gene amplification by FISH predicts poor survival in Barrett's esophagus-associated adenocarcinoma. Hum Pathol 31:35-9, 2000
44. Glick AB, Weinberg WC, Wu IH, Quan W, Yuspa SH: Transforming growth factor beta 1 suppresses genomic instability independent of a G1 arrest, p53, and Rb. Cancer Res 56:3645-50, 1996

45. Wrana JL, Attisano L, Wieser R, Ventura F, Massague J: Mechanism of activation of the TGF-beta receptor. Nature 370:341-7, 1994
46. Souza RF, Garrigue-Antar L, Lei J, Yin J, Appel R, Vellucci VF, Zou TT, Zhou X, Wang S, Rhyu MG, Cymes K, Chan O, Park WS, Krasna MJ, Greenwald BD, Cottrell J, Abraham JM, Simms L, Leggett B, Young J, Harpaz N, Reiss M, Meltzer SJ: Alterations of transforming growth factor-beta 1 receptor type II occur in ulcerative colitis-associated carcinomas, sporadic colorectal neoplasms, and esophageal carcinomas, but not in gastric neoplasms. Hum Cell 9:229-36, 1996
47. Garrigue-Antar L, Souza RF, Vellucci VF, Meltzer SJ, Reiss M: Loss of transforming growth factor-beta type II receptor gene expression in primary human esophageal cancer. Lab Invest 75:263-72, 1996
48. Tanaka S, Mori M, Mafune K, Ohno S, Sugimachi K: A dominant negative mutation of transforming growth factor-beta receptor type II gene in microsatellite stable oesophageal carcinoma. Br J Cancer 82:1557-60, 2000
49. Torquati A, O'Rear L, Longobardi L, Spagnoli A, Richards WO, Daniel Beauchamp R: RUNX3 inhibits cell proliferation and induces apoptosis by reinstating transforming growth factor beta responsiveness in esophageal adenocarcinoma cells. Surgery 136:310-6, 2004
50. Trautmann B, Wittekind C, Strobel D, Meixner H, Keymling J, Gossner L, Ell C, Hahn EG: K-ras point mutations are rare events in premalignant forms of Barrett's oesophagus. Eur J Gastroenterol Hepatol 8:799-804, 1996
51. Casson AG, Wilson SM, McCart JA, O'Malley FP, Ozcelik H, Tsao MS, Chambers AF: ras mutation and expression of the ras-regulated genes osteopontin and cathepsin L in human esophageal cancer. Int J Cancer 72:739-45, 1997
52. Arber N, Shapira I, Ratan J, Stern B, Hibshoosh H, Moshkowitz M, Gammon M, Fabian I, Halpern Z: Activation of c-K-ras mutations in human gastrointestinal tumors. Gastroenterology 118:1045-50, 2000
53. Lord RV, O'Grady R, Sheehan C, Field AF, Ward RL: K-ras codon 12 mutations in Barrett's oesophagus and adenocarcinomas of the oesophagus and oesophagogastric junction. J Gastroenterol Hepatol 15:730-6, 2000
54. Sarbia M, Arjumand J, Wolter M, Reifenberger G, Heep H, Gabbert HE: Frequent c-myc amplification in high-grade dysplasia and adenocarcinoma in Barrett esophagus. Am J Clin Pathol 115:835-40, 2001
55. Moskaluk CA, Hu J, Perlman EJ: Comparative genomic hybridization of esophageal and gastroesophageal adenocarcinomas shows consensus areas of DNA gain and loss. Genes Chromosomes Cancer 22:305-11, 1998
56. Lu SH, Hsieh LL, Luo FC, Weinstein IB: Amplification of the EGF receptor and c-myc genes in human esophageal cancers. Int J Cancer 42:502-5, 1988
57. van Dekken H, Geelen E, Dinjens WN, Wijnhoven BP, Tilanus HW, Tanke HJ, Rosenberg C: Comparative genomic hybridization of cancer of the gastroesophageal junction: deletion of 14Q31-32.1 discriminates between esophageal (Barrett's) and gastric cardia adenocarcinomas. Cancer Res 59:748-52, 1999
58. Persons DL, Croughan WS, Borelli KA, Cherian R: Interphase cytogenetics of esophageal adenocarcinoma and precursor lesions. Cancer Genet Cytogenet 106:11-7, 1998
59. Tselepis C, Morris CD, Wakelin D, Hardy R, Perry I, Luong QT, Harper E, Harrison R, Attwood SE, Jankowski JA: Upregulation of the oncogene c-myc in Barrett's adenocarcinoma: induction of c-myc by acidified bile acid in vitro. Gut 52:174-80, 2003
60. Takekura N, Yasui W, Yoshida K, Tsujino T, Nakayama H, Kameda T, Yokozaki H, Nishimura Y, Ito H, Tahara E: pp60c-src protein kinase activity in human gastric carcinomas. Int J Cancer 45:847-51, 1990
61. Kumble S, Omary MB, Cartwright CA, Triadafilopoulos G: Src activation in malignant and premalignant epithelia of Barrett's esophagus. Gastroenterology 112:348-56, 1997
62. Jankowski J, Coghill G, Hopwood D, Wormsley KG: Oncogenes and onco-suppressor gene in adenocarcinoma of the oesophagus. Gut 33:1033-8, 1992
63. Buskens CJ, Ristimaki A, Offerhaus GJ, Richel DJ, van Lanschot JJ: Role of cyclooxygenase-2 in the development and treatment of oesophageal adenocarcinoma. Scand J Gastroenterol Suppl:87-93, 2003

64. Buttar NS, Wang KK, Anderson MA, Dierkhising RA, Pacifico RJ, Krishnadath KK, Lutzke LS: The effect of selective cyclooxygenase-2 inhibition in Barrett's esophagus epithelium: an in vitro study. J Natl Cancer Inst 94:422-9, 2002
65. Souza RF, Shewmake K, Beer DG, Cryer B, Spechler SJ: Selective inhibition of cyclooxygenase-2 suppresses growth and induces apoptosis in human esophageal adenocarcinoma cells. Cancer Res 60:5767-72, 2000
66. Lagorce C, Paraf F, Vidaud D, Couvelard A, Wendum D, Martin A, Flejou JF: Cyclooxygenase-2 is expressed frequently and early in Barrett's oesophagus and associated adenocarcinoma. Histopathology 42:457-65, 2003
67. Morris CD, Armstrong GR, Bigley G, Green H, Attwood SE: Cyclooxygenase-2 expression in the Barrett's metaplasia-dysplasia-adenocarcinoma sequence. Am J Gastroenterol 96:990-6, 2001
68. Buskens CJ, Van Rees BP, Sivula A, Reitsma JB, Haglund C, Bosma PJ, Offerhaus GJ, Van Lanschot JJ, Ristimaki A: Prognostic significance of elevated cyclooxygenase 2 expression in patients with adenocarcinoma of the esophagus. Gastroenterology 122:1800-7, 2002
69. Kaur BS, Khamnehei N, Iravani M, Namburu SS, Lin O, Triadafilopoulos G: Rofecoxib inhibits cyclooxygenase 2 expression and activity and reduces cell proliferation in Barrett's esophagus. Gastroenterology 123:60-7, 2002
70. Abdalla SI, Lao-Sirieix P, Novelli MR, Lovat LB, Sanderson IR, Fitzgerald RC: Gastrin-induced cyclooxygenase-2 expression in Barrett's carcinogenesis. Clin Cancer Res 10:4784-92, 2004
71. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA: Mutations of the BRAF gene in human cancer. Nature 417:949-54, 2002
72. Brose MS, Volpe P, Feldman M, Kumar M, Rishi I, Gerrero R, Einhorn E, Herlyn M, Minna J, Nicholson A, Roth JA, Albelda SM, Davies H, Cox C, Brignell G, Stephens P, Futreal PA, Wooster R, Stratton MR, Weber BL: BRAF and RAS mutations in human lung cancer and melanoma. Cancer Res 62:6997-7000, 2002
73. Naoki K, Chen TH, Richards WG, Sugarbaker DJ, Meyerson M: Missense mutations of the BRAF gene in human lung adenocarcinoma. Cancer Res 62:7001-3, 2002
74. Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE: Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature 418:934, 2002
75. Sommerer F, Vieth M, Markwarth A, Rohrich K, Vomschloss S, May A, Ell C, Stolte M, Hengge UR, Wittekind C, Tannapfel A: Mutations of BRAF and KRAS2 in the development of Barrett's adenocarcinoma. Oncogene 23:554-8, 2004
76. Fitzgerald RC: Ablative mucosectomy is the procedure of choice to prevent Barrett's cancer. Gut 52:16-7, 2003
77. Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, Yan H, Gazdar A, Powell SM, Riggins GJ, Willson JK, Markowitz S, Kinzler KW, Vogelstein B, Velculescu VE: High frequency of mutations of the PIK3CA gene in human cancers. Science 304:554, 2004
78. Miller CT, Moy JR, Lin L, Schipper M, Normolle D, Brenner DE, Iannettoni MD, Orringer MB, Beer DG: Gene amplification in esophageal adenocarcinomas and Barrett's with high-grade dysplasia. Clin Cancer Res 9:4819-25, 2003
79. Lin L, Miller CT, Contreras JI, Prescott MS, Dagenais SL, Wu R, Yee J, Orringer MB, Misek DE, Hanash SM, Glover TW, Beer DG: The hepatocyte nuclear factor 3 alpha gene, HNF3alpha (FOXA1), on chromosome band 14q13 is amplified and overexpressed in esophageal and lung adenocarcinomas. Cancer Res 62:5273-9, 2002
80. Zou TT, Lei J, Shi YQ, Yin J, Wang S, Souza RF, Kong D, Shimada Y, Smolinski KN, Greenwald BD, Abraham JM, Harpaz N, Meltzer SJ: FHIT gene alterations in esophageal cancer and ulcerative colitis (UC). Oncogene 15:101-5, 1997
81. Michael D, Beer DG, Wilke CW, Miller DE, Glover TW: Frequent deletions of FHIT and FRA3B in Barrett's metaplasia and esophageal adenocarcinomas. Oncogene 15:1653-9, 1997

82. Fang JM, Arlt MF, Burgess AC, Dagenais SL, Beer DG, Glover TW: Translocation breakpoints in FHIT and FRA3B in both homologs of chromosome 3 in an esophageal adenocarcinoma. Genes Chromosomes Cancer 30:292-8, 2001
83. Menin C, Santacatterina M, Zambon A, Montagna M, Parenti A, Ruol A, D'Andrea E: Anomalous transcripts and allelic deletions of the FHIT gene in human esophageal cancer. Cancer Genet Cytogenet 119:56-61, 2000
84. Albrecht B, Hausmann M, Zitzelsberger H, Stein H, Siewert JR, Hopt U, Langer R, Hofler H, Werner M, Walch A: Array-based comparative genomic hybridization for the detection of DNA sequence copy number changes in Barrett's adenocarcinoma. J Pathol 203:780-8, 2004
85. Chen YJ, Chen PH, Lee MD, Chang JG: Aberrant FHIT transcripts in cancerous and corresponding non-cancerous lesions of the digestive tract. Int J Cancer 72:955-8, 1997
86. Maher ER, Kaelin WG, Jr.: von Hippel-Lindau disease. Medicine (Baltimore) 76:381-91, 1997
87. Vamecq J, Latruffe N: Medical significance of peroxisome proliferator-activated receptors. Lancet 354:141-8, 1999
88. Wijnhoven BP, Lindstedt EW, Abbou M, Ijzendoorn Y, de Krijger RR, Tilanus HW, Dinjens WN: Molecular genetic analysis of the von Hippel-Lindau and human peroxisome proliferator-activated receptor gamma tumor-suppressor genes in adenocarcinomas of the gastroesophageal junction. Int J Cancer 94:891-5, 2001
89. Dolan K, Garde J, Gosney J, Sissons M, Wright T, Kingsnorth AN, Walker SJ, Sutton R, Meltzer SJ, Field JK: Allelotype analysis of oesophageal adenocarcinoma: loss of heterozygosity occurs at multiple sites. Br J Cancer 78:950-7, 1998
90. Sanz-Ortega J, Hernandez S, Saez MC, Sierra E, Sanz-Ortega G, Torres A, Balibrea JL, Sanz-Esponera J, Merino MJ: 3p21, 5q21, 9p21 and 17p13.1 allelic deletions are potential markers of individuals with a high risk of developing adenocarcinoma in Barrett's epithelium without dysplasia. Hepatogastroenterology 50:404-7, 2003
91. Raja S, Finkelstein SD, Baksh FK, Gooding WE, Swalsky PA, Godfrey TE, Buenaventura PO, Luketich JD: Correlation between dysplasia and mutations of six tumor suppressor genes in Barrett's esophagus. Ann Thorac Surg 72:1130-5, 2001
92. Dolan K, Garde J, Walker SJ, Sutton R, Gosney J, Field JK: Histological and molecular mapping of adenocarcinoma of the oesophagus and gastroesophageal junction: loss of heterozygosity occurs in histologically normal epithelium in the oesophagus and stomach. Oncol Rep 7:521-8, 2000
93. Bektas N, Donner A, Wirtz C, Heep H, Gabbert HE, Sarbia M: Allelic loss involving the tumor suppressor genes APC and MCC and expression of the APC protein in the development of dysplasia and carcinoma in Barrett esophagus. Am J Clin Pathol 114:890-5, 2000
94. Zhuang Z, Vortmeyer AO, Mark EJ, Odze R, Emmert-Buck MR, Merino MJ, Moon H, Liotta LA, Duray PH: Barrett's esophagus: metaplastic cells with loss of heterozygosity at the APC gene locus are clonal precursors to invasive adenocarcinoma. Cancer Res 56:1961-4, 1996
95. Gonzalez MV, Artimez ML, Rodrigo L, Lopez-Larrea C, Menendez MJ, Alvarez V, Perez R, Fresno MF, Perez MJ, Sampedro A, Coto E: Mutation analysis of the p53, APC, and p16 genes in the Barrett's oesophagus, dysplasia, and adenocarcinoma. J Clin Pathol 50:212-7, 1997
96. Ogasawara S, Tamura G, Maesawa C, Suzuki Y, Ishida K, Satoh N, Uesugi N, Saito K, Satodate R: Common deleted region on the long arm of chromosome 5 in esophageal carcinoma. Gastroenterology 110:52-7, 1996
97. Powell SM, Papadopoulos N, Kinzler KW, Smolinski KN, Meltzer SJ: APC gene mutations in the mutation cluster region are rare in esophageal cancers. Gastroenterology 107:1759-63, 1994
98. Tsuchiya T, Tamura G, Sato K, Endoh Y, Sakata K, Jin Z, Motoyama T, Usuba O, Kimura W, Nishizuka S, Wilson KT, James SP, Yin J, Fleisher AS, Zou T, Silverberg SG, Kong D, Meltzer SJ: Distinct methylation patterns of two APC gene promoters in normal and cancerous gastric epithelia. Oncogene 19:3642-6, 2000
99. Kawakami K, Brabender J, Lord RV, Groshen S, Greenwald BD, Krasna MJ, Yin J, Fleisher AS, Abraham JM, Beer DG, Sidransky D, Huss HT, Demeester TR, Eads C, Laird PW, Ilson DH, Kelsen DP, Harpole D, Moore MB, Danenberg KD, Danenberg PV, Meltzer SJ: Hypermethylated APC DNA in plasma and prognosis of patients with esophageal adenocarcinoma. J Natl Cancer Inst 92:1805-11, 2000

100. Clement G, Bosman FT, Fontolliet C, Benhattar J: Monoallelic methylation of the APC promoter is altered in normal gastric mucosa associated with neoplastic lesions. Cancer Res 64:6867-73, 2004
101. Tarmin L, Yin J, Zhou X, Suzuki H, Jiang HY, Rhyu MG, Abraham JM, Krasna MJ, Cottrell J, Meltzer SJ: Frequent loss of heterozygosity on chromosome 9 in adenocarcinoma and squamous cell carcinoma of the esophagus. Cancer Res 54:6094-6, 1994
102. Gleeson CM, Sloan JM, McGuigan JA, Ritchie AJ, Weber JL, Russell SE: Barrett's oesophagus: microsatellite analysis provides evidence to support the proposed metaplasia-dysplasia-carcinoma sequence. Genes Chromosomes Cancer 21:49-60, 1998
103. Barrett MT, Galipeau PC, Sanchez CA, Emond MJ, Reid BJ: Determination of the frequency of loss of heterozygosity in esophageal adenocarcinoma by cell sorting, whole genome amplification and microsatellite polymorphisms. Oncogene 12:1873-8, 1996
104. Nobukawa B, Abraham SC, Gill J, Heitmiller RF, Wu TT: Clinicopathologic and molecular analysis of high-grade dysplasia and early adenocarcinoma in short- versus long-segment Barrett esophagus. Hum Pathol 32:447-54, 2001
105. Barrett MT, Sanchez CA, Prevo LJ, Wong DJ, Galipeau PC, Paulson TG, Rabinovitch PS, Reid BJ: Evolution of neoplastic cell lineages in Barrett oesophagus. Nat Genet 22:106-9, 1999
106. Esteve A, Martel-Planche G, Sylla BS, Hollstein M, Hainaut P, Montesano R: Low frequency of p16/CDKN2 gene mutations in esophageal carcinomas. Int J Cancer 66:301-4, 1996
107. Muzeau F, Flejou JF, Thomas G, Hamelin R: Loss of heterozygosity on chromosome 9 and p16 (MTS1, CDKN2) gene mutations in esophageal cancers. Int J Cancer 72:27-30, 1997
108. Suzuki H, Zhou X, Yin J, Lei J, Jiang HY, Suzuki Y, Chan T, Hannon GJ, Mergner WJ, Abraham JM, et al.: Intragenic mutations of CDKN2B and CDKN2A in primary human esophageal cancers. Hum Mol Genet 4:1883-7, 1995
109. Barrett MT, Sanchez CA, Galipeau PC, Neshat K, Emond M, Reid BJ: Allelic loss of 9p21 and mutation of the CDKN2/p16 gene develop as early lesions during neoplastic progression in Barrett's esophagus. Oncogene 13:1867-73, 1996
110. Klump B, Hsieh CJ, Holzmann K, Gregor M, Porschen R: Hypermethylation of the CDKN2/p16 promoter during neoplastic progression in Barrett's esophagus. Gastroenterology 115:1381-6, 1998
111. Wong DJ, Barrett MT, Stoger R, Emond MJ, Reid BJ: p16INK4a promoter is hypermethylated at a high frequency in esophageal adenocarcinomas. Cancer Res 57:2619-22, 1997
112. Wong DJ, Paulson TG, Prevo LJ, Galipeau PC, Longton G, Blount PL, Reid BJ: p16(INK4a) lesions are common, early abnormalities that undergo clonal expansion in Barrett's metaplastic epithelium. Cancer Res 61:8284-9, 2001
113. Bian YS, Osterheld MC, Fontolliet C, Bosman FT, Benhattar J: p16 inactivation by methylation of the CDKN2A promoter occurs early during neoplastic progression in Barrett's esophagus. Gastroenterology 122:1113-21, 2002
114. Vieth M, Schneider-Stock R, Rohrich K, May A, Ell C, Markwarth A, Roessner A, Stolte M, Tannapfel A: INK4a-ARF alterations in Barrett's epithelium, intraepithelial neoplasia and Barrett's adenocarcinoma. Virchows Arch 445:135-41, 2004
115. Galipeau PC, Prevo LJ, Sanchez CA, Longton GM, Reid BJ: Clonal expansion and loss of heterozygosity at chromosomes 9p and 17p in premalignant esophageal (Barrett's) tissue. J Natl Cancer Inst 91:2087-95, 1999
116. Maley CC, Galipeau PC, Li X, Sanchez CA, Paulson TG, Reid BJ: Selectively advantageous mutations and hitchhikers in neoplasms: p16 lesions are selected in Barrett's esophagus. Cancer Res 64:3414-27, 2004
117. Suspiro A, Pereira AD, Afonso A, Albuquerque C, Chaves P, Soares J, Leitao CN: Losses of heterozygosity on chromosomes 9p and 17p are frequent events in Barrett's metaplasia not associated with dysplasia or adenocarcinoma. Am J Gastroenterol 98:728-34, 2003
118. Sarbia M, Tekin U, Zeriouh M, Donner A, Gabbert HE: Expression of the RB protein, allelic imbalance of the RB gene and amplification of the CDK4 gene in metaplasias, dysplasias and carcinomas in Barrett's oesophagus. Anticancer Res 21:387-92, 2001
119. Casson AG, Mukhopadhyay T, Cleary KR, Ro JY, Levin B, Roth JA: p53 gene mutations in Barrett's epithelium and esophageal cancer. Cancer Res 51:4495-9, 1991

120. Krishnadath KK, Tilanus HW, van Blankenstein M, Bosman FT, Mulder AH: Accumulation of p53 protein in normal, dysplastic, and neoplastic Barrett's oesophagus. J Pathol 175:175-80, 1995
121. Younes M, Ertan A, Lechago LV, Somoano JR, Lechago J: p53 Protein accumulation is a specific marker of malignant potential in Barrett's metaplasia. Dig Dis Sci 42:697-701, 1997
122. Blount PL, Meltzer SJ, Yin J, Huang Y, Krasna MJ, Reid BJ: Clonal ordering of 17p and 5q allelic losses in Barrett dysplasia and adenocarcinoma. Proc Natl Acad Sci U S A 90:3221-5, 1993
123. Blount PL, Galipeau PC, Sanchez CA, Neshat K, Levine DS, Yin J, Suzuki H, Abraham JM, Meltzer SJ, Reid BJ: 17p allelic losses in diploid cells of patients with Barrett's esophagus who develop aneuploidy. Cancer Res 54:2292-5, 1994
124. Morgan RJ, Newcomb PV, Bailey M, Hardwick RH, Alderson D: Loss of heterozygosity at microsatellite marker sites for tumor suppressor genes in oesophageal adenocarcinoma. Eur J Surg Oncol 24:34-7, 1998
125. Wu TT, Watanabe T, Heitmiller R, Zahurak M, Forastiere AA, Hamilton SR: Genetic alterations in Barrett esophagus and adenocarcinomas of the esophagus and esophagogastric junction region. Am J Pathol 153:287-94, 1998
126. Hamelin R, Flejou JF, Muzeau F, Potet F, Laurent-Puig P, Fekete F, Thomas G: TP53 gene mutations and p53 protein immunoreactivity in malignant and premalignant Barrett's esophagus. Gastroenterology 107:1012-8, 1994
127. Gleeson CM, Sloan JM, McGuigan JA, Ritchie AJ, Russell SE: Base transitions at CpG dinucleotides in the p53 gene are common in esophageal adenocarcinoma. Cancer Res 55:3406-11, 1995
128. Soslow RA, Altorki NK, Yang G, Xie D, Yang CS: mdm-2 expression correlates with wild-type p53 status in esophageal adenocarcinoma. Mod Pathol 12:580-6, 1999
129. Neshat K, Sanchez CA, Galipeau PC, Blount PL, Levine DS, Joslyn G, Reid BJ: p53 mutations in Barrett's adenocarcinoma and high-grade dysplasia. Gastroenterology 106:1589-95, 1994
130. Bian YS, Osterheld MC, Bosman FT, Benhattar J, Fontolliet C: p53 gene mutation and protein accumulation during neoplastic progression in Barrett's esophagus. Mod Pathol 14:397-403, 2001
131. Doak SH, Jenkins GJ, Parry EM, Griffiths AP, Shah V, Baxter JN, Parry JM: Characterisation of p53 status at the gene, chromosomal and protein levels in oesophageal adenocarcinoma. Br J Cancer 89:1729-35, 2003
132. Dolan K, Morris AI, Gosney JR, Field JK, Sutton R: Loss of heterozygosity on chromosome 17p predicts neoplastic progression in Barrett's esophagus. J Gastroenterol Hepatol 18:683-9, 2003
133. Dolan K, Walker SJ, Gosney J, Field JK, Sutton R: TP53 mutations in malignant and premalignant Barrett's esophagus. Dis Esophagus 16:83-9, 2003
134. Reid BJ, Prevo LJ, Galipeau PC, Sanchez CA, Longton G, Levine DS, Blount PL, Rabinovitch PS: Predictors of progression in Barrett's esophagus II: baseline 17p (p53) loss of heterozygosity identifies a patient subset at increased risk for neoplastic progression. Am J Gastroenterol 96:2839-48, 2001
135. Schneider PM, Stoeltzing O, Roth JA, Hoelscher AH, Wegerer S, Mizumoto S, Becker K, Dittler HJ, Fink U, Siewert JR: P53 mutational status improves estimation of prognosis in patients with curatively resected adenocarcinoma in Barrett's esophagus. Clin Cancer Res 6:3153-8, 2000
136. Casson AG, Evans SC, Gillis A, Porter GA, Veugelers P, Darnton SJ, Guernsey DL, Hainaut P: Clinical implications of p53 tumor suppressor gene mutation and protein expression in esophageal adenocarcinomas: results of a ten-year prospective study. J Thorac Cardiovasc Surg 125:1121-31, 2003
137. Ireland AP, Shibata DK, Chandrasoma P, Lord RV, Peters JH, DeMeester TR: Clinical significance of p53 mutations in adenocarcinoma of the esophagus and cardia. Ann Surg 231:179-87, 2000
138. Barrett MT, Schutte M, Kern SE, Reid BJ: Allelic loss and mutational analysis of the DPC4 gene in esophageal adenocarcinoma. Cancer Res 56:4351-3, 1996
139. Wang S, Mori Y, Sato F, Yin J, Xu Y, Zou TT, Olaru A, Kimos MC, Perry K, Selaru FM, Deacu E, Sun M, Shi YC, Shibata D, Abraham JM, Greenwald BD, Meltzer SJ: An LOH and mutational investigation of the ST7 gene locus in human esophageal carcinoma. Oncogene 22:467-70, 2003
140. Sarbia M, Geddert H, Klump B, Kiel S, Iskender E, Gabbert HE: Hypermethylation of tumor suppressor genes (p16INK4A, p14ARF and APC) in adenocarcinomas of the upper gastrointestinal tract. Int J Cancer 111:224-8, 2004

141. Eads CA, Lord RV, Kurumboor SK, Wickramasinghe K, Skinner ML, Long TI, Peters JH, DeMeester TR, Danenberg KD, Danenberg PV, Laird PW, Skinner KA: Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma. Cancer Res 60:5021-6, 2000
142. Brock MV, Gou M, Akiyama Y, Muller A, Wu TT, Montgomery E, Deasel M, Germonpre P, Rubinson L, Heitmiller RF, Yang SC, Forastiere AA, Baylin SB, Herman JG: Prognostic importance of promoter hypermethylation of multiple genes in esophageal adenocarcinoma. Clin Cancer Res 9:2912-9, 2003
143. Eads CA, Lord RV, Wickramasinghe K, Long TI, Kurumboor SK, Bernstein L, Peters JH, DeMeester SR, DeMeester TR, Skinner KA, Laird PW: Epigenetic patterns in the progression of esophageal adenocarcinoma. Cancer Res 61:3410-8, 2001
144. Meltzer SJ, Yin J, Manin B, Rhyu MG, Cottrell J, Hudson E, Redd JL, Krasna MJ, Abraham JM, Reid BJ: Microsatellite instability occurs frequently and in both diploid and aneuploid cell populations of Barrett's-associated esophageal adenocarcinomas. Cancer Res 54:3379-82, 1994
145. Keller G, Rotter M, Vogelsang H, Bischoff P, Becker KF, Mueller J, Brauch H, Siewert JR, Hofler H: Microsatellite instability in adenocarcinomas of the upper gastrointestinal tract. Relation to clinicopathological data and family history. Am J Pathol 147:593-600, 1995
146. Kulke MH, Thakore KS, Thomas G, Wang H, Loda M, Eng C, Odze RD: Microsatellite instability and hMLH1/hMSH2 expression in Barrett esophagus-associated adenocarcinoma. Cancer 91:1451-7, 2001
147. Evans SC, Gillis A, Geldenhuys L, Vaninetti NM, Malatjalian DA, Porter GA, Guernsey DL, Casson AG: Microsatellite instability in esophageal adenocarcinoma. Cancer Lett 212:241-51, 2004
148. Orr-Weaver TL, Weinberg RA: A checkpoint on the road to cancer. Nature 392:223-4, 1998
149. Mogensen MM, Tucker JB, Mackie JB, Prescott AR, Nathke IS: The adenomatous polyposis coli protein unambiguously localizes to microtubule plus ends and is involved in establishing parallel arrays of microtubule bundles in highly polarized epithelial cells. J Cell Biol 157:1041-8, 2002
150. Doak SH, Jenkins GJ, Parry EM, Griffiths AP, Baxter JN, Parry JM: Differential expression of the MAD2, BUB1 and HSP27 genes in Barrett's oesophagus-their association with aneuploidy and neoplastic progression. Mutat Res 547:133-44, 2004
151. Zumbrunn J, Kinoshita K, Hyman AA, Nathke IS: Binding of the adenomatous polyposis coli protein to microtubules increases microtubule stability and is regulated by GSK3 beta phosphorylation. Curr Biol 11:44-9, 2001
152. Levine DS, Reid BJ, Haggitt RC, Rubin CE, Rabinovitch PS: Correlation of ultrastructural aberrations with dysplasia and flow cytometric abnormalities in Barrett's epithelium. Gastroenterology 96:355-67, 1989
153. Reid BJ, Blount PL, Rubin CE, Levine DS, Haggitt RC, Rabinovitch PS: Flow-cytometric and histological progression to malignancy in Barrett's esophagus: prospective endoscopic surveillance of a cohort. Gastroenterology 102:1212-9, 1992
154. Reid BJ, Haggitt RC, Rubin CE, Rabinovitch PS: Barrett's esophagus. Correlation between flow cytometry and histology in detection of patients at risk for adenocarcinoma. Gastroenterology 93:1-11, 1987
155. Reid BJ, Levine DS, Longton G, Blount PL, Rabinovitch PS: Predictors of progression to cancer in Barrett's esophagus: baseline histology and flow cytometry identify low- and high-risk patient subsets. Am J Gastroenterol 95:1669-76, 2000
156. Fang M, Lew E, Klein M, Sebo T, Su Y, Goyal R: DNA abnormalities as marker of risk for progression of Barrett's esophagus to adenocarcinoma: image cytometric DNA analysis in formalin-fixed tissues. Am J Gastroenterol 99:1887-94, 2004
157. Teodori L, Gohde W, Persiani M, Ferrario F, Tirindelli Danesi D, Scarpignato C, Di Tondo U, Alo P, Capurso L: DNA/protein flow cytometry as a predictive marker of malignancy in dysplasia-free Barrett's esophagus: thirteen-year follow-up study on a cohort of patients. Cytometry 34:257-63, 1998
158. James PD, Atkinson M: Value of DNA image cytometry in the prediction of malignant change in Barrett's oesophagus. Gut 30:899-905, 1989
159. Rabinovitch PS, Longton G, Blount PL, Levine DS, Reid BJ: Predictors of progression in Barrett's esophagus III: baseline flow cytometric variables. Am J Gastroenterol 96:3071-83, 2001

160. Rodriguez E, Mathew S, Reuter V, Ilson DH, Bosl GJ, Chaganti RS: Cytogenetic analysis of 124 prospectively ascertained male germ cell tumors. Cancer Res 52:2285-91, 1992
161. Raskind WH, Norwood T, Levine DS, Haggitt RC, Rabinovitch PS, Reid BJ: Persistent clonal areas and clonal expansion in Barrett's esophagus. Cancer Res 52:2946-50, 1992
162. Garewal HS, Sampliner R, Liu Y, Trent JM: Chromosomal rearrangements in Barrett's esophagus. A premalignant lesion of esophageal adenocarcinoma. Cancer Genet Cytogenet 42:281-6, 1989
163. Menke-Pluymers MB, van Drunen E, Vissers KJ, Mulder AH, Tilanus HW, Hagemeijer A: Cytogenetic analysis of Barrett's mucosa and adenocarcinoma of the distal esophagus and cardia. Cancer Genet Cytogenet 90:109-17, 1996
164. Doak SH, Jenkins GJ, Parry EM, D'Souza FR, Griffiths AP, Toffazal N, Shah V, Baxter JN, Parry JM: Chromosome 4 hyperploidy represents an early genetic aberration in premalignant Barrett's oesophagus. Gut 52:623-8, 2003
165. Croft J, Parry EM, Jenkins GJ, Doak SH, Baxter JN, Griffiths AP, Brown TH, Parry JM: Analysis of the premalignant stages of Barrett's oesophagus through to adenocarcinoma by comparative genomic hybridization. Eur J Gastroenterol Hepatol 14:1179-86, 2002
166. Walch AK, Zitzelsberger HF, Bruch J, Keller G, Angermeier D, Aubele MM, Mueller J, Stein H, Braselmann H, Siewert JR, Hofler H, Werner M: Chromosomal imbalances in Barrett's adenocarcinoma and the metaplasia-dysplasia-carcinoma sequence. Am J Pathol 156:555-66, 2000
167. Riegman PH, Vissers KJ, Alers JC, Geelen E, Hop WC, Tilanus HW, van Dekken H: Genomic alterations in malignant transformation of Barrett's esophagus. Cancer Res 61:3164-70, 2001
168. Dolan K, Garde J, Walker SJ, Sutton R, Gosney J, Field JK: LOH at the sites of the DCC, APC, and TP53 tumor suppressor genes occurs in Barrett's metaplasia and dysplasia adjacent to adenocarcinoma of the esophagus. Hum Pathol 30:1508-14, 1999
169. El-Rifai W, Frierson HF, Jr., Moskaluk CA, Harper JC, Petroni GR, Bissonette EA, Jones DR, Knuutila S, Powell SM: Genetic differences between adenocarcinomas arising in Barrett's esophagus and gastric mucosa. Gastroenterology 121:592-8, 2001
170. El-Rifai W, Harper JC, Cummings OW, Hyytinen ER, Frierson HF, Jr., Knuutila S, Powell SM: Consistent genetic alterations in xenografts of proximal stomach and gastro-esophageal junction adenocarcinomas. Cancer Res 58:34-7, 1998
171. van Dekken H, Alers JC, Riegman PH, Rosenberg C, Tilanus HW, Vissers K: Molecular cytogenetic evaluation of gastric cardia adenocarcinoma and precursor lesions. Am J Pathol 158:1961-7, 2001
172. Huang Y, Boynton RF, Blount PL, Silverstein RJ, Yin J, Tong Y, McDaniel TK, Newkirk C, Resau JH, Sridhara R, et al.: Loss of heterozygosity involves multiple tumor suppressor genes in human esophageal cancers. Cancer Res 52:6525-30, 1992
173. Krishnadath KK, Tilanus HW, Alers JC, Mulder AH, van Dekken H: Detection of genetic changes in Barrett's adenocarcinoma and Barrett's esophagus by DNA in situ hybridization and immunohistochemistry. Cytometry 15:176-84, 1994
174. Varis A, Puolakkainen P, Savolainen H, Kokkola A, Salo J, Nieminen O, Nordling S, Knuutila S: DNA copy number profiling in esophageal Barrett adenocarcinoma: comparison with gastric adenocarcinoma and esophageal squamous cell carcinoma. Cancer Genet Cytogenet 127:53-8, 2001
175. Beuzen F, Dubois S, Flejou JF: Chromosomal numerical aberrations are frequent in oesophageal and gastric adenocarcinomas: a study using in-situ hybridization. Histopathology 37:241-9, 2000
176. Walch AK, Zitzelsberger HF, Bink K, Hutzler P, Bruch J, Braselmann H, Aubele MM, Mueller J, Stein H, Siewert JR, Hofler H, Werner M: Molecular genetic changes in metastatic primary Barrett's adenocarcinoma and related lymph node metastases: comparison with nonmetastatic Barrett's adenocarcinoma. Mod Pathol 13:814-24, 2000
177. Sinicrope FA, Roddey G, McDonnell TJ, Shen Y, Cleary KR, Stephens LC: Increased apoptosis accompanies neoplastic development in the human colorectum. Clin Cancer Res 2:1999-2006, 1996
178. Soslow RA, Remotti H, Baergen RN, Altorki NK: Suppression of apoptosis does not foster neoplastic growth in Barrett's esophagus. Mod Pathol 12:239-50, 1999
179. Whittles CE, Biddlestone LR, Burton A, Barr H, Jankowski JA, Warner PJ, Shepherd NA: Apoptotic and proliferative activity in the neoplastic progression of Barrett's oesophagus: a comparative study. J Pathol 187:535-40, 1999

180. Gillen P, McDermott M, Grehan D, Hourihane DO, Hennessy TP: Proliferating cell nuclear antigen in the assessment of Barrett's mucosa. Br J Surg 81:1766-8, 1994
181. Kimos MC, Wang S, Borkowski A, Yang GY, Yang CS, Perry K, Olaru A, Deacu E, Sterian A, Cottrell J, Papadimitriou J, Sisodia L, Selaru FM, Mori Y, Xu Y, Yin J, Abraham JM, Meltzer SJ: Esophagin and proliferating cell nuclear antigen (PCNA) are biomarkers of human esophageal neoplastic progression. Int J Cancer 111:415-7, 2004
182. Lauwers GY, Kandemir O, Kubilis PS, Scott GV: Cellular kinetics in Barrett's epithelium carcinogenic sequence: roles of apoptosis, bcl-2 protein, and cellular proliferation. Mod Pathol 10:1201-8, 1997
183. Jankowski J, McMenemin R, Yu C, Hopwood D, Wormsley KG: Proliferating cell nuclear antigen in oesophageal diseases; correlation with transforming growth factor alpha expression. Gut 33:587-91, 1992
184. Hong MK, Laskin WB, Herman BE, Johnston MH, Vargo JJ, Steinberg SM, Allegra CJ, Johnston PG: Expansion of the Ki-67 proliferative compartment correlates with degree of dysplasia in Barrett's esophagus. Cancer 75:423-9, 1995
185. Polkowski W, van Lanschot JJ, Ten Kate FJ, Baak JP, Tytgat GN, Obertop H, Voorn WJ, Offerhaus GJ: The value of p53 and Ki67 as markers for tumor progression in the Barrett's dysplasia-carcinoma sequence. Surg Oncol 4:163-71, 1995
186. Yacoub L, Goldman H, Odze RD: Transforming growth factor-alpha, epidermal growth factor receptor, and MiB-1 expression in Barrett's-associated neoplasia: correlation with prognosis. Mod Pathol 10:105-12, 1997
187. Rioux-Leclercq N, Turlin B, Sutherland F, Heresbach N, Launois B, Campion JP, Ramee MP: Analysis of Ki-67, p53 and Bcl-2 expression in the dysplasia-carcinoma sequence of Barrett's esophagus. Oncol Rep 6:877-82, 1999
188. Krishnadath KK, Tilanus HW, van Blankenstein M, Hop WC, Teijgeman R, Mulder AH, Bosman FT, van Dekken H: Accumulation of genetic abnormalities during neoplastic progression in Barrett's esophagus. Cancer Res 55:1971-6, 1995
189. Halm U, Tannapfel A, Breitung B, Breidert M, Wittekind CW, Mossner J: Apoptosis and cell proliferation in the metaplasia-dysplasia-carcinoma-sequence of Barrett's esophagus. Hepatogastroenterology 47:962-6, 2000
190. Going JJ, Keith WN, Neilson L, Stoeber K, Stuart RC, Williams GH: Aberrant expression of minichromosome maintenance proteins 2 and 5, and Ki-67 in dysplastic squamous oesophageal epithelium and Barrett's mucosa. Gut 50:373-7, 2002
191. Ouatu-Lascar R, Fitzgerald RC, Triadafilopoulos G: Differentiation and proliferation in Barrett's esophagus and the effects of acid suppression. Gastroenterology 117:327-35, 1999
192. Wetscher GJ, Schwelberger H, Unger A, Offner FA, Profanter C, Glaser K, Klingler A, Gadenstaetter M, Klinger P: Reflux-induced apoptosis of the esophageal mucosa is inhibited in Barrett's epithelium. Am J Surg 176:569-73, 1998
193. Katada N, Hinder RA, Smyrk TC, Hirabayashi N, Perdikis G, Lund RJ, Woodward T, Klingler PJ: Apoptosis is inhibited early in the dysplasia-carcinoma sequence of Barrett esophagus. Arch Surg 132:728-33, 1997
194. Hughes SJ, Nambu Y, Soldes OS, Hamstra D, Rehemtulla A, Iannettoni MD, Orringer MB, Beer DG: Fas/APO-1 (CD95) is not translocated to the cell membrane in esophageal adenocarcinoma. Cancer Res 57:5571-8, 1997
195. Younes M, Schwartz MR, Finnie D, Younes A: Overexpression of Fas ligand (FasL) during malignant transformation in the large bowel and in Barrett's metaplasia of the esophagus. Hum Pathol 30:1309-13, 1999
196. Younes M, Lechago J, Ertan A, Finnie D, Younes A: Decreased expression of Fas (CD95/APO1) associated with goblet cell metaplasia in Barrett's esophagus. Hum Pathol 31:434-8, 2000
197. Reed JC: Bcl-2 and the regulation of programmed cell death. J Cell Biol 124:1-6, 1994
198. Goldblum JR, Rice TW: bcl-2 protein expression in the Barrett's metaplasia-dysplasia-carcinoma sequence. Mod Pathol 8:866-9, 1995
199. Beardsmore DM, Verbeke CS, Davies CL, Guillou PJ, Clark GW: Apoptotic and proliferative indexes in esophageal cancer: predictors of response to neoadjuvant therapy [corrected]. J Gastrointest Surg 7:77-86; discussion 86-7, 2003

200. van der Woude CJ, Jansen PL, Tiebosch AT, Beuving A, Homan M, Kleibeuker JH, Moshage H: Expression of apoptosis-related proteins in Barrett's metaplasia-dysplasia-carcinoma sequence: a switch to a more resistant phenotype. Hum Pathol 33:686-92, 2002
201. Chatelain D, Flejou JF: High-grade dysplasia and superficial adenocarcinoma in Barrett's esophagus: histological mapping and expression of p53, p21 and Bcl-2 oncoproteins. Virchows Arch 442:18-24, 2003
202. Iravani S, Zhang HQ, Yuan ZQ, Cheng JQ, Karl RC, Jove R, Coppola D: Modification of insulin-like growth factor 1 receptor, c-Src, and Bcl-XL protein expression during the progression of Barrett's neoplasia. Hum Pathol 34:975-82, 2003
203. Raouf AA, Evoy DA, Carton E, Mulligan E, Griffin MM, Reynolds JV: Loss of Bcl-2 expression in Barrett's dysplasia and adenocarcinoma is associated with tumor progression and worse survival but not with response to neoadjuvant chemoradiation. Dis Esophagus 16:17-23, 2003
204. Bond GL, Hu W, Bond EE, Robins H, Lutzker SG, Arva NC, Bargonetti J, Bartel F, Taubert H, Wuerl P, Onel K, Yip L, Hwang SJ, Strong LC, Lozano G, Levine AJ: A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. Cell 119:591-602, 2004
205. Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC, Altieri DC: Control of apoptosis and mitotic spindle checkpoint by survivin. Nature 396:580-4, 1998
206. Kawasaki H, Altieri DC, Lu CD, Toyoda M, Tenjo T, Tanigawa N: Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. Cancer Res 58:5071-4, 1998
207. Sarela AI, Macadam RC, Farmery SM, Markham AF, Guillou PJ: Expression of the antiapoptosis gene, survivin, predicts death from recurrent colorectal carcinoma. Gut 46:645-50, 2000
208. Adida C, Berrebi D, Peuchmaur M, Reyes-Mugica M, Altieri DC: Anti-apoptosis gene, survivin, and prognosis of neuroblastoma. Lancet 351:882-3, 1998
209. Swana HS, Grossman D, Anthony JN, Weiss RM, Altieri DC: Tumor content of the antiapoptosis molecule survivin and recurrence of bladder cancer. N Engl J Med 341:452-3, 1999
210. Nemoto T, Kitagawa M, Hasegawa M, Ikeda S, Akashi T, Takizawa T, Hirokawa K, Koike M: Expression of IAP family proteins in esophageal cancer. Exp Mol Pathol 76:253-9, 2004
211. Lord RV, Salonga D, Danenberg KD, Peters JH, DeMeester TR, Park JM, Johansson J, Skinner KA, Chandrasoma P, DeMeester SR, Bremner CG, Tsai PI, Danenberg PV: Telomerase reverse transcriptase expression is increased early in the Barrett's metaplasia, dysplasia, adenocarcinoma sequence. J Gastrointest Surg 4:135-42, 2000
212. Morales CP, Lee EL, Shay JW: In situ hybridization for the detection of telomerase RNA in the progression from Barrett's esophagus to esophageal adenocarcinoma. Cancer 83:652-9, 1998
213. Reid BJ, Sanchez CA, Blount PL, Levine DS: Barrett's esophagus: cell cycle abnormalities in advancing stages of neoplastic progression. Gastroenterology 105:119-29, 1993
214. Roncalli M, Bosari S, Marchetti A, Buttitta F, Bossi P, Graziani D, Peracchia A, Bonavina L, Viale G, Coggi G: Cell cycle-related gene abnormalities and product expression in esophageal carcinoma. Lab Invest 78:1049-57, 1998
215. Arber N, Lightdale C, Rotterdam H, Han KH, Sgambato A, Yap E, Ahsan H, Finegold J, Stevens PD, Green PH, Hibshoosh H, Neugut AI, Holt PR, Weinstein IB: Increased expression of the cyclin D1 gene in Barrett's esophagus. Cancer Epidemiol Biomarkers Prev 5:457-9, 1996
216. Arber N, Gammon MD, Hibshoosh H, Britton JA, Zhang Y, Schonberg JB, Roterdam H, Fabian I, Holt PR, Weinstein IB: Overexpression of cyclin D1 occurs in both squamous carcinomas and adenocarcinomas of the esophagus and in adenocarcinomas of the stomach. Hum Pathol 30:1087-92, 1999
217. Kuwahara M, Hirai T, Yoshida K, Yamashita Y, Hihara J, Inoue H, Toge T: p53, p21(Waf1/Cip1) and cyclin D1 protein expression and prognosis in esophageal cancer. Dis Esophagus 12:116-9, 1999
218. Morgan RJ, Newcomb PV, Hardwick RH, Alderson D: Amplification of cyclin D1 and MDM-2 in oesophageal carcinoma. Eur J Surg Oncol 25:364-7, 1999
219. Shimada Y, Imamura M, Shibagaki I, Tanaka H, Miyahara T, Kato M, Ishizaki K: Genetic alterations in patients with esophageal cancer with short- and long-term survival rates after curative esophagectomy. Ann Surg 226:162-8, 1997

220. Bani-Hani K, Martin IG, Hardie LJ, Mapstone N, Briggs JA, Forman D, Wild CP: Prospective study of cyclin D1 overexpression in Barrett's esophagus: association with increased risk of adenocarcinoma. J Natl Cancer Inst 92:1316-21, 2000
221. Geddert H, Heep HJ, Gabbert HE, Sarbia M: Expression of cyclin B1 in the metaplasia-dysplasia-carcinoma sequence of Barrett esophagus. Cancer 94:212-8, 2002
222. Sarbia M, Bektas N, Muller W, Heep H, Borchard F, Gabbert HE: Expression of cyclin E in dysplasia, carcinoma, and nonmalignant lesions of Barrett esophagus. Cancer 86:2597-601, 1999
223. Lin L, Aggarwal S, Glover TW, Orringer MB, Hanash S, Beer DG: A minimal critical region of the 8p22-23 amplicon in esophageal adenocarcinomas defined using sequence tagged site-amplification mapping and quantitative polymerase chain reaction includes the GATA-4 gene. Cancer Res 60:1341-7, 2000
224. Hanas JS, Lerner MR, Lightfoot SA, Raczkowski C, Kastens DJ, Brackett DJ, Postier RG: Expression of the cyclin-dependent kinase inhibitor p21(WAF1/CIP1) and p53 tumor suppressor in dysplastic progression and adenocarcinoma in Barrett esophagus. Cancer 86:756-63, 1999
225. Moskaluk CA, Heitmiller R, Zahurak M, Schwab D, Sidransky D, Hamilton SR: p53 and p21(WAF1/CIP1/SDI1) gene products in Barrett esophagus and adenocarcinoma of the esophagus and esophagogastric junction. Hum Pathol 27:1211-20, 1996
226. Singh SP, Lipman J, Goldman H, Ellis FH, Jr., Aizenman L, Cangi MG, Signoretti S, Chiaur DS, Pagano M, Loda M: Loss or altered subcellular localization of p27 in Barrett's associated adenocarcinoma. Cancer Res 58:1730-5, 1998
227. Bailey T, Biddlestone L, Shepherd N, Barr H, Warner P, Jankowski J: Altered cadherin and catenin complexes in the Barrett's esophagus-dysplasia-adenocarcinoma sequence: correlation with disease progression and dedifferentiation. Am J Pathol 152:135-44, 1998
228. Krishnadath KK, Tilanus HW, van Blankenstein M, Hop WC, Kremers ED, Dinjens WN, Bosman FT: Reduced expression of the cadherin-catenin complex in oesophageal adenocarcinoma correlates with poor prognosis. J Pathol 182:331-8, 1997
229. Wijnhoven BP, de Both NJ, van Dekken H, Tilanus HW, Dinjens WN: E-cadherin gene mutations are rare in adenocarcinomas of the oesophagus. Br J Cancer 80:1652-7, 1999
230. Corn PG, Heath EI, Heitmiller R, Fogt F, Forastiere AA, Herman JG, Wu TT: Frequent hypermethylation of the 5' CpG island of E-cadherin in esophageal adenocarcinoma. Clin Cancer Res 7:2765-9, 2001
231. Morin PJ: beta-catenin signaling and cancer. Bioessays 21:1021-30, 1999
232. Kikuchi A: Regulation of beta-catenin signaling in the Wnt pathway. Biochem Biophys Res Commun 268:243-8, 2000
233. Osterheld MC, Bian YS, Bosman FT, Benhattar J, Fontolliet C: Beta-catenin expression and its association with prognostic factors in adenocarcinoma developed in Barrett esophagus. Am J Clin Pathol 117:451-6, 2002
234. Choi YW, Heath EI, Heitmiller R, Forastiere AA, Wu TT: Mutations in beta-catenin and APC genes are uncommon in esophageal and esophagogastric junction adenocarcinomas. Mod Pathol 13:1055-9, 2000
235. Bian YS, Osterheld MC, Bosman FT, Fontolliet C, Benhattar J: Nuclear accumulation of beta-catenin is a common and early event during neoplastic progression of Barrett esophagus. Am J Clin Pathol 114:583-90, 2000
236. Wijnhoven BP, Nollet F, De Both NJ, Tilanus HW, Dinjens WN: Genetic alterations involving exon 3 of the beta-catenin gene do not play a role in adenocarcinomas of the esophagus. Int J Cancer 86:533-7, 2000
237. Wielenga VJ, Smits R, Korinek V, Smit L, Kielman M, Fodde R, Clevers H, Pals ST: Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT pathway. Am J Pathol 154:515-23, 1999
238. Bottger TC, Youssef V, Dutkowski P, Maschek H, Brenner W, Junginger T: Expression of CD44 variant proteins in adenocarcinoma of Barrett's esophagus and its relation to prognosis. Cancer 83:1074-80, 1998
239. Lagorce-Pages C, Paraf F, Dubois S, Belghiti J, Flejou JF: Expression of CD44 in premalignant and malignant Barrett's oesophagus. Histopathology 32:7-14, 1998

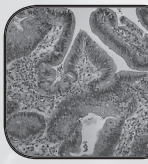
240. Menges M, Goebel R, Pueschel W, Zeitz M, Stallmach A: Expression of CD44v5 and -v6 in Barrett's carcinoma is not increased compared to that in nondysplastic Barrett's mucosa. Exp Mol Pathol 72:207-12, 2002
241. Keppler D, Sameni M, Moin K, Mikkelsen T, Diglio CA, Sloane BF: Tumor progression and angiogenesis: cathepsin B & Co. Biochem Cell Biol 74:799-810, 1996
242. Owonikoko T, Rees M, Gabbert HE, Sarbia M: Intratumoral genetic heterogeneity in Barrett adenocarcinoma. Am J Clin Pathol 117:558-66, 2002
243. Prevo LJ, Sanchez CA, Galipeau PC, Reid BJ: p53-mutant clones and field effects in Barrett's esophagus. Cancer Res 59:4784-7, 1999
244. Maley CC, Galipeau PC, Li X, Sanchez CA, Paulson TG, Blount PL, Reid BJ: The combination of genetic instability and clonal expansion predicts progression to esophageal adenocarcinoma. Cancer Res 64:7629-33, 2004

Part II

Molecular Biology

Chapter 3

Wnt signaling pathway in adenocarcinomas of the gastro-esophageal junction



Submitted

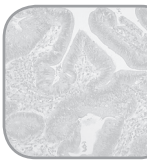
Linetta B Koppert
Bas PL Wijnhoven
Mascha van Noort
Herman van Dekken
Hugo W Tilanus
Winand NM Dinjens

ABSTRACT

We aimed to investigate the contribution and possible prognostic role of the Wnt signaling pathway in gastro-esophageal junction (GEJ) adenocarcinomas. An immunohistochemical study of the diverse components of the Wnt pathway was undertaken in 164 GEJ adenocarcinomas and in 5 human GEJ adenocarcinoma cell lines, in which we formerly demonstrated that nuclear β-catenin expression correlates with enhanced TCF-mediated transcription of a reporter gene. Expression of β-catenin, phospho-β-catenin Ser33/37/Thr41, phospho-β-catenin Thr41/Ser45, dephosphorylated 'active' β-catenin, LEF1, TCF4, and the Wnt target genes CD44V6 and EphB2 was analysed in formalin-fixed, paraffin embedded tumor tissues and correlated to clinicopathological factors and survival. Thirty-five % of tumors expressed nuclear β-catenin in more than 10% of tumor cells, which correlated positively with elevated cytoplasmic (p=0.04) and reduced membranous (p=0.001) β-catenin as well as with elevated nuclear LEF1 expression (p=0.03). Membranous EphB2 as well as cytoplasmic EphB2 expression significantly correlated with better tumor differentiation grade (p=0.05 and p=0.04) and with better overall survival (p=0.04 and p=0.04 respectively), which was not substantiated in multivariable analysis. Activated Wnt signaling might probably be of less importance in GEJ adenocarcinomas than previously thought. However, expression of the Wnt target gene EphB2 correlated to prognosis of GEJ adenocarcinoma patients.

INTRODUCTION

Adenocarcinomas of the gastro-esophageal junction (GEJ) show a rapidly increasing incidence in the western world since the last three decades [1,2]. Little is known about the molecular mechanisms underlying the origin of these tumors. As in colorectal carcinomas the wingless-type (Wnt) pathway is suggested to play a key role in GEJ adenocarcinomas as well. Nuclear β-catenin expression, generally believed to indicate activated Wnt signaling, is reported in up to 60% of GEJ adenocarcinomas [3-7].



Wnt signaling has importance in a number of developmental processes in vertebrates and invertebrates, i.e. regulation of cell fate specification, proliferation and differentiation in various tissues [8]. Additionally, Wnt signaling is able to contribute to tumorigenesis by altering the state and activity of β-catenin [9], a protein that was first described in humans as a member of the cell membrane-bound adherens complex [10]. Later β-Catenin was found to participate also in cell-signaling which involves translocation of the protein from the cytoplasm to the nucleus through activated Wnt signaling [11,12]. β-Catenin levels are regulated by a protein complex containing the tumor suppressor gene product adenomatous polyposis coli (APC), serine/threonine protein kinase glycogen synthase kinase-3β (GSK3β) and Axin/conductin and by casein kinase Iα [13-19]. Degradation of free cytosolic β-catenin is consecutively promoted via phosphorylation of NH_2-terminal β-catenin sequences at Ser45 (by casein kinase Iα) and subsequently at Thr41, Ser37 and Ser33 (by the GSK3β/APC/Axin complex) followed by ubiquination and proteasomal degradation [20]. Upon Wnt signaling the degradation complex is inhibited. Non-phosphorylated β-catenin accumulates in the cytoplasm and translocates to the nucleus where it complexes with the transcription regulator proteins T-Cell factor 4/Lymphoid Enhancer-binding Factor 1 (TCF4/LEF1) [14,21]. TCF4/LEF1 proteins cooperate with β-catenin to activate transcription of target genes including i.e. c-myc, cyclin D1, immunoglobulin transcription factor-2 (ITF-2), although many of the target genes remain unknown [22-24]. CD44V6 and ephrin receptors B2 and B3 (EphB2, EphB3) also function as potential TCF4/LEF1 target genes [25,26].

In carcinogenesis, β-catenin signaling, besides Wnt induced, can also be the result of increased β-catenin levels caused by impaired β-catenin degradation due to lack of phosphorylation of β-catenin. In several tumor types mutations at phosphorylation sites in β-catenin or inactivating mutations in APC or Axin leading to inhibition of β-catenin phosphorylation and decreased degradation have been described [27-30]. However, in GEJ adenocarcinomas very few inactivating mutations leading to β-catenin stabilisation have been identified in APC or Axin, neither β-catenin phosphorylation residue mutations were identified and therefore the mechanism of Wnt activation in these tumors remains unknown [4,31-33]. Interestingly, Staal and colleagues showed Wnt signals to be transduced via N-terminally dephosphorylated β-catenin using an antibody specific for β-catenin non-phosphorylated at residues Ser37 and Thr41 [34,35]. To obtain more insight in a possible role of Wnt activation in GEJ adenocarcinomas

we investigated by immunohistochemistry the expression of total-, phosphorylated- and non-phosphorylated β-catenin, the β-catenin signaling partners TCF4 and LEF1 and the Wnt target genes CD44V6 and EphB2 in a large series GEJ adenocarcinomas. The results were compared with clinicopathological data and survival.

MATERIALS AND METHODS

Patients and Tissue Samples

Routine formalin-fixed and paraffin-embedded tissues derived from 164 patients with an adenocarcinoma of the gastro-esophageal junction were retrieved from the files of the Department of Pathology, Erasmus MC, Rotterdam, The Netherlands. All patients (141 men; 23 women) had received transhiatal resection of the tumor with restoration of continuity of the gastrointestinal tract by a gastric tube with cervical anastomosis between 1987 and 2002. Patient's mean age at the time of diagnosis was 64.7 year (range 44-84 years). Adenocarinomas arising from distal esophagus or gastric cardia, i.e. the gastro-esophageal junction, were included. Tumors arising from the fundus or corpus of the stomach and infiltrating the gastric cardia or distal esophagus were excluded. Histological diagnosis and staging were established following the TNM proposed by the International Union Against Cancer according to standard criteria classification (TNM-stage I, n=15; TNM-stage II, n=41; TNM-stage III, n=86; TNM-stage IV, n=22). Every tumor was examined histologically for differentiation (well differentiated, n=9; moderately differentiated, n=73; poorly differentiated, n=82).

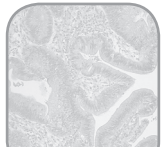
Antibodies and immunohistochemical staining

To detect the localization of β-catenin, phospho-β-catenin (Ser33/37/Thr41), phospho-β-catenin (Thr41/Ser45), LEF1, TCF4, CD44V6 and EphB2, antibodies were used as described in Table 3.1. To detect dephosphorylated β-catenin, a monoclonal antibody active β-catenin, which recognises β-catenin when Ser37 and Thr41 are both non-phosphorylated, was used, as described by Van Noort et al., from here referred to as 'active β-catenin' [35]. Four-μm thick paraffin sections were mounted on 3-aminopropyl-triethoxysilane (APES)-coated glass slides. Immunohistochemical staining was carried out by a standard avidin biotin immunoperoxidase technique, using a commercially available kit (Labvision, Fremont, USA). Deparaffinized sections were treated with methanol containing 3% H_2O_2 for 20 minutes. After washing with phosphate-buffered saline (PBS), blocking serum was applied for 5 minutes. Then, primary antibodies were allowed to react as described in Table 3.1. After washing in PBS, biotin-conjugated secondary antibody was applied for 10 minutes followed by peroxidase-marked streptavidin. After rinsing in PBS, peroxidase was visualized by diaminobenzidine hydrochloride (Fluka, Neu-Ulm, Germany) with 0.03% H_2O_2 solution for 10 minutes. The slides were then counterstained with Mayer's Haematoxylin and dehydrated in alcohol before mounting. Ex-

Table 3.1. Primary antibodies used

Antibody	Manufacturer	Working dilution, incubation time	Validation
β-catenin (m)	Transduction Laboratories, Lexington, KY, USA	1:200, 30 minutes RT	Ougolkov et al. [61], Koch et al. [43]
Phospho β-catenin (recognizes when phosphorylation at Ser33/37/Thr41, p)	Cell Signaling Technology, Beverly, MA, USA	1:150, overnight 4°C	Kielhorn et al. [38], Chung et al. [37]
Phospho β-catenin (recognizes when phosphorylation at Thr41/Ser45, p)	Cell Signaling Technology, Beverly, MA, USA	1:25, 90 minutes RT	Hagen et al. [20]
Active β-catenin (recognizes when dephosphorylation at Ser37 and Thr41, m)	Upstate, Waltham, MA, USA	1:100, overnight RT	Staal et al. [34]
LEF1 (m)	Exalpha Biologicals, Boston, MA, USA	1:400, overnight 4°C	Behrens et al. [62]
TCF4 (m)	Upstate Biotechnology, Lake Placid, NY, USA	1:2000, 30 minutes RT	Barker et al. [63]
CD44V6 (m)	Bender MedSystems, Vienna, Austria	1:1000, 60 minutes RT	Joo et al. [58]
Ephrin B2 (m)	ITK Diagnostics, Uithoorn, The Netherlands	1:400, overnight 4°C	Battle et al. [52]

m: monoclonal antibody, p: polyclonal antibody, RT: room temperature



pression of antibodies was evaluated by highpower microscopic examination (400X) of the entire tissue section. As negative control we used normal mouse immunoglobulins and normal rabbit serum instead of the antibodies as described in Table 3.1. Normal gastric mucosas from the same resection specimens were used as internal positive controls.

Cell lines

As controls, sections of formalin fixed and paraffin embedded cultured cells were stained with β-catenin antibodies. In earlier experiments we performed a TCF/β-catenin reporter gene assay with pTOPGLOW and pFOPGLOW constructs [36]. Esophageal adenocarcinoma cell lines JROECL19 and JROECL33 showed 350- and 18-fold increase in transcriptional activity of the pTOPGLOW reporter as compared to the negative control pFOPGLOW. Esophageal adenocarcinoma cell lines SKGT-4, TE-7 and OACP4C showed no enhanced transcription of the pTOPGLOW reporter [36]. Sections of formalin fixed and paraffin embedded cultured cells were stained with β-catenin, phospho-β-catenin (Ser33/37/Thr41), phospho-β-catenin (Thr41/Ser45) and 'active β-catenin' antibodies. Moreover APC-mutant colon adenocarcinoma cell line SW480 with a 385-fold increase in transcriptional activity was immunohistochemically investigated.

Quantitation of immunostaining

Nuclear, cytoplasmic and membranous staining of antibodies was determined separately for each specimen. Concerning cytoplasmic and membranous staining, sections were considered negative when immunoreactive cells were absent and positive when immunoreactive cells, irrespective of the amount, were present. Nuclear expression levels of β-catenin, phospho-β-catenin (Thr41/Ser45), phospho-β-catenin (Ser33/37/Thr41), active β-catenin, LEF1, TCF4 and EphB2 were initially graded into 5 subgroups: (a) no detectable expression (0, negative); (b) expression detected in less than 10% of tumor cells (1+); expression detected in 10-30% of tumor cells (2+); expression detected in 30-80% of tumor cells (3+); expression detected in >80% of tumor cells (4+). Eventually, interpretation of staining score was defined as positive when >10% of tumor cells stained, and as negative when none or <10% of tumor cells stained.

Statistics

Significance testing for discrete variables was performed with the χ^2-test or the Fisher's exact test when appropriate. Reported P values are two-sided. $P<0.05$ was considered statistically significant. Follow-up was carried out until June 2003 for all patients. Survival rates were calculated according to the Kaplan-Meier method and differences in survival were assessed using the log rank test. Multivariate analysis using the Cox proportional hazards model was carried out to assess the independent prognostic significance of variables on overall survival.

RESULTS

Immunohistochemistry

Cell line JROECL19 (350-fold increase of transcriptional activity) expressed nuclear β-catenin, nuclear phospho-β-catenin (Thr41/Ser45) and nuclear active β-catenin, as did the APC mutant colon cancer cell line SW480 which served as a positive control (Table 3.2). Cell line JROECL33 (18-fold increase of transcriptional activity) showed nuclear β-catenin expression (weak) and nuclear phospho-β-catenin (Thr41/Ser45) expression (Table 3.2). Cell line OACP4C (no enhanced transcriptional activity) showed absence of nuclear β-catenin, nuclear phospho-β-catenin (Ser33/37/Thr41) and nuclear active β-catenin expression (Table 3.2).

Table 3.2. ß-catenin expression in GEJ adenocarcinoma-derived cell lines JROECL19, JROECL33, OACP4, TE7, KGT4 and in APC mutant colorectal cancer cell line SW480.

	ß-catenin	Phospho ß-catenin Ser33/37/Thr41	Phospho ß-catenin Thr41/Ser45[a]	Active ß-catenin
SW480 (APC mutation, signaling on[b])	+ nucleus + cytoplasm	- nucleus	+ nucleus	++ nucleus + cytoplasm
JROECL19 (signaling on[b])	+ nucleus + cytoplasm + membrane	- nucleus	+ nucleus	+/- nucleus
JROECL33 (signaling on[b], although weak)	+/- nucleus +/- cytoplasm +/- membrane	- nucleus	+ nucleus	- nucleus
OACP4 (signaling off[b])	- nucleus	- nucleus	No data	- nucleus
TE7 (signaling off[b])	No data	No data	No data	- nucleus + membrane
SKGT4 (signaling off[b])	No data	No data	No data	- nucleus + membrane

[a] Strongly expressed in dividing cells (M-phase of cell cycle); [b] Shown by a TCF/ ß-catenin reporter gene assay performed with pTOPGLOW and p FOPGLOW constructs in a former study (Koppert et al. [36])

Typical immunostaining patterns for the antibodies used are shown in Figure 3.1. In general, with all antibodies used we saw remarkable intratumor heterogeneity in staining. We noticed a very strong staining pattern of phospho-β-catenin (Thr41/Ser45) in mitotic cells (Figure 3.1G, H).

Using scoring criteria for nuclear staining as described in Materials and Methods, expression data are reported in Table 3.3. Fifty-eight out of 164 tumors (35%) expressed nuclear β-catenin in >10% of tumor cells. Nuclear β-catenin correlated positively with elevated cytoplasmic (p=0.04) and reduced membranous (p=0.001) β-catenin. Moreover, nuclear β-catenin correlated positively with elevated nuclear LEF1 expression (p=0.03, data not shown).

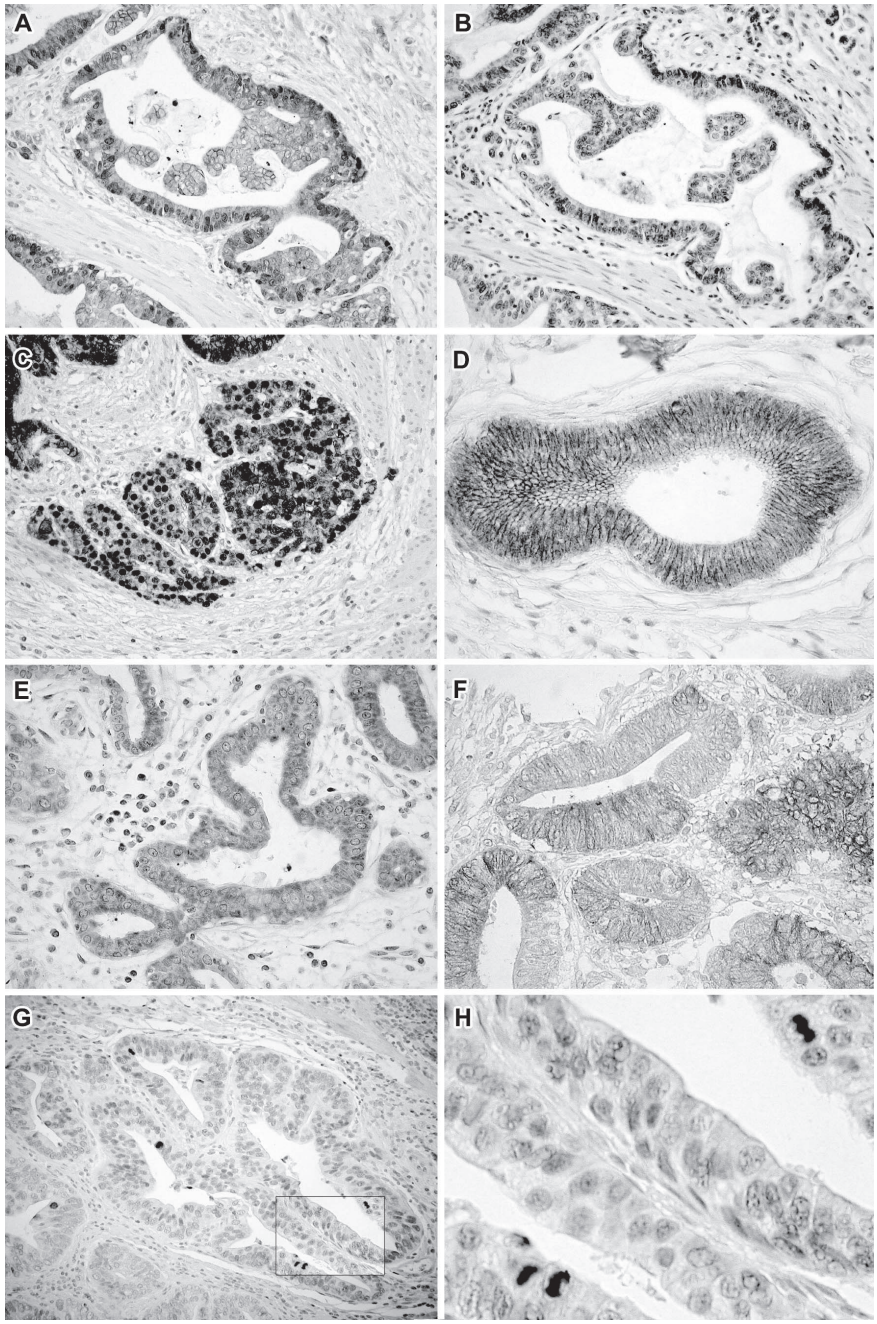
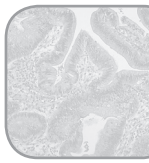


Figure 3.1. Immunohistochemistry of β-catenin in GEJ adenocarcinomas (Magnification x 200 (A., B., C., G.) and magnification x 400 (D., E., F., H.). A. Nuclear and cytoplasmic expression of β-catenin in the tumor cells. B. Nuclear active β-catenin staining in same GEJ tumor tissue as mentioned in A. C. Strong nuclear active β-catenin. D. Active β-catenin in membrane and cytoplasm of GEJ adenocarcinoma. E. EphB2 staining in cytoplasm of the tumor cells. F. Membranous EphB2 staining. G. Nuclear phospho β-catenin (Thr41/Ser45) with strong staining patterns in mitotic cells (insert H.). *Also see color figures page 291.*

Table 3.3. Results staining, correlation with TNM-stage and histology grade.

		Staining (%)	TNM-stage		P-value	Histology grade		P-value
			I, II	III, IV		good, moderate	poor	
β-catenin								
Nuclear	<10%	106 (65)	38	68		55	51	
	>10%	48 (35)	18	40	0.5	27	31	0.5
Cytoplasmic	-	102 (62)	36	66		56	46	
	+	62 (38)	20	42	0.7	26	36	0.1
Nuclear and/or cytoplasm	-	72 (44)	24	48		40	32	
	+	92 (56)	32	60	0.8	42	50	0.2
	-	31 (19)	8	23		15	16	
Membranous	+	133 (81)	48	85	0.3	67	66	0.8
Phβ-c 33/37/41								
Nuclear	<10%	132 (81)	47	85		67	65	
	>10%	31 (19)	9	22	0.5	15	16	0.8
Cytoplasmic	-	143 (88)	49	94		73	70	
	+	20 (12)	7	13	0.9	9	11	0.6
Phβ-c 41/45								
Nuclear	<10%	38 (25)	13	25		19	19	
	>10%	114 (75)	38	76	0.9	56	58	0.9
Cytoplasmic	-	50 (33)	16	34		24	26	
	+	102 (67)	35	67	0.8	51	51	0.8
Membranous	-	146 (96)	47	99		72	74	
	+	6 (4)	4	2	0.08	3	3	0.9
Active β-catenin								
Nuclear	<10%	137 (84)	49	88		69	68	
	>10%	26 (16)	7	19	0.4	12	14	0.7
Cytoplasmic	-	112 (69)	37	75		52	60	
	+	51 (31)	19	32	0.6	29	22	0.2
Nuclear and/or cytoplasm	-	100 (61)	34	66		46	54	
	+	64 (39)	22	42	0.9	36	28	0.2
	-	74 (45)	24	50		32	42	
Membranous	+	89 (55)	32	57	0.6	49	40	0.1
LEF1								
Nuclear	<10%	123 (77)	39	84		59	64	
	>10%	37 (23)	15	22	0.3	20	17	0.5
Cytoplasmic	-	95 (59)	23	72		41	54	
	+	65 (41)	31	34	0.002	38	27	0.06
TCF4								
Nuclear	<10%	54 (34)	16	38		30	24	
	>10%	105 (66)	39	66	0.3	49	56	0.3
Cytoplasmic	-	104 (65)	31	73		54	50	
	+	55 (35)	24	31	0.08	25	30	0.4
CD44V6								
Membranous	-	30 (18)	11	19		14	16	
	+	133 (82)	45	88	0.8	68	65	0.7
EphB2								
Cytoplasmic	-	56 (35)	14	42		22	34	
	+	103 (65)	40	63	0.08	58	45	0.04
Membranous	-	120 (75)	39	81		55	65	
	+	39 (25)	15	24	0.5	25	14	0.05



Correlations with clinicopathological parameters and multivariate analysis

Absence of cytoplasmic LEF1 expression significantly correlated with unfavorable TNM stage (p=0.002, Table 3.3). Cytoplasmic EphB2 as well as membranous EphB2 expression significantly correlated with better tumor differentiation grade (p=0.04 and p=0.05 respectively, Table 3.3).

Survival Analysis

In univariate analysis, significantly better survival was shown for patients with membranous EphB2 staining as compared to patients without membranous EphB2 staining (p=0.04, Table 3.4, Figure 3.2). A significant better survival was also shown for patients with cytoplasmic EphB2 staining as compared to patients without cytoplasmic EphB2 staining (p=0.04, Table 3.4). These significant outcomes were not substantiated in multivariable analysis, with adjustments for age, gender, TNM stage and differentiation grade (Table 3.4). When patients with EphB2 cytoplasmic and/or membranous expression were pooled, EphB2 expression reached a Hazard Ratio of 0.6 (95% Confidence Interval 0.4-1.0, data not shown). Only TNM stage turned out to be an independent predictor of overall survival in multivariable Cox's proportional hazards analysis (p<0.001, Table 3.4).

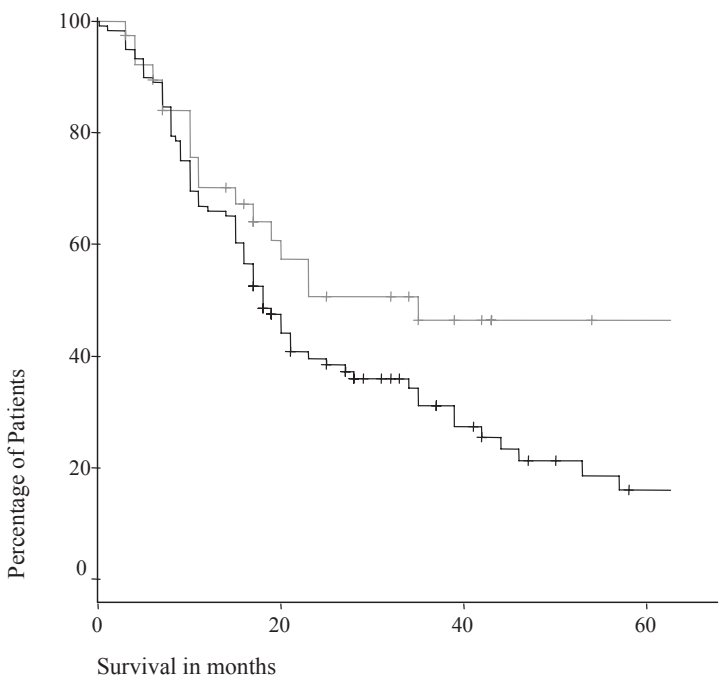


Figure 3.2. Overall survival of patients with membranous EphB2 staining (gray line n=39) versus patients with absence of membranous EphB2 staining (black line n=120), P logrank = 0.04. Five-year overall survival percentages were 46% and 16% respectively.

Table 3.4. Multivariable analysis of potential prognostic factors with overall survival in potentially curative resected GEJ adenocarcinomas. Multivariable analysis was performed using a Cox proportional hazards model.

Factor	Univariate 5-yr overall survival (%)	P-value	Hazard Ratio	95% Confidence Interval	P-value
Age[a]	23	-	1.01	1.0-1.04	0.3
Gender (M, F)					
M[b]	24		1	-	-
F	15	0.8	0.8	0.4-1.4	0.4
TNM-stage					
I, II[b]	43		1	-	-
III, IV	11	<0.0001	2.7	1.6-4.5	<0.001
Differentiation grade					
Well/Moderate[b]	31		1	-	-
Poor	13	0.06	1.4	0.9-2.1	0.1
Cytoplasmic EphB2					
Negative[b]	11		1	-	-
Positive	30	0.04	0.8	0.5-1.2	0.3
Membranous EphB2					
Negative[b]	16		1	-	-
Positive	46	0.04	0.7	0.4-1.2	0.2

[a] Age was included as a continous variable in the multivariable analysis. [b] Reference category.

DISCUSSION

Current models of Wnt signaling state that accumulation of nuclear β-catenin forms the key signaling event. In our series, 35% of GEJ adenocarcinomas (58/164) show nuclear β-catenin expression in >10% of tumor cells, being less than reported in literature [3-7]. This percentage increased to 56% when nuclear and/or cytoplasmic β-catenin expression was taken into account. Staal and collegues postulate that not the accumulation of β-catenin per se but the lack of phosphorylation at both Ser37 and Thr41 of this increased β-catenin forms the key signaling step [34]. They showed that Wnt signals are transduced via N-terminally dephosphorylated β-catenin using an antibody specific for β-catenin non-phosphorylated at residues Ser37 and Thr41 [34,35]. Only 16% of GEJ adenocarcinomas in our series express dephosphorylated β-catenin in >10% of tumor cells determined by the specific antibody for 'active' β-catenin which detects β-catenin when residues Ser37 and Thr41 are not phosphorylated both [35]. This percentage went up to 39 when nuclear and/or cytoplasmic expression was taken into account. According to the findings of Staal et al., this would imply that GEJ adenocarcinomas occasionally show activated Wnt signaling and that the conventional 'pan' β-catenin antibody does not represent the 'signal transducing' form of β-catenin.

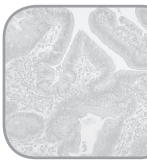
By using esophageal adenocarcinoma cell lines with available TCF reporter assay data, we aimed to affirm this assumption in an earlier study [36]. Nuclear β-catenin expression (as vi-

sualized by the conventional 'pan' β-catenin antibody) correlated with high TCF transcriptional activity (SW480, JROEC19, JROEC33). High TCF transcriptional activity in JROEC19 also correlated with nuclear 'active' β-catenin expression, though weak, as was the case in the control cell line SW480, known to have a homozygous inactivating APC mutation (Table 2). Also nuclear phospho-β-catenin (Thr41/Ser45) expression seemed to correlate with high TCF transcriptional activity in cell lines SW480, JROEC19, JROEC33. We hypothesize that casein kinase Iα still functions in these tumor cells, resulting in phosphorylation of β-catenin at position Ser45, but that these cells lack β-catenin phosphorylation by the GSK3β/APC/Axin complex, causing absence of phospho-β-catenin (Ser33/37/Thr41) expression. The immunohistochemical results in the colorectal cancer cell line SW480, with an inactive GSK3β/APC/Axin complex, support this assumption.

Notably, in 19 of 31 cases (61%) with nuclear phospho-β-catenin (Ser33/37/Thr41) none or less than 10% of tumor cells showed nuclear β-catenin, which could probably be due to antibody affinities. We could not establish an improved survival in patients with nuclear phospho-β-catenin (Ser33/37/Thr41), as reported in a large series of colorectal carcinoma patients [37]. Conversely, nuclear expression of phospho-β-catenin (Ser33/37/Thr41) in malignant melanomas has been described to correlate with poor outcome, lending evidence for differential behaviours in different tumors [38]. This could not be established in our series either.

To date only APC and β-catenin exon 3 mutation analysis in GEJ adenocarcinomas have been performed and very few mutations were found [4,7,31-33]. We investigated Axin1 mutations in all 10 exons in 17 GEJ adenocarcinomas with strong nuclear β-catenin expression and did not identify mutations [36]. Mutations in other components therefore should be considered, as well as activation of the canonical Wnt pathway or deregulated expression of pathway components responsible for Wnt pathway activation in these tumors [39,40]. A proper candidate for a mutation is β-transducing repeat-containing protein (β-TrCP), an F-box protein that is involved in β-catenin degradation. β-TrCP mutations are however rarely found in human tumors which are wild type for β-catenin and APC. β-TrCP mutations only have been described in two prostate cancer samples, of which one had nuclear β-catenin expression [41]. Despite the role of β-TrCP in β-catenin turnover, it is unlikely that β-TrCP might function as a tumor suppressor [42]. Within hepatoblastomas, increased expression of Wnt-inducible genes such as Axin2, Dkk-1, NKD-1 and β-TrCP was seen to be a common event [43]. These so called Wnt antagonists act as inhibitors of the Wnt pathway and their overexpression indicates Wnt pathway activation in hepatoblastomas, most likely because genetic alterations disrupt the multiprotein complex that controls β-catenin stability [43]. Epigenetic silencing of the phosphorylation complex might be another possibility for Wnt pathway activation. Methylation of APC has been described by Clément and colleagues during the neoplastic progression of Barrett's esophagus [44]. Additionally, it is possible that the Alzheimer's disease linked gene presenilin 1 (PS1) is deficient in these tumors. PS1 has been described as a negative regulator of the Wnt pathway [45]. Presinilin deficiency might reduce the pool of phospho-β-catenin

(Ser33/37/Thr41) available for ubiquitination by failing to target phospho-β-catenin (Ser45) for further phosphorylation and degradation [46]. Interestingly, Kang et al. also report extensive nuclear localization of phospho-β-catenin (Ser45) in tumors with either PS1 deficiency or activating β-catenin mutations and propose that Ser45-phosphorylated β-catenin might be signaling competent [46]. To our opinion rather the β-catenin mutation or PS1 deficiency than the presence of the phosphorylated protein seems responsible for Wnt-pathway activation in these tumors since phosphorylation means ubiquination and therefore degradation of β-catenin. In skin tumor tissues the absence of PS1 was associated with elevated nuclear β-catenin and upregulated β-catenin/LEF-dependent signaling [47].



We noticed a very strong staining pattern of phospho-β-catenin (Thr41/Ser45) in mitotic cells (Figure 3.1G, H). Interestingly, a third function of β-catenin, besides participation in cell-cell adhesion and Wnt-stimulated transcriptional activation, was recently described by Kaplan et al. [48]. They show evidence that β-catenin regulates the process of mitotic spindle formation by ensuring that components of the mitotic machinery are assembled in the correct place and at correct time for cell division to proceed efficiently [48].

The EphB2 receptor has a role in cell positioning in the intestine and has been described as a target gene of the β-catenin/TCF4 complex [26,49]. Moreover EphB2 is overexpressed in several human carcinomas, including tumors of the gastrointestinal tract, suggesting that this gene may indeed be involved in carcinogenesis [50,51]. Loss of EphB2 expression was recently described to be a critical step in colorectal cancer progression, including the onset of tumor invasion, dedifferentiation and metastasis [52,53]. Moreover EphB2 was identified as a prognostic factor in colorectal cancer with shorter mean duration of survival when EphB2 expression was lost [54]. Also in carcinomas of the small intestine and stomach EphB2 inactivation/downregulation may be relevant for tumor progression [55]. Loss of EphB2 membranous expression was also associated with poorer survival as well as with poorer tumor differentiation grade in our series. Since it is generally believed that localization on the cell membrane is necessary for the function of the ephrin receptor/ephrin system, cytoplasmic presence or absence of EphB2 is less likely to confer significance in tumor progression [51,56]. However, in our series cytoplasmic EphB2 staining was observed frequently, mostly apart from membranous EphB2 staining. And loss of cytoplasmic EphB2 staining was significantly correlated with poorer tumor differentiation grade as well as survival. Since membranous EphB2 is also expressed in the normal intestine, the observed better survival and tumor differentiation grade in patients with membranous expression could be the mere result of a proper functioning receptor instead of an activated Wnt pathway [52].

Cell surface glycoprotein CD44 expression is part of a genetic program controlled by the β-catenin/TCF4 signaling pathway and suggest a role for CD44 in the generation and turnover of epithelial cells [25]. CD44V6, a variant isoform of CD44, is considered to be implicated in carcinogenesis [57-59]. In Barrett's related adenocarcinoma CD44V6 expression is described to be correlated with aggressive pathological features [60]. In our series, 133 out of 163 tumors

had membranous CD44V6 expression, which was not correlated to either TNM-stage, tumor differentiation grade or patient survival. Membranous CD44V6 expression did not correlate with (any form, either phosphorylated or dephosphorylated) nuclear β-catenin expression. We therefore are not able to judge about a possible Wnt target gene function of CD44V6 in GEJ adenocarcinomas.

In summary, Wnt pathway activation, as classically represented by nuclear β-catenin expression, was present in 35% of our large series of GEJ adenocarcinomas. Moreover, since Wnt pathway activation might be caused exclusively by β-catenin which is dephosphorylated at Ser37 and Thr41, this pathway might be even far less important in GEJ adenocarcinomas than previously thought. However, immunohistochemical analysis of diverse contributors and targets of the Wnt pathway in a large series of GEJ adenocarcinomas showed loss of expression of the Wnt target gene EphB2 to be significantly correlated with worse survival and poor tumor differentiation grade.

ACKNOWLEDGMENTS

We would like to thank Paola Alberici for critical reading of the manuscript. We are grateful to Conny Vollebregt-Uiterwijk for kindly and dedicated assistance in collecting the data. We wish to thank Anke ter Brugge, Petra Lodder and Esther Hasper for technical assistance and Frank van der Panne for photographic work.

REFERENCES

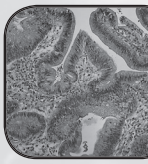
1. Wijnhoven BP, Louwman MW, Tilanus HW, Coebergh JW: Increased incidence of adenocarcinomas at the gastro-oesophageal junction in Dutch males since the 1990s. Eur J Gastroenterol Hepatol 14:115-22, 2002
2. Devesa SS, Blot WJ, Fraumeni JF, Jr.: Changing patterns in the incidence of esophageal and gastric carcinoma in the United States. Cancer 83:2049-53, 1998
3. Bailey T, Biddlestone L, Shepherd N, Barr H, Warner P, Jankowski J: Altered cadherin and catenin complexes in the Barrett's esophagus-dysplasia-adenocarcinoma sequence: correlation with disease progression and dedifferentiation. Am J Pathol 152:135-44, 1998
4. Bian YS, Osterheld MC, Bosman FT, Fontolliet C, Benhattar J: Nuclear accumulation of beta-catenin is a common and early event during neoplastic progression of Barrett esophagus. Am J Clin Pathol 114:583-90, 2000
5. Osterheld MC, Bian YS, Bosman FT, Benhattar J, Fontolliet C: Beta-catenin expression and its association with prognostic factors in adenocarcinoma developed in Barrett esophagus. Am J Clin Pathol 117:451-6, 2002
6. Washington K, Chiappori A, Hamilton K, Shyr Y, Blanke C, Johnson D, Sawyers J, Beauchamp D: Expression of beta-catenin, alpha-catenin, and E-cadherin in Barrett's esophagus and esophageal adenocarcinomas. Mod Pathol 11:805-13, 1998
7. Wijnhoven BP, Nollet F, De Both NJ, Tilanus HW, Dinjens WN: Genetic alterations involving exon 3 of the beta-catenin gene do not play a role in adenocarcinomas of the esophagus. Int J Cancer 86:533-7, 2000
8. Funayama N, Fagotto F, McCrea P, Gumbiner BM: Embryonic axis induction by the armadillo repeat domain of beta-catenin: evidence for intracellular signaling. J Cell Biol 128:959-68, 1995
9. Miller JR, Moon RT: Signal transduction through beta-catenin and specification of cell fate during embryogenesis. Genes Dev 10:2527-39, 1996
10. Kemler R, Ozawa M: Uvomorulin-catenin complex: cytoplasmic anchorage of a Ca2+-dependent cell adhesion molecule. Bioessays 11:88-91, 1989
11. Morin PJ: beta-catenin signaling and cancer. Bioessays 21:1021-30, 1999
12. Kikuchi A: Regulation of beta-catenin signaling in the Wnt pathway. Biochem Biophys Res Commun 268:243-8, 2000
13. Liu C, Li Y, Semenov M, Han C, Baeg GH, Tan Y, Zhang Z, Lin X, He X: Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell 108:837-47, 2002
14. Peifer M, Polakis P: Wnt signaling in oncogenesis and embryogenesis--a look outside the nucleus. Science 287:1606-9, 2000
15. Polakis P: Wnt signaling and cancer. Genes Dev 14:1837-51, 2000
16. Kishida S, Yamamoto H, Ikeda S, Kishida M, Sakamoto I, Koyama S, Kikuchi A: Axin, a negative regulator of the wnt signaling pathway, directly interacts with adenomatous polyposis coli and regulates the stabilization of beta-catenin. J Biol Chem 273:10823-6, 1998
17. Hart MJ, de los Santos R, Albert IN, Rubinfeld B, Polakis P: Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. Curr Biol 8:573-81, 1998
18. Sakanaka C, Weiss JB, Williams LT: Bridging of beta-catenin and glycogen synthase kinase-3beta by axin and inhibition of beta-catenin-mediated transcription. Proc Natl Acad Sci U S A 95:3020-3, 1998
19. Behrens J, Jerchow BA, Wurtele M, Grimm J, Asbrand C, Wirtz R, Kuhl M, Wedlich D, Birchmeier W: Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. Science 280:596-9, 1998
20. Hagen T, Vidal-Puig A: Characterisation of the phosphorylation of beta-catenin at the GSK-3 priming site Ser45. Biochem Biophys Res Commun 294:324-8, 2002
21. Bienz M, Clevers H: Linking colorectal cancer to Wnt signaling. Cell 103:311-20, 2000
22. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW: Identification of c-MYC as a target of the APC pathway. Science 281:1509-12, 1998
23. Tetsu O, McCormick F: Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 398:422-6, 1999

24. Kolligs FT, Nieman MT, Winer I, Hu G, Van Mater D, Feng Y, Smith IM, Wu R, Zhai Y, Cho KR, Fearon ER: ITF-2, a downstream target of the Wnt/TCF pathway, is activated in human cancers with beta-catenin defects and promotes neoplastic transformation. Cancer Cell 1:145-55, 2002
25. Wielenga VJ, Smits R, Korinek V, Smit L, Kielman M, Fodde R, Clevers H, Pals ST: Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT pathway. Am J Pathol 154:515-23, 1999
26. Batlle E, Henderson JT, Beghtel H, van den Born MM, Sancho E, Huls G, Meeldijk J, Robertson J, van de Wetering M, Pawson T, Clevers H: Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. Cell 111:251-63, 2002
27. Kobayashi M, Honma T, Matsuda Y, Suzuki Y, Narisawa R, Ajioka Y, Asakura H: Nuclear translocation of beta-catenin in colorectal cancer. Br J Cancer 82:1689-93, 2000
28. Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW: Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science 275:1787-90, 1997
29. Sparks AB, Morin PJ, Vogelstein B, Kinzler KW: Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. Cancer Res 58:1130-4, 1998
30. Webster MT, Rozycka M, Sara E, Davis E, Smalley M, Young N, Dale TC, Wooster R: Sequence variants of the axin gene in breast, colon, and other cancers: an analysis of mutations that interfere with GSK3 binding. Genes Chromosomes Cancer 28:443-53, 2000
31. Choi YW, Heath EI, Heitmiller R, Forastiere AA, Wu TT: Mutations in beta-catenin and APC genes are uncommon in esophageal and esophagogastric junction adenocarcinomas. Mod Pathol 13:1055-9, 2000
32. Gonzalez MV, Artimez ML, Rodrigo L, Lopez-Larrea C, Menendez MJ, Alvarez V, Perez R, Fresno MF, Perez MJ, Sampedro A, Coto E: Mutation analysis of the p53, APC, and p16 genes in the Barrett's oesophagus, dysplasia, and adenocarcinoma. J Clin Pathol 50:212-7, 1997
33. Powell SM, Papadopoulos N, Kinzler KW, Smolinski KN, Meltzer SJ: APC gene mutations in the mutation cluster region are rare in esophageal cancers. Gastroenterology 107:1759-63, 1994
34. Staal FJ, Noort Mv M, Strous GJ, Clevers HC: Wnt signals are transmitted through N-terminally dephosphorylated beta-catenin. EMBO Rep 3:63-8, 2002
35. van Noort M, Meeldijk J, van der Zee R, Destree O, Clevers H: Wnt signaling controls the phosphorylation status of beta-catenin. J Biol Chem 277:17901-5, 2002
36. Koppert LB, van der Velden AW, van de Wetering M, Abbou M, van den Ouweland AM, Tilanus HW, Wijnhoven BP, Dinjens WN: Frequent loss of the AXIN1 locus but absence of AXIN1 gene mutations in adenocarcinomas of the gastro-oesophageal junction with nuclear beta-catenin expression. Br J Cancer 90:892-9, 2004
37. Chung GG, Provost E, Kielhorn EP, Charette LA, Smith BL, Rimm DL: Tissue microarray analysis of beta-catenin in colorectal cancer shows nuclear phospho-beta-catenin is associated with a better prognosis. Clin Cancer Res 7:4013-20, 2001
38. Kielhorn E, Provost E, Olsen D, D'Aquila TG, Smith BL, Camp RL, Rimm DL: Tissue microarray-based analysis shows phospho-beta-catenin expression in malignant melanoma is associated with poor outcome. Int J Cancer 103:652-6, 2003
39. Li Y, Welm B, Podsypanina K, Huang S, Chamorro M, Zhang X, Rowlands T, Egeblad M, Cowin P, Werb Z, Tan LK, Rosen JM, Varmus HE: Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. Proc Natl Acad Sci U S A 100:15853-8, 2003
40. Uematsu K, He B, You L, Xu Z, McCormick F, Jablons DM: Activation of the Wnt pathway in non small cell lung cancer: evidence of dishevelled overexpression. Oncogene 22:7218-21, 2003
41. Gerstein AV, Almeida TA, Zhao G, Chess E, Shih Ie M, Buhler K, Pienta K, Rubin MA, Vessella R, Papadopoulos N: APC/CTNNB1 (beta-catenin) pathway alterations in human prostate cancers. Genes Chromosomes Cancer 34:9-16, 2002
42. Fuchs SY, Spiegelman VS, Kumar KG: The many faces of beta-TrCP E3 ubiquitin ligases: reflections in the magic mirror of cancer. Oncogene 23:2028-36, 2004
43. Koch A, Waha A, Hartmann W, Hrychyk A, Schuller U, Waha A, Wharton KA, Jr., Fuchs SY, von Schweinitz D, Pietsch T: Elevated expression of Wnt antagonists is a common event in hepatoblastomas. Clin Cancer Res 11:4295-304, 2005

44. Clement G, Braunschweig R, Pasquier N, Bosman FT, Benhattar J: Alterations of the Wnt signaling pathway during the neoplastic progression of Barrett's esophagus. Oncogene, 2006
45. Soriano S, Kang DE, Fu M, Pestell R, Chevallier N, Zheng H, Koo EH: Presenilin 1 negatively regulates beta-catenin/T cell factor/lymphoid enhancer factor-1 signaling independently of beta-amyloid precursor protein and notch processing. J Cell Biol 152:785-94, 2001
46. Kang DE, Soriano S, Xia X, Eberhart CG, De Strooper B, Zheng H, Koo EH: Presenilin couples the paired phosphorylation of beta-catenin independent of axin: implications for beta-catenin activation in tumorigenesis. Cell 110:751-62, 2002
47. Xia X, Qian S, Soriano S, Wu Y, Fletcher AM, Wang XJ, Koo EH, Wu X, Zheng H: Loss of presenilin 1 is associated with enhanced beta-catenin signaling and skin tumorigenesis. Proc Natl Acad Sci U S A 98:10863-8, 2001
48. Kaplan DD, Meigs TE, Kelly P, Casey PJ: Identification of a role for beta-catenin in the establishment of a bipolar mitotic spindle. J Biol Chem 279:10829-32, 2004
49. van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Batlle E, Coudreuse D, Haramis AP, Tjon-Pon-Fong M, Moerer P, van den Born M, Soete G, Pals S, Eilers M, Medema R, Clevers H: The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 111:241-50, 2002
50. Kiyokawa E, Takai S, Tanaka M, Iwase T, Suzuki M, Xiang YY, Naito Y, Yamada K, Sugimura H, Kino I: Overexpression of ERK, an EPH family receptor protein tyrosine kinase, in various human tumors. Cancer Res 54:3645-50, 1994
51. Liu W, Ahmad SA, Jung YD, Reinmuth N, Fan F, Bucana CD, Ellis LM: Coexpression of ephrin-Bs and their receptors in colon carcinoma. Cancer 94:934-9, 2002
52. Batlle E, Bacani J, Begthel H, Jonkheer S, Gregorieff A, van de Born M, Malats N, Sancho E, Boon E, Pawson T, Gallinger S, Pals S, Clevers H: EphB receptor activity suppresses colorectal cancer progression. Nature 435:1126-30, 2005
53. Guo DL, Zhang J, Yuen ST, Tsui WY, Chan AS, Ho C, Ji J, Leung SY, Chen X: Reduced expression of EphB2 that parallels invasion and metastasis in colorectal tumors. Carcinogenesis, 2005
54. Jubb AM, Zhong F, Bheddah S, Grabsch HI, Frantz GD, Mueller W, Kavi V, Quirke P, Polakis P, Koeppen H: EphB2 is a prognostic factor in colorectal cancer. Clin Cancer Res 11:5181-7, 2005
55. Lugli A, Spichtin H, Maurer R, Mirlacher M, Kiefer J, Huusko P, Azorsa D, Terracciano L, Sauter G, Kallioniemi OP, Mousses S, Tornillo L: EphB2 expression across 138 human tumor types in a tissue microarray: high levels of expression in gastrointestinal cancers. Clin Cancer Res 11:6450-8, 2005
56. Liu W, Jung YD, Ahmad SA, McCarty MF, Stoeltzing O, Reinmuth N, Fan F, Ellis LM: Effects of overexpression of ephrin-B2 on tumor growth in human colorectal cancer. Br J Cancer 90:1620-6, 2004
57. Chen XY, Wang ZC, Li H, Cheng XX, Sun Y, Wang XW, Wu ML, Liu J: Nuclear translocations of beta-catenin and TCF4 in gastric cancers correlate with lymph node metastasis but probably not with CD44 expression. Hum Pathol 36:1294-301, 2005
58. Joo M, Lee HK, Kang YK: Expression of E-cadherin, beta-catenin, CD44s and CD44v6 in gastric adenocarcinoma: relationship with lymph node metastasis. Anticancer Res 23:1581-8, 2003
59. Masaki T, Goto A, Sugiyama M, Matsuoka H, Abe N, Sakamoto A, Atomi Y: Possible contribution of CD44 variant 6 and nuclear beta-catenin expression to the formation of budding tumor cells in patients with T1 colorectal carcinoma. Cancer 92:2539-46, 2001
60. Lagorce-Pages C, Paraf F, Dubois S, Belghiti J, Flejou JF: Expression of CD44 in premalignant and malignant Barrett's oesophagus. Histopathology 32:7-14, 1998
61. Ougolkov A, Zhang B, Yamashita K, Bilim V, Mai M, Fuchs SY, Minamoto T: Associations among beta-TrCP, an E3 ubiquitin ligase receptor, beta-catenin, and NF-kappaB in colorectal cancer. J Natl Cancer Inst 96:1161-70, 2004
62. Behrens J, von Kries JP, Kuhl M, Bruhn L, Wedlich D, Grosschedl R, Birchmeier W: Functional interaction of beta-catenin with the transcription factor LEF-1. Nature 382:638-42, 1996
63. Barker N, Huls G, Korinek V, Clevers H: Restricted high level expression of Tcf-4 protein in intestinal and mammary gland epithelium. Am J Pathol 154:29-35, 1999

Chapter 4

Frequent loss of the AXIN1 locus but absence of AXIN1 gene mutations in adenocarcinomas of the gastro-esophageal junction with nuclear beta-catenin expression



British Journal of Cancer, 2004 Feb 23;90(4):892-9

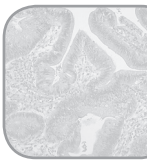
Linetta B Koppert
Albertina W van der Velden
Marc van de Wetering
Mustaffa Abbou
AMW van den Ouweland
Hugo W Tilanus
Bas PL Wijnhoven
Winand NM Dinjens

ABSTRACT

Up to 60% of gastro-esophageal junction (GEJ) adenocarcinomas show nuclear β-catenin expression, pointing to activated T-cell factor (TCF)/β-catenin driven gene transcription. We demonstrate in 5 human GEJ adenocarcinoma cell lines that nuclear β-catenin expression indeed correlates with enhanced TCF-mediated transcription of a reporter gene. In several tumor types, TCF/β-catenin activation is caused by mutations in either adenomatous polyposis coli (APC), β-catenin exon 3, AXIN1, AXIN2 or β-transducin repeat-containing protein (β-TrCP). In GEJ adenocarcinomas very few APC and β-catenin mutations have been found. Therefore the mechanism of Wnt pathway activation remains unclear. In the present study we did not find AXIN1 gene mutations in 17 GEJ tumors with nuclear β-catenin expression (without β-catenin exon 3 mutations). Six intragenic single nucleotide polymorphisms (SNPs) were identified. One of these, the AXIN1 gene T1942C SNP has a frequency of 21% but is only very recently described despite numerous AXIN1 gene mutational studies. We provide evidence why this SNP was missed in single strand conformation polymorphism (SSCP) analyses. The AXIN1 gene G2063A variation was previously described as a gene mutation but we demonstrate that this is a polymorphism. With these 6 SNPs loss of heterozygosity (LOH) was found in 11 of 15 (73%) informative tumors. To investigate a possible AXIN1 gene dosage effect in GEJ tumors expressing nuclear β-catenin, AXIN1 locus LOH was determined in 20 tumors expressing membranous and no nuclear β-catenin. LOH was found in 10 of 13 (77%) informative cases. AXIN1 protein immunohistochemistry revealed cytoplasmic expression in all tumors irrespective of the presence of AXIN1 locus LOH. These data indicate that nuclear β-catenin expression is indicative for activated Wnt signaling and that neither AXIN1 gene mutations nor AXIN1 locus LOH are involved in Wnt pathway activation in GEJ adenocarcinomas.

INTRODUCTION

The incidence of adenocarcinoma of the gastro-esophageal junction (GEJ), i.e. distal esophagus and gastric cardia, is rising in the Western world [1-3]. Patients with GEJ adenocarcinoma have a poor prognosis with 5-year survival rates of less than 25% [4]. Despite the common occurrence of this malignancy, relatively little is known about the molecular mechanisms underlying the genesis and the progression of these tumors.



Numerous studies focussed on cell-cell adhesion in GEJ adenocarcinomas since defective cell-cell adhesion is an important feature in epithelial tumor initiation and progression [5-10]. Aberrant expression of components of the E-cadherin-catenin complex, the prime mediator of epithelial cell-cell adhesion, has been found frequently in GEJ adenocarcinomas. In addition, E-cadherin-catenin complex aberrations appeared to have prognostic value in these tumors [11-16].

Recently, the E-cadherin-catenin complex component β-catenin has been demonstrated to play a dual role in the tumorigenic process [17-22]. β-Catenin, originally described to function in epithelial cell-cell adhesion, is implicated also in the Wnt signaling cascade as a transcriptional activator. In the absence of Wnt signals β-catenin is located at the plasma membrane, linked to E-cadherin, and functions in cell-cell adhesion. Excess cytoplasmic β-catenin is sequestered in a protein complex comprised of adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK-3β) and AXIN1 or AXIN2. In this complex β-catenin is phosphorylated by GSK-3β and then targeted by β-transducin repeat-containing protein (β-TrCP) to proteasomal degradation. Activated Wnt signaling inhibits the phosphorylation of β-catenin, thereby preventing its degradation. The impaired β-catenin degradation leads to an increase in cytoplasmic β-catenin and its translocation to the nucleus. Nuclear β-catenin forms heterodimers with members of the T-cell factor (TCF) family of transcription factors and activates genes containing TCF-binding sites [19,20].

It has been demonstrated that β-catenin/TCF activated gene transcription can induce neoplastic transformation [23-25] and the β-catenin/TCF target genes comprise the oncogenes c-myc [26], cyclin D1 [27] and ITF-2 [23]. Impaired degradation of β-catenin in tumors has been reported to be caused by inactivating mutations in APC, AXIN1, AXIN2 or β-TrCP or oncogenic mutations in β-catenin exon 3 [5,19,20,28-33]. These mutations are in most cases mutually exclusive [28,29,31,34-36] .

Several studies have reported nuclear β-catenin expression in up to 60% of GEJ adenocarcinomas [11,12,15,16,37]. This nuclear expression of β-catenin can be regarded as an indication for activated, oncogenic, β-catenin/TCF transcription. However, mutation analysis of APC and β-catenin in GEJ adenocarcinomas revealed mutations in only less than 7 and 3% of cases respectively [12,38-40]. In accordance with these data, we recently did not find β-catenin exon 3 mutations in a series of 69 GEJ adenocarcinomas [37]. Inactivation of the AXIN1 gene has been demonstrated to induce β-catenin/TCF transcription and AXIN1 gene mutations have been described in hepatocellular carcinomas, hepatoblastomas, colorectal cancers, ovarian endo-

metrioid adenocarcinomas and in sporadic medulloblastomas [29,31,33,41-47]. In addition, reduced protein expression of AXIN1 has recently been reported to correlate with tumor progression in esophageal squamous cell carcinoma [48]. These results prompted us to search for genomic aberrations in the AXIN1 gene in GEJ adenocarcinomas. From the previously investigated series of 69 GEJ adenocarcinomas, 17 tumors with prominent nuclear β-catenin expression were selected for mutation and loss of heterozygosity (LOH) analysis. The entire coding region including the exon-intron boundaries of the AXIN1 gene was analysed for genetic alterations by single strand conformation polymorphism (SSCP) analysis. The presence of 6 intragenic single nucleotide polymorphisms (SNPs) was used to detect LOH. These 6 SNPs were also used to perform AXIN1 gene LOH analysis in 20 tumors with strong membranous β-catenin expression. In addition, AXIN1 protein expression was investigated by immunohistochemistry in all 37 tumor samples.

MATERIALS AND METHODS

TCF/β-catenin reporter gene assay

All cell lines, JROECL19, JROEL33, SKGT-4, TE-7, OACP4C and SW480 were cultured in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. Transcriptional activation mediated by TCF/β-catenin protein complexes was determined by transient transfection of the cell lines with either the pTOPGLOW or pFOPGLOW reporter constructs as described previously [49]. The pTOPGLOW and pFOPGLOW constructs contain a multimerized wildtype or mutant TCF binding motive, respectively, upstream of a luciferase gene. Cells were grown to 50-80% confluency in six-well plates and transfection was performed with 1μg of purified constructs each, using Fugene-6 (Boehringer, Mannheim, Germany). Transfection efficiencies were determined by cotransfection of a pRL-TK reporter construct (Promega, Madison, WI, USA) that contained the Renilla luciferase gene under control of the herpes simplex virus thymidine kinase promotor. Cells were harvested 24 h after transfection. Activity of both luciferases was measured sequentially in each sample using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). TCF-mediated gene transcription was defined by the ratio of pTOPGLOW to pFOPGLOW luciferase activities. The luciferase activity of the internal control reporter was used to correct for differences in transfection efficiency.

Tumor and cell line DNA samples

In a previous study 69 GEJ adenocarcinoma samples, consisting of 54 primary tumors, 4 lymph node metastases, 9 xenografts and 2 in vitro cell lines were investigated for β-catenin expression and β-catenin exon 3 mutations [37]. From all cases tumor and normal DNA was isolated from frozen samples by standard proteinase K digestion and phenol/chloroform extraction. After β-catenin immunohistochemistry on 5μm paraffin sections parts of the tumor

with intense nuclear reactivity were isolated by microdissection from consecutive unstained sections. From the microdissected fragments DNA was isolated with standard proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation. No β-catenin exon 3 aberrations were found in these samples. For the present study the DNA from 17 tumors with strong nuclear β-catenin expression was used. Fifteen samples were originated from primary GEJ adenocarcinomas and 2 were from GEJ adenocarcinoma derived cell lines JROECL19 and JROECL33 [50]. In addition, the DNA's from 20 tumors with strong membranous β-catenin expression were investigated for LOH with the 6 SNPs. These 20 samples comprised 17 primary tumors and the established cell lines OACP4C, SKGT-4 and TE-7 [51-53]. Cell lines JROECL19 and 33 were obtained from the European Collection of Cell Cultures (ECACC, Wiltshire, United Kingdom), SKGT-4 and TE-7 were kind gifts from D. Schrump, NIH, Bethesda, USA and T. Kudo, Tohoku University, Sendai, Japan, respectively, OACP4C was established at our own institute. From cell lines JROECL19 and 33 patient's normal tissue was kindly provided by Dr. S.J. Darnton, Birmingham Heartlands Hospital, Birmingham, UK. From cell line OACP4C patient's normal tissue was obtained from our pathology archive and from cell lines SKGT-4 and TE-7 patient's normal tissue was not available. To determine SNP frequencies in the normal population we used DNA isolated from 161 healthy Caucasian blood donor volunteers.

Mutation analysis of the AXIN1 gene

The 17 pairs of tumor and normal DNA from tumors with nuclear β-catenin expression were screened for aberrations in the AXIN1 gene. The entire coding sequence, including the exon-intron boundaries was investigated by PCR-SSCP using the previously described 23 sets of primers with slight modifications [54]. All amplifications were performed in 15μL PCR containing 50-100ng DNA, 1.5mM $MgCl_2$, 0.02mM dATP, 0.2 mM dGTP, dCTP and dTTP each, 0.8μCi of [^{32}P]dATP (Amersham Biosciences, Buckinghamshire, UK), 20pmol of each primer and 0.3U AmpliTaq Gold polymerase (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). AmpliTaq Gold polymerase was used because this enzyme is superior to other DNA polymerases with regard to amplification of DNA retrieved from routine formalin fixed and paraffin embedded tissues. To the PCRs with primer sets 4, 10, 12, 13, 14, 15, 18, 21 and 23 DMSO (5%) was added to increase the amplification efficiency. The PCRs were performed for 35 cycles of 95^{0}C for 30 sec, 55^{0}C (primer sets 10 and 14 at 58^{0}C and 60^{0}C, respectively) for 45 sec and 72^{0}C for 1 min. The PCR products were diluted 1:4 with loading buffer (95% formamide, 10mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol), heated for 5 min, cooled on ice and electrophoresed in 6% polyacrylamide gels containing 10% glycerol at 7W overnight at room temperature, in 1x TBE running buffer. Gels were dried and exposed to X-ray films at –80^{0}C. Results were evaluated by visual inspection. With the used SSCP conditions the PCR products from primer sets 18, 19 and 21 resulted in simple banding patterns, inefficient for the detection of aberrations. To increase the DNA aberration detection efficiency these PCR products were electrophoresed without glycerol at 4^{0}C for 6 hours resulting in more complex

banding patterns. For each variant SSCP pattern identified by SSCP analysis, the genomic DNA samples were reamplified for bidirectional direct sequencing with the amplification primers. In 20 DNA samples from tumors with strong membranous β-catenin expression LOH was determined by SSCP analysis of 6 detected SNPs. These SNPs are at positions (according to GenBank accession no. AF009674) A94C, C874T, intron 4+17 G→A (nucleotide position identified from exon-intron boundary), G1396A, T1942C and G2063A. Amplification of these SNPs was performed with primer sets 1, 7, 11, 12 and 17, respectively.

β-catenin and AXIN1 immunohistochemistry

In all 37 tumor samples immunostaining for β-catenin and AXIN1 was performed on 5μm paraffin sections with a mouse anti-human β-catenin monoclonal antibody (Transduction Laboratories, Lexington, KY, USA; 1:200, 30 min, room temperature) [37] and a rabbit polyclonal anti-human AXIN1 antibody (Zymed Laboratories, San Francisco, CA, USA; 1:25, 30 min, room temperature), respectively. After deparaffinisation and treatment with methanol/H_2O_2, antigen retrieval was performed in citrate buffer for 15 min prior to incubation with the β-catenin antibody. No antigen retrieval was necessary for the AXIN1 immunohistochemistry. Immunoreactivity was made visible by a standard avidin biotin immunoperoxidase technique, using a commercially available kit (Labvision, Fremont, CA, USA) and diaminobenzidine hydrochloride (Fluka, Neu-Ulm, Germany). As negative controls normal mouse immunoglobulins and normal rabbit serum were used.

RESULTS

TCF/β-catenin reporter gene assay

The TCF/β-catenin reporter gene assay was performed with the pTOPGLOW and pFOPGLOW constructs. Cell lines JROECL19 and JROECL33, both with nuclear β-catenin expression (JROECL19, Figure 4.1c), showed 350- and 18-fold increase in transcriptional activity of the pTOPGLOW reporter as compared to the negative control pFOPGLOW (Figure 4.2). Cell lines SKGT-4, TE-7 and OACP4C, all with membranous β-catenin immunoreactivity (TE-7, Figure 4.1d), showed no enhanced transcription of the pTOPGLOW reporter (Figure 4.2).

Mutation analysis of the AXIN1 gene

SSCP analysis of the AXIN1 gene in 17 tumor/normal DNA pairs from tumors with strong nuclear β-catenin expression (Figure 4.1a, 4.1c) revealed 6 different aberration patterns. All these aberrations were also present in the corresponding normal DNA. Sequencing of the aberrant samples identified 6 different SNPs. These were at positions (according to GenBank accession no. AF009674) A94C, C874T, intron 4+17 G→A, G1396A, T1942C and G2063A (Figure 4.3).

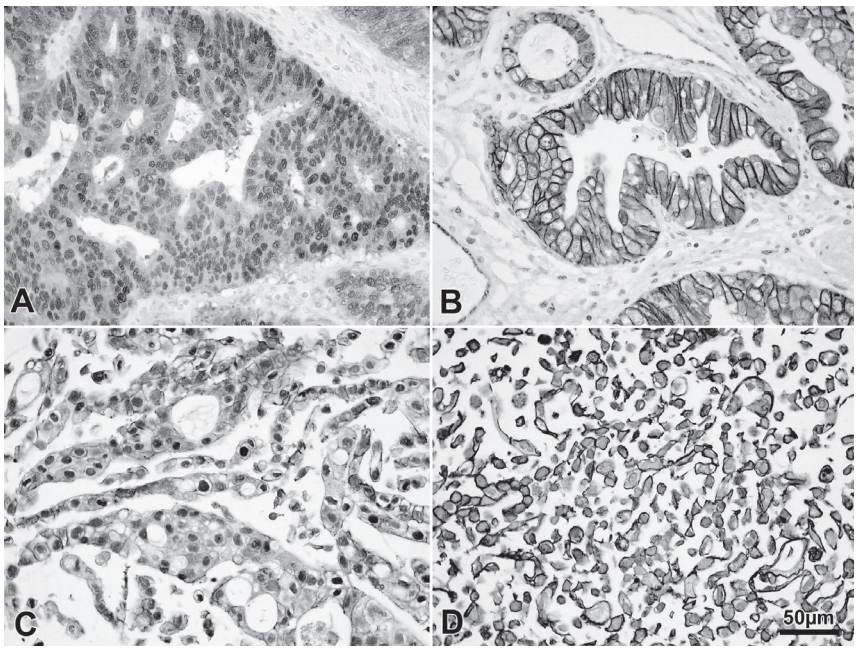


Figure 4.1 Immunohistochemistry of β-catenin in GEJ adenocarcinomas and cell lines. (β-catenin antibody, DAB and haematoxylin counterstain, magnification x 400). A. GEJ adenocarcinoma. Strong nuclear expression of β-catenin in the tumor cells. B. GEJ adenocarcinoma. Prominent membranous expression of β-catenin. C. Cell line JROECL19. Strong nuclear expression of β-catenin. D. Cell line TE-7. Membranous expression of β-catenin. *Also see color figures page 291.*

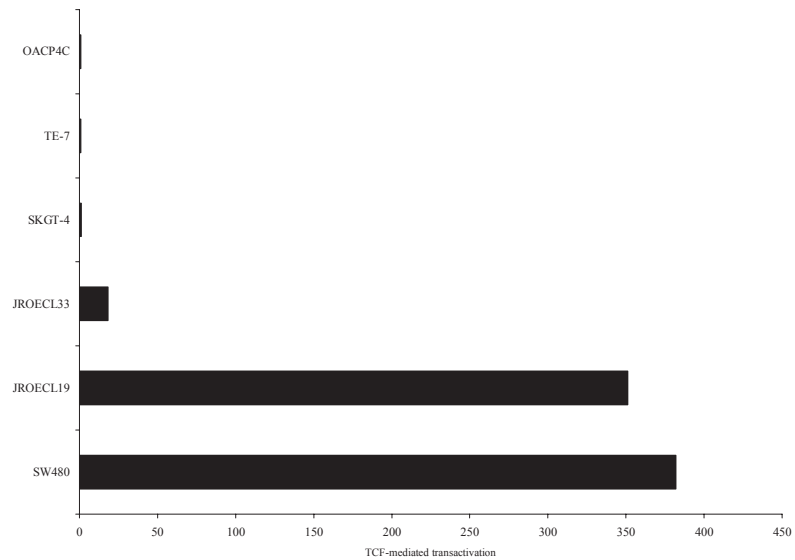


Figure 4.2 TCF-mediated transcriptional activation in GEJ adenocarcinoma cell lines. Constitutive transcriptional activation was detected in cell line JROECL33 and JROECL19. APC mutant colorectal cancer cell line SW480 served as a positive control. TCF-mediated transcriptional activity was defined as the ratio of pTOPGLOW:pFOPGLOW luciferase activities, each corrected for pRL-TK luciferase activities and where no transactivation equals 1.

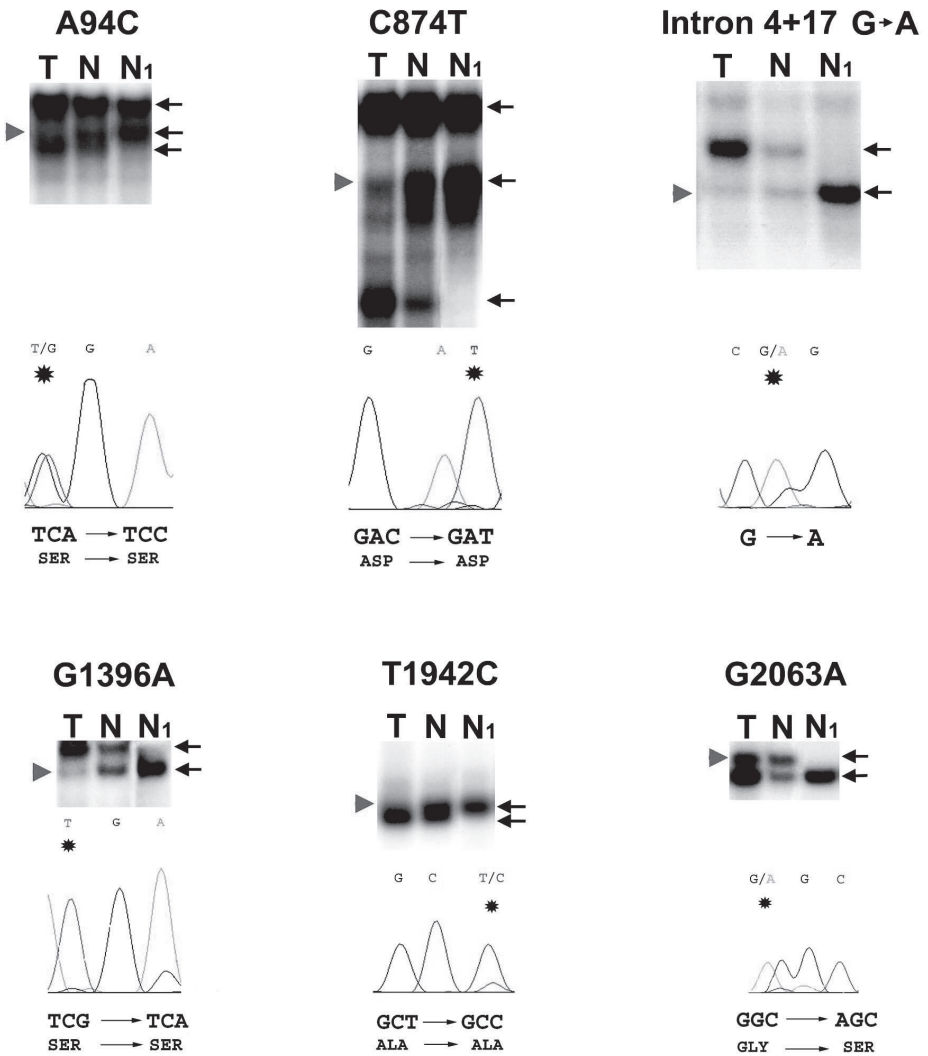


Figure 4.3 PCR-SSCP and sequencing analyses of the SNPs in tumors (T) and corresponding normal DNA (N), compared with DNA from individuals without SNPs (N1). Shown are informative cases with LOH. Black arrows point to allelic patterns. Red arrow heads point to deleted alleles in the tumor DNA. The sequencing chromatograms below each autoradiograph show the alterations (note the substituted nucleotide marked by an asterisk), which all represent SNPs. SNPs A94C and G1396A are annotated in the reverse complementary direction, whereas the SNPs C874, intron 4+17 G→A, T1942C and G2063A are annotated in forward direction. *Also see color figures page 291.*

Table 4.1 Patterns of allelic loss in GEJ adenocarcinomas with nuclear ß-catenin expression

Case	A94C	C874T	Intron 4 + 17 G□A	G1396A	T1942C	G2063A
1	Loss	HN	Loss	HN	HN	HN
2	HN	No loss	HN	No loss	Loss	HN
3	HN	HN	HN	HN	HN	HN
4	HP	HN	Loss	HN	HN	HN
5	Loss	HN	HN	HN	HN	HN
6	HN	HP	HN	No loss	HN	HN
7	HN	HP	HN	No loss	HN	HN
8	Loss	HN	Loss	No loss	No loss	HN
9	HN	Loss	HN	HN	HN	HN
10	No loss	HN	No loss	No loss	HN	HN
11	HN	HN	HN	HN	HN	Loss
12	HN	HN	HN	HN	HN	HN
13	HN	HN	HN	Loss	Loss	HN
14	HN	HN	HN	Loss	HN	HN
15	HN	Loss	HN	Loss	HN	HN
Frequency	6/34 (18%)	8/34 (24%)	4/34 (12%)	9/34 (26%)	4/34 (12%)	1/34 (3%)
Frequency NP	ND	ND	ND	ND	69/322 (21%)	9/322 (3%)

HN (Homozygous Normal)
HP (Homozygous Polymorphism)
NP Normal Population
ND (No Data)

Fifteen of the 17 cases appeared to be heterozygous for at least one polymorphism and LOH was observed in 11 (73%) cases (Table 4.1). The polymorphisms were used to investigate LOH in 20 GEJ adenocarcinoma cases with strong membranous β-catenin expression (Figure 4.1b, 4.1d). Also in this series all 6 polymorphisms were present and in 10 of 13 (77%) informative cases LOH was found (Table 4.2).

The T1942C and G2063A SNPs were found to have population frequencies of 21% and 3%, respectively (Tables 4.1 and 4.2). The relatively frequent SNP T1942C was only described very recently in two independent studies [46,47] and remained undetected in 10 AXIN1 gene mutation studies [29,31,43,44,48,54-58]. In most of these studies, as in ours, SSCP analyses were used. To determine whether the SSCP conditions have influence on the detection of the T1942C SNP we amplified DNA samples with AmpliTaq Gold and with Promega Taq DNA polymerases (Promega, Madison, WI, USA) in both AmpliTaq Gold and Promega Taq buffers. The AmpliTaq Gold buffer consists of 15 mM Tris-HCl (pH 8.0), 50 mM KCl and 1.5 mM $MgCl_2$ and the Promega buffer of 10 mM Tris-HCl (pH 9.0), 50 mM KCL, 1.5 mM $MgCl_2$ and 0.1%Triton X-100. As demonstrated in Figure 4 the T1942C SNP is only clearly visible after amplification with the AmpliTaq buffer.

Table 4.2 Patterns of allelic loss in GEJ adenocarcinomas without nuclear ß-catenin expression

Case	A94C	C874T	Intron 4 + 17 G→A	G1396A	T1942C	G2063A
1	No loss	HP	Loss	HN	Loss	HN
2	HN	Loss	HN	HN	Loss	HN
3	HN	HN	HN	Loss	Loss	HN
4	HN	HN	HN	HN	Loss	HN
5	HN	HP	HN	HN	Loss	HN
6	HN	HP	HN	Loss	HN	Loss
7	HN	No loss	HN	HN	HN	HN
8	No loss	HP	No loss	HN	HN	HN
9	HN	HN	HN	HN	HN	HN
10	HN	HN	HN	HN	HN	HN
11	Loss	HP	Loss	HN	HN	HN
12	Loss	Loss	HN	Loss	Loss	HN
13	HN	HP	HN	HN	HP	HN
14	HN	HN	HN	HN	HN	HN
15	HP	HP	HP	HN	HN	HN
16	No loss	HP	No loss	HN	No loss	HN
17	HN	Loss	HN	HN	HN	HN
Frequency	7/40 (18%)	20/40 (50%)	6/40 (15%)	5/40 (13%)	9/40 (23%)	2/40 (5%)
Frequency NP	ND	ND	ND	ND	69/322 (21%)	9/322 (3%)

HN (Homozygous Normal)
HP (Homozygous Polymorphism)
NP Normal Population
ND (No Data)

β-catenin and AXIN1 immunohistochemistry

As mentioned before 37 GEJ adenocarcinoma samples were investigated, 17 with strong nuclear β-catenin expression and 20 cases with normal membranous reactivity (Figure 4.1). Eleven of the 15 (73%) informative cases with nuclear β-catenin expression and 10 of 13 (77%) informative cases with membranous β-catenin immunoreactivity showed loss of the AXIN1 gene locus. In all tumor samples AXIN1 protein expression was confined to the cytoplasm of the tumor cells exclusively (Figure 4.5). No consistent differences in AXIN1 protein expression were observed between tumors with and without AXIN1 locus loss, irrespective of nuclear or membranous β-catenin expression (Figure 4.5).

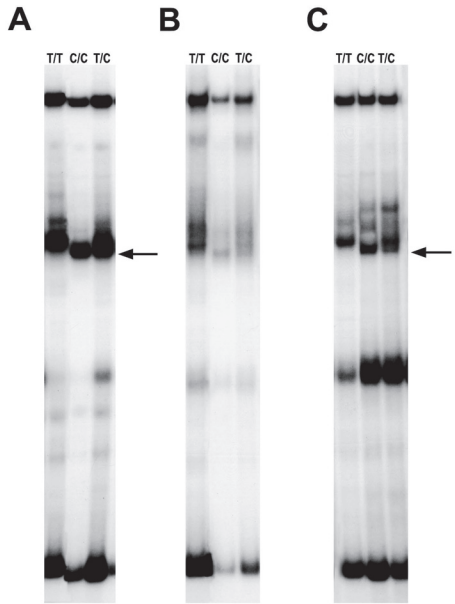


Figure 4.4 T1942C PCR-SSCP. Three DNA samples, 1942T/T (homozygous normal); 1942C/C (homozygous polymorphic); 1942T/C (heterozygous), were amplified with AmpliTaq Gold polymerase and AmpliTaq Gold buffer (panel A), Promega Taq and Promega buffer (panel B), Promega Taq and AmpliTaq Gold buffer (panel C) and with AmpliTaq Gold and Promega buffer (no PCR products obtained). All samples were amplified and SSCP-electrophoresed in the same experiment. Arrows point to the polymorphic SSCP fragment. Note that the polymorphic fragments are clearly visible only after amplification in AmpliTaq Gold buffer (panels A and C).

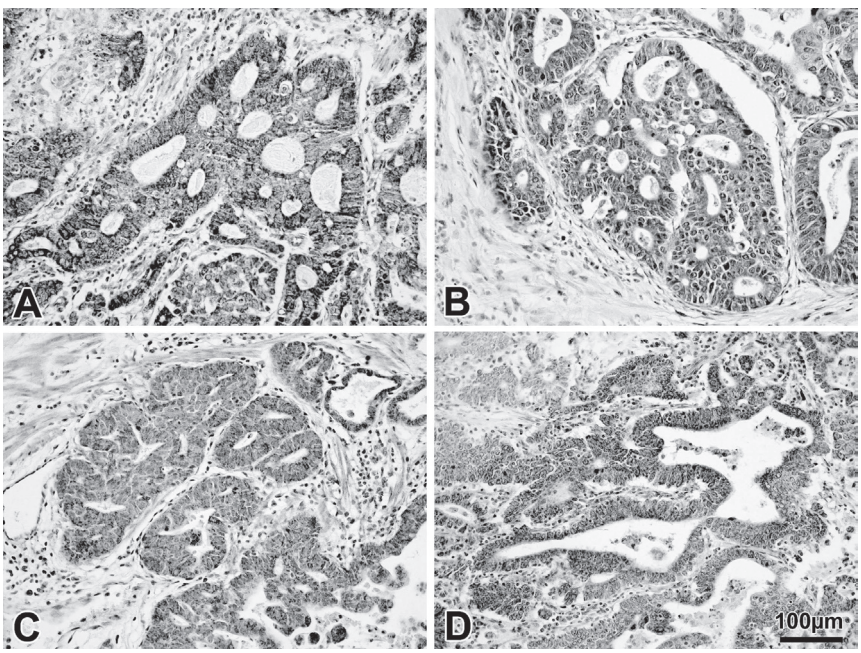


Figure 4.5 Immunohistochemistry of AXIN1 in GEJ adenocarcinomas. (AXIN1 antibody, DAB and haematoxylin counterstain, magnification x 200). A. and B. GEJ adenocarcinomas with nuclear β-catenin expression without (A.) and with (B.) AXIN1 locus LOH. C. and D. GEJ adenocarcinomas with membranous β-catenin expression without (C.) and with (D.) AXIN1 locus LOH. Note the strong cytoplasmic AXIN1 expression in the tumor cells in all 4 cases. *Also see color figures page 291.*

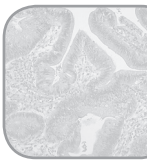
DISCUSSION

Nuclear β-catenin expression has been reported in up to 60% of GEJ adenocarcinomas [11,12,15,16,37]. In the present study we used the nuclear expression of β-catenin in GEJ adenocarcinomas as indication for activated Wnt signaling. This presupposition is substantiated by our finding of highly enhanced TCF-mediated transcriptional activity in the cell lines JROECL19 and JROECL33, both with nuclear β-catenin expression. None of the 3 cell lines without nuclear but with membranous β-catenin expression had increased transcription of the TOPGLOW reporter. Activated Wnt signaling in tumors is caused by increased levels of β-catenin which can be the result of mutations in APC, β-catenin, AXIN1, AXIN2 or β-TrCP [5,19,20,28-33]. To date only APC and β-catenin mutation analysis in GEJ adenocarcinomas has been performed and only few mutations were found [12,37-40]. Therefore, the mechanism of Wnt activation remains obscure in these tumors. These results prompted us to investigate GEJ adenocarcinoma samples with nuclear β-catenin expression for mutations in the AXIN1 gene. None of the tumors in the present study showed β-catenin exon 3 mutations [37]. In 17 GEJ adenocarcinoma samples no AXIN1 gene mutations were found. We therefore conclude that Wnt activation in GEJ tumors is not caused by AXIN1 gene mutations.

Six previously described polymorphisms in the AXIN1 gene were detected. All the detected DNA variations were also found in patients' constitutional DNAs indicating that they are truly polymorphisms and not somatic mutations. The G2063A SNP in exon 6 results in a substitution from glycine to serine. This polymorphism is described by Webster et al. [42] although these authors annotated the polymorphism to exon 7 and regarded it as a silent polymorphism. Furthermore, in a recent study by Taniguchi et al. [29] the G2063A polymorphism is found in one hepatocellular carcinoma and in one hepatoblastoma. Patients' normal tissues were not investigated and because the SNP was not found in their 147 control individuals they regarded it as an AXIN1 mutation. We found a G2063A allele frequency of 4% and 3% in GEJ adenocarcinoma patients and healthy controls, respectively, indicating that this AXIN1 gene variant is a SNP and not a mutation. In addition, we found a silent T1942C SNP (ala-ala) in exon 6 with an allele frequency of 18% and 21% in GEJ adenocarcinoma patients and healthy controls, respectively. This frequent SNP was only recently described, with comparable allele frequencies, in 2 independent studies [46,47] and remained undetected in 10 AXIN1 gene mutation investigations with in total 670 DNA samples [29,31,33,43,44,48,54,55,57,58]. We demonstrate that detection of the T1942C SNP by SSCP analysis is influenced by the PCR buffer characteristics. This finding suggests that also other SSCP parameters (gel composition, running buffer, running temperature, etc.) can have influence on the SNP detection.

The AXIN1 gene polymorphisms were used to determine LOH. Fifteen of 17 cases with nuclear β-catenin expression appeared to be heterozygous for at least one polymorphism and from these in 11 (73%) clear LOH was observed. This frequent loss of the AXIN1 gene could point to a dosage effect where the presence of 50% of the AXIN1 protein is insufficient

for proper β-catenin degradation and subsequently leads to Wnt-activation. To gather more information about this possibility we investigated AXIN1 gene LOH with the detected SNPs in a series of 20 tumors from our previous study with strong, normal membranous β-catenin expression. These included 3 cell lines (OACP4C, SKGT-4 and TE-7) without enhanced TCF-mediated transcriptional activity. In this series the T1942C and G2063A SNPs were detected in 4 and 2 samples, respectively. Thirteen cases were informative and in 10 (77%) clear LOH was observed.



By AXIN1 immunohistochemistry no differences were observed between tumors with and without AXIN1 locus loss. It's known that with the semiquantitative immunohistochemical method a twofold reduction in protein expression cannot be detected. In esophageal squamous cell carcinoma reduced protein and RNA expression has recently been described by Nakajima et al. [48]. These investigators suggest AXIN1 gene silencing by promotor methylation, in addition to allelic losses, as mechanism for AXIN1 downregulation. Our immunohistochemical results indicate that AXIN1 gene silencing does not occur in GEJ adenocarcinomas.

All the above-mentioned results indicate that AXIN1 gene haplo-insufficiency is not sufficient for Wnt-activation. This is in accordance with studies that demonstrated bi-allelic inactivation of the AXIN1 gene in several tumor types [29,31,33,41], although heterozygous AXIN1 gene inactivation has also been described [42,45]. Chromosome 16p loss has been reported in up to 40% in GEJ adenocarcinomas [59] and the presence of a tumor suppressor gene on 16p13.3 has been suggested by Takamochi et al. [60]. The frequent AXIN1 locus LOH in our study could point to a gene dosage effect independent of Wnt pathway activation. Furthermore, it cannot be excluded that an as yet to be defined tumor suppressor gene on 16p13.3 is the actual target for LOH in GEJ adenocarcinomas.

Since mutations in APC, β-catenin and AXIN1 do not play a major role in the frequent TCF/β-catenin activation in GEJ adenocarcinomas other components should be considered. AXIN2 gene mutations have been described in 11 colorectal cancers [32], one endometrioid ovarian adenocarcinoma [44] and two hepatocellular carcinomas [29]. Twelve of these 14 tumor samples had nuclear β-catenin expression indicating activated Wnt signaling. The AXIN2 gene mutations appear to be present exclusively in tumors with a microsatellite instable phenotype since all AXIN2 mutant colorectal carcinomas and the ovarian endometrioid adenocarcinoma were microsatellite instable [29,44]. The AXIN2 gene is an improbable candidate for Wnt activation in GEJ adenocarcinomas because microsatellite instability has been described in only less than 6% in these tumors [37,61]. Another candidate is β-TrCP, involved in β-catenin degradation. Recently, β-TrCP mutations have been described in two prostate cancer samples, of which one had nuclear β-catenin expression [28].

Activated Wnt signaling can also be the result of activation of the canonical Wnt pathway by secreted Wnts or by expression of the Wnt receptors frizzled [62,63]. In cell line cultures this would imply the presence of an autocrine Wnt/frizzled loop. Furthermore, tumor necrosis factor-α has recently been demonstrated to induce TCF/β-catenin mediated transcription in

a GEJ adenocarcinoma cell line [64]. These findings indicate that next to mutational activation of the Wnt pathway, gene expression alterations should be considered also as driving force behind Wnt activation in GEJ adenocarcinomas.

In summary, our results demonstrate that nuclear β-catenin expression in GEJ adenocarcinoma cell lines correlates with TCF-mediated transcription activation and so with activated Wnt signaling. The frequent nuclear localization of β-catenin in GEJ adenocarcinomas cannot be attributed by AXIN1 gene mutations. The mechanism of Wnt activation in these tumors remains to be established. In addition, the role of the frequent LOH of the AXIN1 locus in GEJ adenocarcinomas deserves further investigation.

ACKNOWLEDGMENTS

We would like to acknowledge Frank van der Panne for photographic work.

REFERENCES

1. Blot WJ, Devesa SS, Kneller RW, Fraumeni JF, Jr.: Rising incidence of adenocarcinoma of the esophagus and gastric cardia. Jama 265:1287-9, 1991
2. Botterweck AA, Schouten LJ, Volovics A, Dorant E, van Den Brandt PA: Trends in incidence of adenocarcinoma of the oesophagus and gastric cardia in ten European countries. Int J Epidemiol 29:645-54, 2000
3. Powell J, McConkey CC: The rising trend in oesophageal adenocarcinoma and gastric cardia. Eur J Cancer Prev 1:265-9, 1992
4. Tilanus HW, Hop WC, Langenhorst BL, van Lanschot JJ: Esophagectomy with or without thoracotomy. Is there any difference? J Thorac Cardiovasc Surg 105:898-903, 1993
5. Hajra KM, Fearon ER: Cadherin and catenin alterations in human cancer. Genes Chromosomes Cancer 34:255-68, 2002
6. Hirohashi S: Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. Am J Pathol 153:333-9, 1998
7. Jankowski JA, Bruton R, Shepherd N, Sanders DS: Cadherin and catenin biology represent a global mechanism for epithelial cancer progression. Mol Pathol 50:289-90, 1997
8. Nollet F, Berx G, van Roy F: The role of the E-cadherin/catenin adhesion complex in the development and progression of cancer. Mol Cell Biol Res Commun 2:77-85, 1999
9. Van Aken E, De Wever O, Correia da Rocha AS, Mareel M: Defective E-cadherin/catenin complexes in human cancer. Virchows Arch 439:725-51, 2001
10. Wijnhoven BP, Dinjens WN, Pignatelli M: E-cadherin-catenin cell-cell adhesion complex and human cancer. Br J Surg 87:992-1005, 2000
11. Bailey T, Biddlestone L, Shepherd N, Barr H, Warner P, Jankowski J: Altered cadherin and catenin complexes in the Barrett's esophagus-dysplasia-adenocarcinoma sequence: correlation with disease progression and dedifferentiation. Am J Pathol 152:135-44, 1998
12. Bian YS, Osterheld MC, Bosman FT, Fontolliet C, Benhattar J: Nuclear accumulation of beta-catenin is a common and early event during neoplastic progression of Barrett esophagus. Am J Clin Pathol 114:583-90, 2000
13. Jian WG, Darnton SJ, Jenner K, Billingham LJ, Matthews HR: Expression of E-cadherin in oesophageal carcinomas from the UK and China: disparities in prognostic significance. J Clin Pathol 50:640-4, 1997
14. Krishnadath KK, Tilanus HW, van Blankenstein M, Hop WC, Kremers ED, Dinjens WN, Bosman FT: Reduced expression of the cadherin-catenin complex in oesophageal adenocarcinoma correlates with poor prognosis. J Pathol 182:331-8, 1997
15. Osterheld MC, Bian YS, Bosman FT, Benhattar J, Fontolliet C: Beta-catenin expression and its association with prognostic factors in adenocarcinoma developed in Barrett esophagus. Am J Clin Pathol 117:451-6, 2002
16. Washington K, Chiappori A, Hamilton K, Shyr Y, Blanke C, Johnson D, Sawyers J, Beauchamp D: Expression of beta-catenin, alpha-catenin, and E-cadherin in Barrett's esophagus and esophageal adenocarcinomas. Mod Pathol 11:805-13, 1998
17. Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, Vogelstein B, Clevers H: Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science 275:1784-7, 1997
18. Resnik E: beta-Catenin--one player, two games. Nat Genet 16:9-11, 1997
19. Polakis P: Wnt signaling and cancer. Genes Dev 14:1837-51, 2000
20. Bienz M, Clevers H: Linking colorectal cancer to Wnt signaling. Cell 103:311-20, 2000
21. Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW: Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science 275:1787-90, 1997
22. Rubinfeld B, Robbins P, El-Gamil M, Albert I, Porfiri E, Polakis P: Stabilization of beta-catenin by genetic defects in melanoma cell lines. Science 275:1790-2, 1997
23. Kolligs FT, Nieman MT, Winer I, Hu G, Van Mater D, Feng Y, Smith IM, Wu R, Zhai Y, Cho KR, Fearon ER: ITF-2, a downstream target of the Wnt/TCF pathway, is activated in human cancers with beta-catenin defects and promotes neoplastic transformation. Cancer Cell 1:145-55, 2002

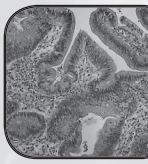
24. Miyoshi K, Rosner A, Nozawa M, Byrd C, Morgan F, Landesman-Bollag E, Xu X, Seldin DC, Schmidt EV, Taketo MM, Robinson GW, Cardiff RD, Hennighausen L: Activation of different Wnt/beta-catenin signaling components in mammary epithelium induces transdifferentiation and the formation of pilar tumors. Oncogene 21:5548-56, 2002
25. Aoki M, Hecht A, Kruse U, Kemler R, Vogt PK: Nuclear endpoint of Wnt signaling: neoplastic transformation induced by transactivating lymphoid-enhancing factor 1. Proc Natl Acad Sci U S A 96:139-44, 1999
26. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW: Identification of c-MYC as a target of the APC pathway. Science 281:1509-12, 1998
27. Tetsu O, McCormick F: Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 398:422-6, 1999
28. Gerstein AV, Almeida TA, Zhao G, Chess E, Shih Ie M, Buhler K, Pienta K, Rubin MA, Vessella R, Papadopoulos N: APC/CTNNB1 (beta-catenin) pathway alterations in human prostate cancers. Genes Chromosomes Cancer 34:9-16, 2002
29. Taniguchi K, Roberts LR, Aderca IN, Dong X, Qian C, Murphy LM, Nagorney DM, Burgart LJ, Roche PC, Smith DI, Ross JA, Liu W: Mutational spectrum of beta-catenin, AXIN1, and AXIN2 in hepatocellular carcinomas and hepatoblastomas. Oncogene 21:4863-71, 2002
30. Kinzler KW, Vogelstein B: Lessons from hereditary colorectal cancer. Cell 87:159-70, 1996
31. Yokota N, Nishizawa S, Ohta S, Date H, Sugimura H, Namba H, Maekawa M: Role of Wnt pathway in medulloblastoma oncogenesis. Int J Cancer 101:198-201, 2002
32. Liu W, Dong X, Mai M, Seelan RS, Taniguchi K, Krishnadath KK, Halling KC, Cunningham JM, Boardman LA, Qian C, Christensen E, Schmidt SS, Roche PC, Smith DI, Thibodeau SN: Mutations in AXIN2 cause colorectal cancer with defective mismatch repair by activating beta-catenin/TCF signaling. Nat Genet 26:146-7, 2000
33. Satoh S, Daigo Y, Furukawa Y, Kato T, Miwa N, Nishiwaki T, Kawasoe T, Ishiguro H, Fujita M, Tokino T, Sasaki Y, Imaoka S, Murata M, Shimano T, Yamaoka Y, Nakamura Y: AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. Nat Genet 24:245-50, 2000
34. Gayet J, Zhou XP, Duval A, Rolland S, Hoang JM, Cottu P, Hamelin R: Extensive characterization of genetic alterations in a series of human colorectal cancer cell lines. Oncogene 20:5025-32, 2001
35. Huang H, Mahler-Araujo BM, Sankila A, Chimelli L, Yonekawa Y, Kleihues P, Ohgaki H: APC mutations in sporadic medulloblastomas. Am J Pathol 156:433-7, 2000
36. Sparks AB, Morin PJ, Vogelstein B, Kinzler KW: Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. Cancer Res 58:1130-4, 1998
37. Wijnhoven BP, Nollet F, De Both NJ, Tilanus HW, Dinjens WN: Genetic alterations involving exon 3 of the beta-catenin gene do not play a role in adenocarcinomas of the esophagus. Int J Cancer 86:533-7, 2000
38. Choi YW, Heath EI, Heitmiller R, Forastiere AA, Wu TT: Mutations in beta-catenin and APC genes are uncommon in esophageal and esophagogastric junction adenocarcinomas. Mod Pathol 13:1055-9, 2000
39. Gonzalez MV, Artimez ML, Rodrigo L, Lopez-Larrea C, Menendez MJ, Alvarez V, Perez R, Fresno MF, Perez MJ, Sampedro A, Coto E: Mutation analysis of the p53, APC, and p16 genes in the Barrett's oesophagus, dysplasia, and adenocarcinoma. J Clin Pathol 50:212-7, 1997
40. Powell SM, Papadopoulos N, Kinzler KW, Smolinski KN, Meltzer SJ: APC gene mutations in the mutation cluster region are rare in esophageal cancers. Gastroenterology 107:1759-63, 1994
41. Laurent-Puig P, Legoix P, Bluteau O, Belghiti J, Franco D, Binot F, Monges G, Thomas G, Bioulac-Sage P, Zucman-Rossi J: Genetic alterations associated with hepatocellular carcinomas define distinct pathways of hepatocarcinogenesis. Gastroenterology 120:1763-73, 2001
42. Webster MT, Rozycka M, Sara E, Davis E, Smalley M, Young N, Dale TC, Wooster R: Sequence variants of the axin gene in breast, colon, and other cancers: an analysis of mutations that interfere with GSK3 binding. Genes Chromosomes Cancer 28:443-53, 2000
43. Shimizu Y, Ikeda S, Fujimori M, Kodama S, Nakahara M, Okajima M, Asahara T: Frequent alterations in the Wnt signaling pathway in colorectal cancer with microsatellite instability. Genes Chromosomes Cancer 33:73-81, 2002

44. Wu R, Zhai Y, Fearon ER, Cho KR: Diverse mechanisms of beta-catenin deregulation in ovarian endometrioid adenocarcinomas. Cancer Res 61:8247-55, 2001
45. Dahmen RP, Koch A, Denkhaus D, Tonn JC, Sorensen N, Berthold F, Behrens J, Birchmeier W, Wiestler OD, Pietsch T: Deletions of AXIN1, a component of the WNT/wingless pathway, in sporadic medulloblastomas. Cancer Res 61:7039-43, 2001
46. Baeza N, Masuoka J, Kleihues P, Ohgaki H: AXIN1 mutations but not deletions in cerebellar medulloblastomas. Oncogene 22:632-6, 2003
47. Miao J, Kusafuka T, Udatsu Y, Okada A: Sequence variants of the Axin gene in hepatoblastoma. Hepatol Res 25:174-179, 2003
48. Nakajima M, Fukuchi M, Miyazaki T, Masuda N, Kato H, Kuwano H: Reduced expression of Axin correlates with tumor progression of oesophageal squamous cell carcinoma. Br J Cancer 88:1734-9, 2003
49. van de Wetering M, Barker N, Harkes IC, van der Heyden M, Dijk NJ, Hollestelle A, Klijn JG, Clevers H, Schutte M: Mutant E-cadherin breast cancer cells do not display constitutive Wnt signaling. Cancer Res 61:278-84, 2001
50. Rockett JC, Larkin K, Darnton SJ, Morris AG, Matthews HR: Five newly established oesophageal carcinoma cell lines: phenotypic and immunological characterization. Br J Cancer 75:258-63, 1997
51. de Both NJ, Wijnhoven BP, Sleddens HF, Tilanus HW, Dinjens WN: Establishment of cell lines from adenocarcinomas of the esophagus and gastric cardia growing in vivo and in vitro. Virchows Arch 438:451-6, 2001
52. Altorki N, Schwartz GK, Blundell M, Davis BM, Kelsen DP, Albino AP: Characterization of cell lines established from human gastric-esophageal adenocarcinomas. Biologic phenotype and invasion potential. Cancer 72:649-57, 1993
53. Nishihira T, Hashimoto Y, Katayama M, Mori S, Kuroki T: Molecular and cellular features of esophageal cancer cells. J Cancer Res Clin Oncol 119:441-9, 1993
54. Lin YM, Kato T, Satoh S, Nakamura Y, Furukawa Y: Identification of novel polymorphisms in the AXIN1++ and CDX-2 genes. J Hum Genet 45:254-6., 2000
55. Webster MT, Rozycka M, Sara E, Davis E, Smalley M, Young N, Dale TC, Wooster R: Sequence variants of the axin gene in breast, colon, and other cancers: an analysis of mutations that interfere with GSK3 binding. Genes Chromosomes Cancer 28:443-53., 2000
56. Satoh S, Daigo Y, Furukawa Y, Kato T, Miwa N, Nishiwaki T, Kawasoe T, Ishiguro H, Fujita M, Tokino T, Sasaki Y, Imaoka S, Murata M, Shimano T, Yamaoka Y, Nakamura Y: AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. Nat Genet 24:245-50., 2000
57. Dahmen RP, Koch A, Denkhaus D, Tonn JC, Sorensen N, Berthold F, Behrens J, Birchmeier W, Wiestler OD, Pietsch T: Deletions of AXIN1, a component of the WNT/wingless pathway, in sporadic medulloblastomas. Cancer Res 61:7039-43., 2001
58. Miao J, Kusafuka T, Udatsu Y, Okada A: Axin, the main component of the Wnt signaling pathway, is not mutated in kidney tumors in children. Int J Mol Med 9:377-9, 2002
59. Gleeson CM, Sloan JM, McGuigan JA, Ritchie AJ, Weber JL, Russell SE: Barrett's oesophagus: microsatellite analysis provides evidence to support the proposed metaplasia-dysplasia-carcinoma sequence. Genes Chromosomes Cancer 21:49-60, 1998
60. Takamochi K, Ogura T, Suzuki K, Kawasaki H, Kurashima Y, Yokose T, Ochiai A, Nagai K, Nishiwaki Y, Esumi H: Loss of heterozygosity on chromosomes 9q and 16p in atypical adenomatous hyperplasia concomitant with adenocarcinoma of the lung. Am J Pathol 159:1941-8, 2001
61. Gleeson CM, Sloan JM, McGuigan JA, Ritchie AJ, Weber JL, Russell SE: Ubiquitous somatic alterations at microsatellite alleles occur infrequently in Barrett's-associated esophageal adenocarcinoma. Cancer Res 56:259-63, 1996
62. Tanaka S, Akiyoshi T, Mori M, Wands JR, Sugimachi K: A novel frizzled gene identified in human esophageal carcinoma mediates APC/beta-catenin signals. Proc Natl Acad Sci U S A 95:10164-9, 1998
63. Mizushima T, Nakagawa H, Kamberov YG, Wilder EL, Klein PS, Rustgi AK: Wnt-1 but not epidermal growth factor induces beta-catenin/T-cell factor-dependent transcription in esophageal cancer cells. Cancer Res 62:277-82, 2002

64. Tselepis C, Perry I, Dawson C, Hardy R, Darnton SJ, McConkey C, Stuart RC, Wright N, Harrison R, Jankowski JA: Tumor necrosis factor-alpha in Barrett's oesophagus: a potential novel mechanism of action. Oncogene 21:6071-81, 2002

Chapter 5

Identification of a 7.1 mega base pairs minimal deletion at 14q31.1-32.11 in adenocarcinomas of the gastro-esophageal junction



Human Pathology, 2006 May;37(5):534-41

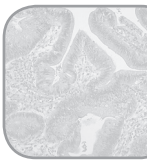
Winand NM Dinjens
Linetta B Koppert
David A Dezentjé
Mustaffa Abbou
Eddy S van Ballegooijen
Hein FBM Sleddens
Herman van Dekken
Hugo W Tilanus
Bas PL Wijnhoven

ABSTRACT

A recent evaluation by comparative genomic hybridization (CGH) demonstrated chromosome 14q31-32.1 to be frequently deleted in adenocarcinomas of the gastro-esophageal junction (GEJ). This suggests the presence of a tumor suppressor gene in the deleted region. In the present study we have performed a detailed loss of heterozygosity (LOH) analysis in 34 GEJ adenocarcinomas and one tumor-corresponding dysplastic Barrett's epithelium sample with 37 polymorphic microsatellite markers. Thirty-five markers are in the 14q24.3-32.33 region with a mean distance of 800 kilo base pairs. Fourteen of 34 tumor samples (41%) showed loss of 14q markers. We identified a minimal region of allelic loss of 7,105,440 base pairs between markers D14S1000 and D14S256 at cytogenetic location 14q31.1-32.11. Within this region markers D14S1035, D14S55, D14S1037, D14S1022, D14S1052, D14S974, D14S73, D14S1033, D14S67, D14S68 and D14S1058 showed loss in all informative tumors with 14q loss. The region between markers D14S1000 and D14S256 contains 7 known genes. The identification of this minimal deletion and the data base information on the genes present in this region facilitate the search for the candidate tumor suppressor gene(s).

INTRODUCTION

Adenocarcinomas arising from the lower esophagus or the gastric cardia are commonly referred to as adenocarcinoma of the gastro-esophageal junction (GEJ). As such, they can be regarded as a single entity [1,2]. These tumors have shown a rapid increase in incidence over the past decades [1,3]. The most important risk factor is gastro-esophageal reflux leading to the replacement of the normal stratified squamous epithelium by columnar epithelium. This condition is known as Barrett's esophagus. The prognosis for patients with adenocarcinoma of the GEJ is poor. Five-year survival after surgery with curative intent is approximately 20-25% [4,5]. Little is known about the genetic alterations involved in the progression of Barrett's epithelium towards dysplasia and invasive adenocarcinoma.



Recently, we and others identified chromosomal aberrations in GEJ tumors by comparative genomic hybridisation (CGH) [6,8]. Loss of chromosome 14q31-32.1 was detected in a significant number of cases, suggesting the presence of a tumor suppressor gene in that region [6]. Deletion of part of chromosome 14 has also been reported in other malignancies such as colorectal carcinoma [9,12], adenocarcinoma of the stomach [13,14], gastrointestinal tract stromal cell tumors [15], ovarian [16], bladder [17], pancreaticobiliary [18], breast [19], head and neck [20,21], renal cell carcinoma [22,23], malignant mesothelioma [24], neuroblastoma [25], meningioma [26], esophageal squamous cell carcinoma [27] and oligodendroglial and astrocytic tumors [28].

The goal of the present study was to define the common region of chromosome 14q31-32.1 loss in GEJ adenocarcinoma in more detail by loss of heterozygosity (LOH) analysis using 37 polymorphic microsatellite markers. From 34 patients 15 primary tumors, 15 xenografts from 10 primary tumors and 5 lymph node metastases, and 4 in vitro GEJ adenocarcinoma cell lines were used. From one patient in addition to a cell line derived from the primary tumor, a cell line established from a lymph node metastasis was investigated. Furthermore, from one patient the primary tumor and the dysplastic epithelium adjacent to the tumor were investigated. The xenografts and cell lines facilitate detection of homozygous deletions.

MATERIALS AND METHODS

Patients

We analyzed 36 tissue samples from 34 patients. All patients were diagnosed with adenocarcinoma of the gastro-esophageal junction, *i.e.* distal esophagus or the gastric cardia, and underwent transhiatal resection of the esophagus and the proximal stomach with curative intent as described [2].

Tumor samples: frozen tumor tissue, xenografts and cell lines

The tissue samples were obtained from the resection specimens and used according to the Code of Proper Secondary Use of Human Tissue in the Netherlands established by the Dutch Federation of Medical Scientific Societies (http://www.fmwv.nl/gedragcodes/goedgebruik/CodeProperSecondaryUseOfHumanTissue.pdf). Immediately after surgery small pieces of tumor, dysplasia and adjacent normal mucosa were taken, snap frozen and stored in liquid nitrogen until further analysis. Specimens included 15 primary tumors (coded Ba) and one dysplastic epithelium sample (Ba10d) adjacent to an adenocarcinoma, 10 primary tumor nude mouse xenografts (with code P-X), 5 xenografts from lymph node metastases (with code M-X) and 5 in vitro cell lines [29,30] (Figure 5.1). All xenografts were used in the first or second passage. Two of the in vitro cell lines are derived from a primary tumor (P4C) and a lymph node metastasis (M4C) from the same patient. Two human cell lines JROECL19 and -33, established by Rockett et al [30] were obtained from the European Collection of Animal Cell Cultures (ECACC) and 3 cell lines (P4C, M4C and M5C) were established in our laboratory [29]. Sixteen of the used tumors were previously investigated by CGH [6]. Eight samples showed 14q loss in the CGH study and all these 8 demonstrated 14q LOH. Of the 8 tumors with no 14q loss in CGH, two demonstrated partial 14q LOH. The remaining 6 tumors without CGH 14q loss did also not show 14q LOH. In our investigated cases there is a generally good concordance between 14q CGH and LOH results, with LOH analysis somewhat more sensitive in detecting DNA loss, as expected.

DNA extraction

DNA from cell lines was isolated according to standard procedures. Genomic DNA from xenografts and tumor samples was isolated from consecutive 5 μm cryostat tissue sections by overnight proteinase K digestion at 55 °C followed by phenol extraction and ethanol precipitation. DNA pellets were dissolved in TE (10 mM Tris/HCl pH 7.8; 1 mM EDTA). From the tumor and dysplastic tissue cryostat sections were microdissected to obtain tissue fragments containing at least 75% neoplastic cells. From all tumors and xenografts corresponding normal DNA was isolated from frozen normal lymph node, normal esophagus or normal stomach tissue. From cell lines JROECL19 and -33 corresponding normal DNA was isolated from formalin fixed and paraffin embedded normal tissue (kind gift of Dr. S.J. Darnton). From the in vitro cell lines P4C/M4C and from the xenografts M53X1, P35X1, M30X1 and M55X1 corresponding primary tumor DNA was isolated after microdissection of cryostat sections. Normal mouse DNA, isolated from mouse liver, was used as negative control for the xenograft samples.

Microsatellite marker selection

Thirty-seven polymorphic microsatellite markers with a high degree of heterozygosity were originally selected from the Généthon human genetic linkage map [31], from The Genome Database (http://www.gdb.org) and from the published complete chromosome 14 sequence

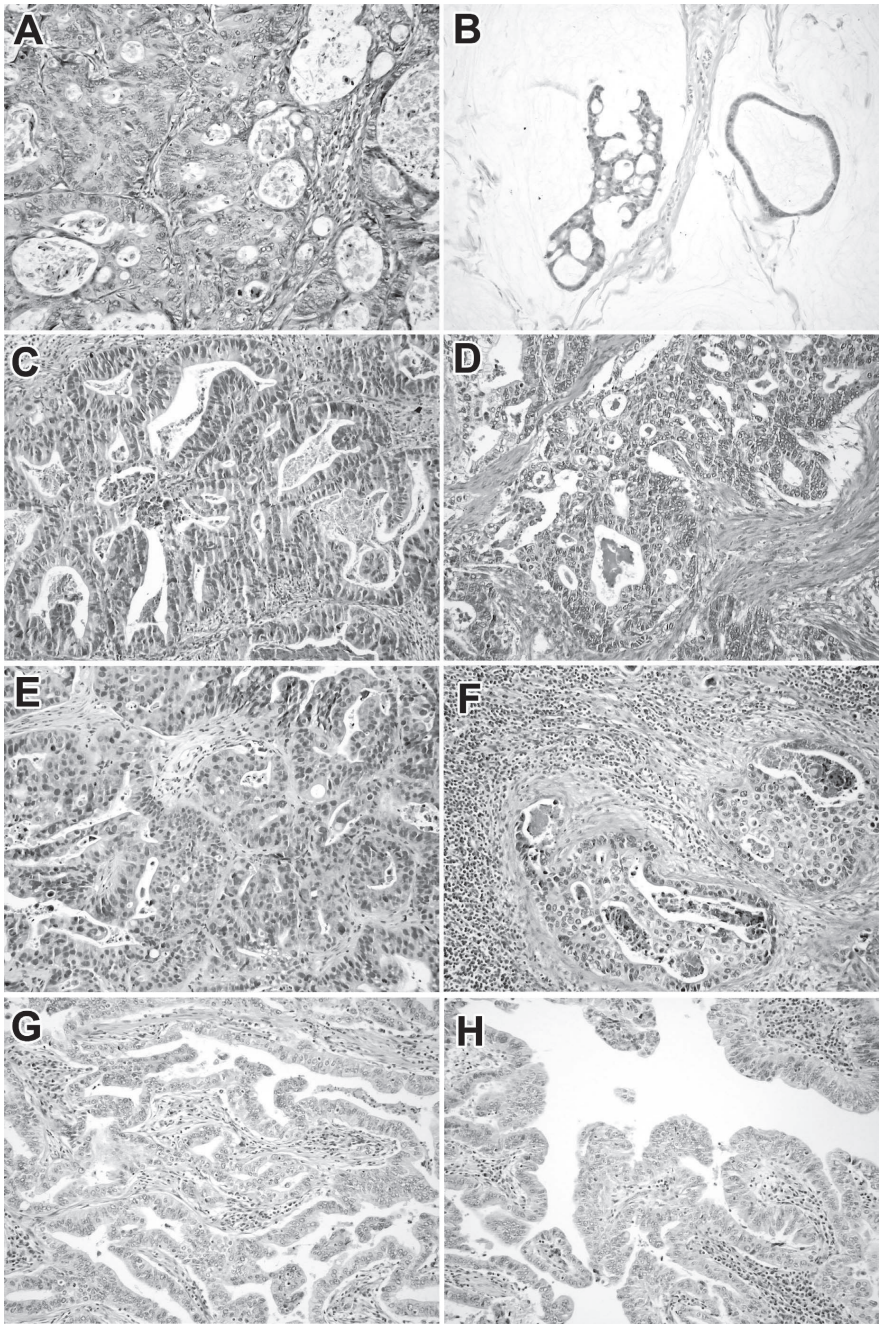


Figure 5.1 Histology (H&E stained sections) of investigated tumors. A: adenocarcinoma of the gastric cardia with 14q LOH (Ba7). B: adenocarcinoma of the gastro-esophageal junction without 14q LOH (Ba1). C and D: primary tumor derived xenograft P35X1 (C) and corresponding primary tumor (D). Note comparable tumor histology. E and F: lymphnode metastasis derived xenograft M55X1 (E) and corresponding lymphnode metastasis (F). Note comparable tumor histology. G and H: esophageal adenocarcinoma (G, Ba10) and from the same patient dysplastic Barrett's epithelium (H, Ba10d). Original magnifications x200.

(http://www.genoscope.cns.fr) [32]. The exact position of the markers was retrieved from the UCSC database (http://genome.ucsc.edu/; freeze May 2004, Build 35) [33]. Thirty-five markers are located in the region 14q24.1-32.33, spanning the region 14q31-32.1, and 2 markers are at 14q11.2. All 37 markers are CA/TG dinucleotide repeats and their location is indicated by the position of the first nucleotide of the CA/TG repeat (C or T) in the chromosome 14 sequence (http://genome.ucsc.edu/; freeze May 2004, Build 35; Figure 5.2). In the 14q24.1-32.33 region the marker distance varies between 13.7 kb (kilo base pairs; markers D14S250 and D14S78; Figure 5.2) and 2,157 kb (markers D14S985 and D14S293; Figure 5.2). We used the primers from the database for marker amplification, except for marker CCC1 for which we used the primers: 5'-GGCAGTTAAGAAGACACAGC-3' and 5'-GCCATAAGCCTGAAGATTGG-3'.

PCR amplification

PCR amplification was performed in a 15 μl reaction volume containing 0.1 μg of genomic DNA, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 0.02 mM dATP and 0.8 μCi α-^{32}P-dATP (Amersham, Buckinghamshire, UK), 20 pmol of each primer, 0.4 U Taq polymerase (Promega, Madison, WI, USA) and 2-4 mM $MgCl_2$. Amplification proceeded during 35 cycles in a Biometra thermal cycler (Biometra, Göttingen, Germany) with the following parameters: denaturing at 95°C for 30 sec, annealing at either 50°C or 55°C for 45 sec and extension at 72°C for 60 sec. Reactions concluded with a final extension at 72°C for 10 min.

Electrophoresis and interpretation of loss of heterozygosity (LOH)

Just prior to gel electrophoresis 5 μl of the PCR amplification products were diluted with 5 μl of loading buffer (95% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol, pH 8.0). Then the PCR products were denatured at 95°C for 5 min and loaded onto adjacent lanes of a 6% denaturing polyacrylamide gel containing 42% ureum. Electrophoresis was performed at 65 W for 2 to 3 hrs, depending on the size of the amplified DNA fragment. Gels were dried and exposed to X-ray films.

The signals derived from tumor and corresponding normal DNA were compared by visual inspection, independently performed by at least three investigators (W.N.M.D., D.A.D., M.A., H.F.B.M. and B.P.L.W.). DNA samples with controversial results were reamplified and in a number of cases we isolated and amplified DNA from a seperate part of the tumor. A case was classified as informative and having undergone allelic loss (LOH), informative and no LOH, not informative, or as having undergone an allelic shift (microsatellite instability, MSI).

RESULTS

From 34 patients 35 tumor samples and one dysplastic Barrett's epithelium were analyzed for LOH using 37 polymorphic microsatellite markers. Thirty-five markers cover chromosome

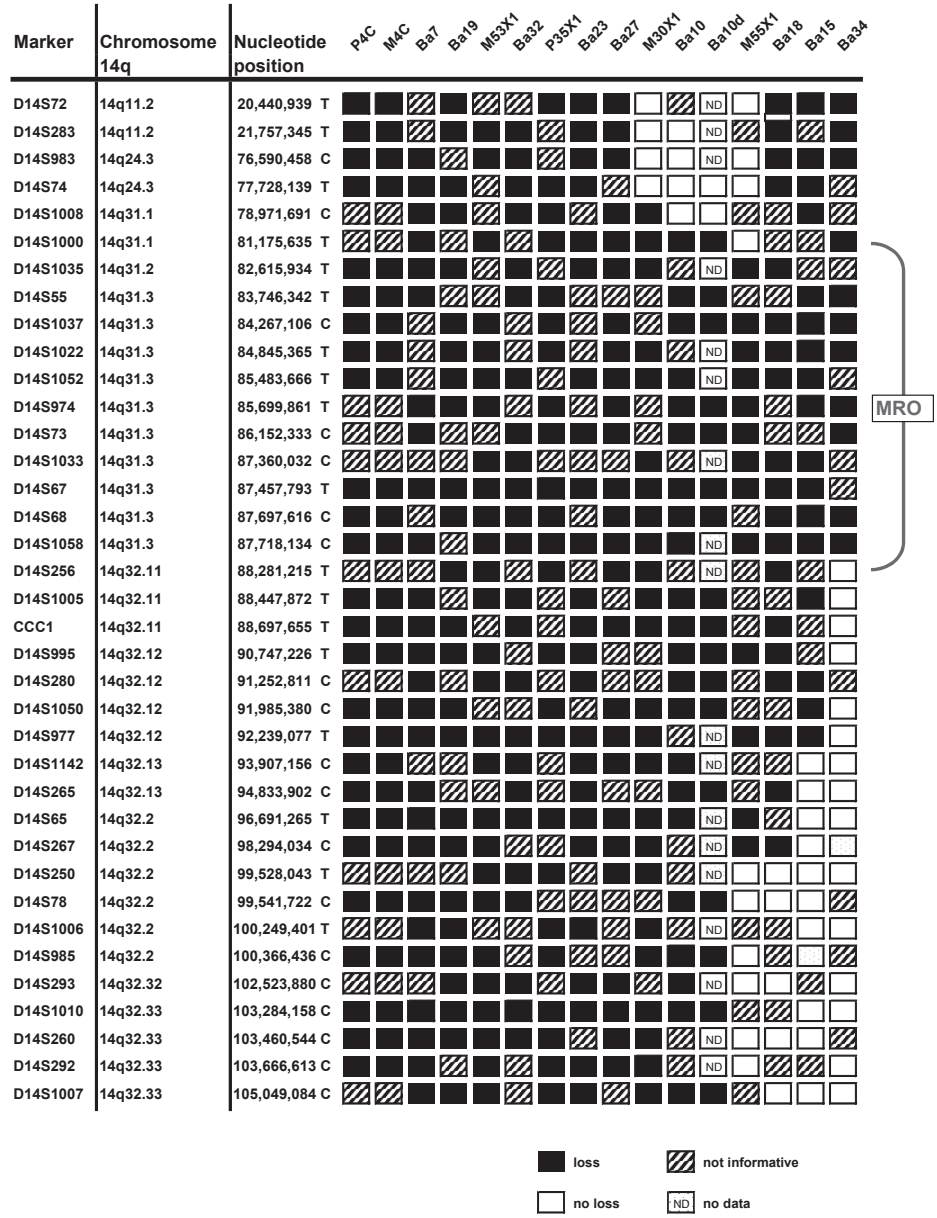


Figure 5.2 Chromosome 14 LOH results. All samples with loss of 14q markers are shown. P4C and M4C are in vitro cell lines from a primary tumor and a metastasis, respectively, from the same patient. P35X1 is a xenograft from a primary tumor and M30X1, M53X1 and M55X1 are xenografts from metastases. The Ba numbers are primary tumors and Ba10d is the dysplastic epithelium sample belonging to primary tumor Ba10. Nucleotide positions from the first nucleotide (C or T) of the CA or TG repeats are indicated by the position of the first nucleotide of the CA/TG repeat (C or T) in the chromosome 14 sequence (http://genome.ucsc.edu/; freeze May 2004, Build 35) [33]. White squares: no loss; black squares: loss; striped squares: not informative; ND: no data. The solid bar indicates the minimal region of overlapping deletion (MRO).

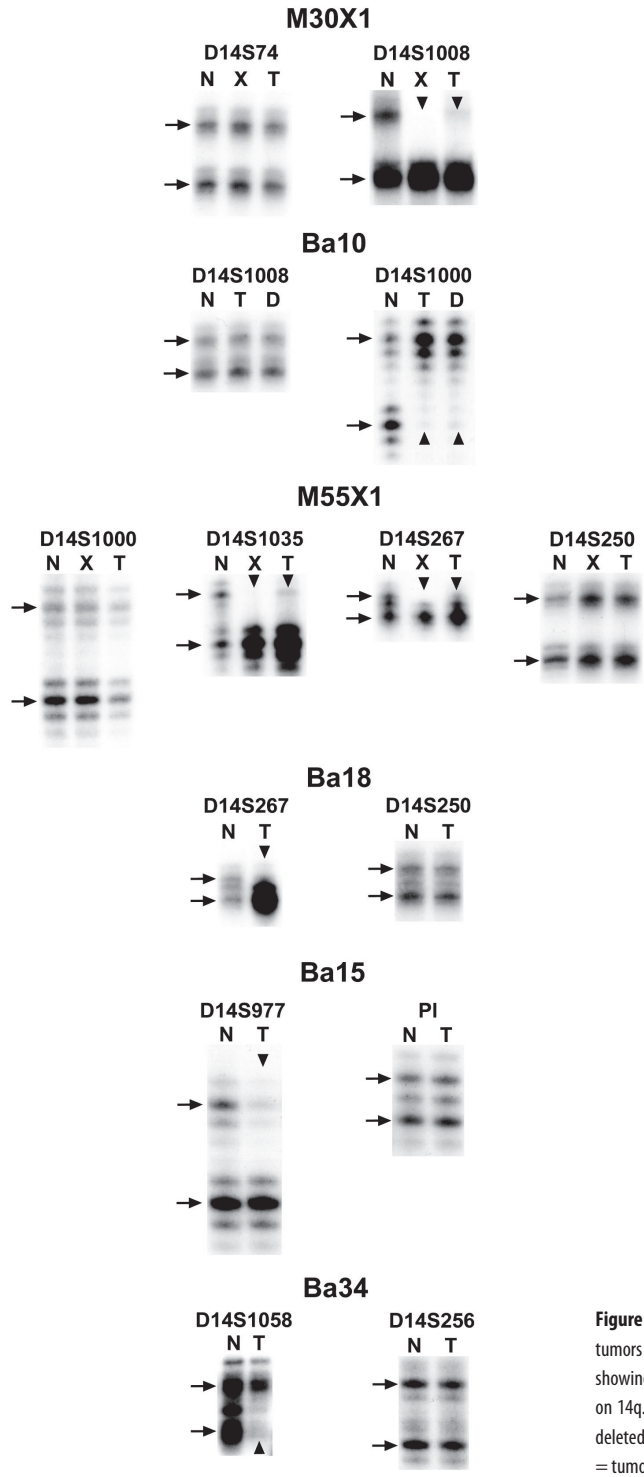
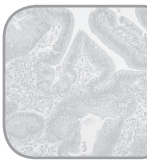


Figure 5.3 Autoradiograms from 6 representative tumors (M30X1, Ba10, M55X1, Ba18, Ba15 and Ba34) showing allelic band patterns from critical markers on 14q. Arrows indicate alleles; arrowheads point to deleted allele. N = normal DNA, X = xenograft DNA, T = tumor DNA and D = Barrett's dysplasia DNA.

region 14q24.3-14q32.33. The relevant results are summarized in Figure 5.2. We regard cell lines P4C and M4C as one sample since they are derived from the same patient and since they showed identical results. No LOH was found in 18 tumors and 2 tumors showed widespread MSI. LOH of 14q was observed in 14 of 34 tumors (41%). Eight tumors (P4C/M4C, Ba7, Ba19, M53X1, Ba32, P35X1, Ba23 and Ba27) showed loss of all informative markers for the entire region 14q11-qter. In 6 tumors both loss and retention of heterozygosity was seen at informative markers. These patterns were used for defining the minimal region of overlapping deletion (MRO). With all xenograft and cell line DNA samples PCR products were obtained with all markers. Seven markers amplified also normal mouse DNA, but with a significant size difference from human DNA. The corresponding primary tumors from cell lines P4C and M4C and from xenografts M53X1, P35X1, M30X1 and M55X1 were investigated with the boundary determining markers D14S72, D14S74, D14S1008, D14S1000, D14S1035, D14S267, D14S250 and D14S292. The cell lines and xenografts and their corresponding primary tumors showed the same LOH pattern in all cases (Figure 5.3).



We defined a region of loss which was proximally bounded by marker D14S1000 and distally by marker D14S256, the size being maximally 7,105,440 base pairs (determined from the position of the first base down stream of the reverse D14S1000 primer (81, 175, 728) and the last base up stream of the forward D14S256 primer (88, 281, 168) (base positions from the 14q sequence at http://genome.ucsc.edu/; freeze May 2004, Build 35). Informative LOH results are shown in Figure 5.3. Xenograft M55X1, with an interstitial deletion, was critical for defining the centromeric boundary, whereas tumor Ba34 defined the telomeric boundary. This region has been cytogenetically assigned to 14q31.1-32.11 and it was deleted in all tumors that showed loss of 14q markers. Deletion of D14S1035, D14S55, D14S1037, D14S1022, D14S1052, D14S974, D14S73, D14S1033, D14S67, D14S68 and D14S1058 was found in all informative tumors with 14q loss. There was no correlation between LOH on 14q and the location of the tumor (esophagus or gastric cardia). GEJ tumors with 14q loss tended to have a higher tumor stage and more frequently distant metastases as compared to the tumors without 14q loss (χ^2 test $p = 0.13$ and $p = 0.076$, respectively).

DISCUSSION

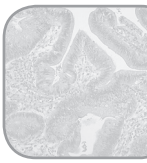
In this study we confirmed our previous finding that allelic loss on chromosome 14q31-32.1 occurs in more than 40% of all GEJ adenocarcinomas [6]. This strongly suggests that this region contains one or more tumor suppressor genes involved in GEJ tumorigenesis. Furthermore, we identified the commonly deleted region of allelic loss. This is located at cytogenetic location 14q31.1-32.11 between markers D14S1000 and marker D14S256, with a size of about 7.1 Mb. In the 15 xenografts and 5 cell lines we did not detect homozygous deletions, since with all markers tested PCR products were obtained. Two cell lines (from one patient) and 4 xeno-

grafts (1 from a primary tumor and 3 from metastases) showed 14q LOH. The corresponding primary tumors showed the same LOH patterns indicating that 14q LOH was not an ex vivo artefact.

Genome-wide allelic loss analysis in colorectal carcinomas reported an overall mean LOH frequency of about 20% which is regarded as non specific, "background", allelic loss [34,35]. The finding of more than 40% 14q311-32.11 allelic loss in adenocarcinomas of the GEJ points to possible involvement of this region in the genesis of these tumors. Furthermore, loss of 14q31-32 has been reported in several other carcinomas like colorectal adenocarcinoma [9-12], adenocarcinoma of the stomach [13,14], ovarian carcinoma [16], bladder carcinoma [17], pancreaticobiliary [18], breast [19] and head and neck carcinoma [20,21] and renal cell carcinoma [22,23]. Loss of 14q was reported to correlate with early age of onset, advanced stage, high grade and poor outcome of the disease [9,11,12,17,20-2]. In our series we also found a trend that loss of 14q is more common in advanced stage tumors and in tumors with distant metastases. These findings indicate that inactivation of the putative 14q31-32 tumor suppressor gene is involved in disease progression. Furthermore, in concordance with this is our finding of no 14q loss (with markers D14S1035, D14S1037, D14S1052, D14S67 and D14S1058) in 11 Barrett's dysplasia samples, including 6 high grades (data not shown). However, the 14q loss found in tumor Ba10 was also detected in the tumor-adjacent dysplastic Barrett's epithelium (Ba10d, Figures 5.2 and 5.3).

To our knowledge no clear candidate 14q31.1-32.11 tumor suppressor genes have been found to date. Seven known genes are present in the region between D14S1000 and D14S256 (derived from: http://genome.ucsc.edu/; freeze May 2004, Build 35) [33]. From centromeric to telomeric these are: fibronectin leucine rich transmembrane protein 2 (FLRT2), galactosylceramidase (GALC), G-protein-coupled receptor 65 (GPR65) or T-cell death-associated protein 8 (TDAG8), potassium channel subfamily K member 10 (KCNK10), spermatogenesis associated 7 (SPATA7), protein tyrosine phosphatase non receptor type 21 (PTPN21) and echinoderm microtubule associated protein like 5 (EML5 or EMAP-2). From current literature data it can be deduced that 3 of the genes have potential as tumor suppressor genes. The function of FLRT2 has been suggested to be in cell adhesion [36] and as such this protein could have a tumor suppressor activity. However, recently evidence has been obtained that FLRT2 has potential as an oncogene as the protein is involved in fibroblast growth factor (FGF) signaling [37]. GPR65/TDAG8 was originally described to function in activation-induced T-cell death [38]. However, recently GPR65/TDAG8 has been found to have oncogenic capacity and the protein is over-expressed in several human cancers [39]. PTPN21 is a tyrosine phosphatase and recently inactivating mutations in PTPN family members have been described in colorectal cancer although no mutations were found in PTPN21 [40]. From the other identified 14q31.1-32.11 genes GALC, KCNK10, SPATA7 and EML5/EMAP-2 no tumor suppressor function is known to date.

The relevance of all the abovementioned genes in GEJ adenocarcinogenesis has to be determined. In addition, it cannot be excluded that the region between markers D14S1000 and D14S256 contains other tumor suppressor genes yet to be discovered. Despite our data, it is also possible that the 14q GEJ adenocarcinoma tumor suppressor gene(s) reside outside the detected MRO. And finally it should be recognized that even frequent loss of a certain chromosomal region can be a bystander phenomenon and not causally related to the tumorigenic process.



In our previous CGH study we found that 14q loss occurred significantly more frequent in esophageal adenocarcinomas than in gastric cardia tumors [6]. In the present study this relationship was not confirmed. Also in a larger CGH study (van Dekken, unpublished results) and in a microsatellite study no relation was found between the location of the tumor and loss of 14q [41]. Besides phenotypical similarities, adenocarcinomas of the esophagus and gastric cardia apparently also have similar genotypes, as suggested by others [42,43].

In conclusion, our study has contributed to unravelling the complex molecular alterations present in adenocarcinomas of the GEJ. We have comprehensively mapped the deletions of chromosome 14q that were previously demonstrated in this disease. The reliability of our findings has been greatly enhanced by the use of xenografts and in vitro cell cultures. We have determined an overlapping region of LOH, possibly harboring a GEJ adenocarcinoma suppressor gene. This result in combination with the available data base information on the genes present in this region can facilitate the identification of the candidate tumor suppressor gene(s).

ACKNOWLEDGMENTS

We thankfully acknowledge the foundation Gastrostart of the Dutch Society for Gastroenterology. We thank Frank van der Panne for the photographic work.

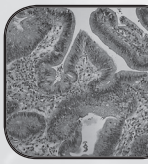
REFERENCES

1. Dolan K, Sutton R, Walker SJ, et al. New classification of oesophageal and gastric carcinomas derived from changing patterns in epidemiology. Br J Cancer 1999;80:834-42.
2. Wijnhoven BP, Siersema PD, Hop WC, et al. Adenocarcinomas of the distal oesophagus and gastric cardia are one clinical entity. Br J Surg 1999;86:529-35
3. Devesa SS, Blot WJ, Fraumeni JF. Changing patterns in the incidence of esophageal and gastric carcinoma in the United States. Cancer 1998;83:2049-53
4. Menke-Pluymers MB, Schoute NW, Mulder AH, et al. Outcome of surgical treatment of adenocarcinoma in Barrett's oesophagus. Gut 1992;33:1454-8
5. DeMeester TR. Esophageal carcinoma: current controversies. Semin Surg 1997;13:217-33
6. van Dekken H, Geelen E, Dinjens WN, et al. Comparative genomic hybridization of cancer of the gastroesophageal junction: deletion of 14q31-32.1 discriminates between esophageal (Barrett's) and gastric cardia adenocarcinomas. Cancer Res 1998;59:748-52
7. Moskaluk CA, Hu J, Perlman EJ. Comparative genomic hybridization of esophageal and gastroesophageal adenocarcinomas shows consensus areas of DNA gain and loss. Genes Chromosomes Cancer 1998;22:305-11
8. Du Plessis L, Dietzsch E, Van Gele M, et al. Mapping of novel regions of DNA gain and loss by comparative genomic hybridization in esophageal carcinoma in the Black and Colored populations of South Africa. Cancer Res 1999;59:1877-83
9. Young J, Leggett B, Ward M, et al. Frequent loss of heterozygosity on chromosome 14 occurs in advanced colorectal carcinomas. Oncogene 1993;3:671-5
10. Bando T, Kato Y, Ihara Y, et al. Loss of heterozygosity of 14q32 in colorectal carcinoma. Cancer Genet Cytogenet 1999;111:161-5
11. Weber TK, Conroy J, Keitz B, et al. Genome-wide allelotyping indicates increased loss of heterozygosity on 9p and 14q in early age of onset colorectal cancer. Cytogenet Cell Genet 1999;86:142-7
12. Thorstensen L, Qvist H, Nesland JM, et al. Identification of two potential suppressor gene regions on chromosome arm 14q that are commonly lost in advanced colorectal carcinomas. Scand J Gastroenterol 2001;12:1327-31
13. Tamura G, Sakata K, Nishizuka S, et al. Allelotype of adenoma and differentiated adenocarcinoma of the stomach. J Pathol 1996;180:371-7
14. Nishizuka S, Tamura G, Terashima M, et al. Loss of heterozygosity during the development and progression of differentiated adenocarcinoma of the stomach. J Pathol 1998;85:38-43
15. El-Rifai W, Sarlomo-Rikala M, Andersson LC, et al. High-resolution deletion mapping of chromosome 14 in stromal tumors of the gastrointestinal tract suggests two distinct tumor suppressor loci. Genes Chromosomes Cancer 2000;27:387-91
16. Bandera CA, Takahashi H, Behbakht K, et al. Deletion mapping of two potential chromosome 14 tumor suppressor gene loci in ovarian carcinoma. Cancer Res 1996;57:513-5
17. Chang WY, Cairns P, Schoenberg MP, et al. Novel suppressor loci on chromosome 14q in primary bladder cancer. Cancer Res 1995;55:3246-9
18. Iacobuzio-Donahue CA, van der Heijden MS, Baumgartner MR, et al. Large-scale allelotype of pancreaticobiliary carcinoma provides quantitative estimates of genome-wide allelic loss. Cancer Res 2004;64:871-5
19. Wang ZC, Lin M, Wei LJ, et al. Loss of heterozygosity and its correlation with expression profiles in subclasses of invasive breast cancers. Cancer Res 2004;64:64-71
20. Lee DJ, Koch WM, Yoo G, et al. Impact of chromosome 14q loss on survival in primary head and neck squamous cell carcinoma. Clin Cancer Res 1997;3:501-5
21. Bockmuhl U, Schluns K, Schmidt S, et al. Chromosomal alterations during metastasis formation of head and neck squamous cell carcinoma. Genes Chromosomes Cancer 2002;33:29-35
22. Schullerus D, Herbers J, Chudek J, et al. Loss of heterozygosity at chromosomes 8p, 9p, and 14q is associated with stage and grade of non-papillary renal cell carcinomas. J Pathol 1997;183:151-5
23. Alimov A, Sundelin B, Wang N, et al. Loss of 14q31-q32.2 in renal cell carcinoma is associated with high malignancy grade and poor survival. Int J Oncol 2004;25:179-85

24. Pylkkanen L, Sainio M, Ollikainen T, et al. Concurrent LOH at multiple loci in human malignant mesothelioma with preferential loss of NF2 gene region. Oncol Rep 2002;9:955-9
25. Takayama H, Suzuki T, Mugishima H, et al. Deletion mapping of chromosomes 14q and 1p in human neuroblastoma. Oncogene 1992;7:1185-9
26. Weber RG, Bostrom J, Wolter M, et al. Analysis of genomic alterations in benign, atypical, and anaplastic meningiomas: toward a genetic model of meningioma progression. Proc Natl Acad Sc USA 1997;94:14719-24
27. Ihara Y, Kato Y, Bando T, et al. Allelic imbalance of 14q32 in esophageal carcinoma. Cancer Genet Cytogenet 2002;135:177-81
28. Dichamp C, Taillibert S, Aguirre-Cruz L, et al. Loss of 14q chromosome in oligodendroglial and astrocytic tumors. J Neurooncol 2004;67:281-5
29. Rockett JC, Larkin K, Darnton SJ, et al. Five newly established oesophageal carcinoma cell lines: phenotypic and immunological characterization. Br J Cancer 1997;75:258-63
30. de Both NJ, Wijnhoven BP, Sleddens HF, et al. Establishment of cell lines from adenocarcinomas of the esophagus and gastric cardia growing in vivo and in vitro. Virchows Archiv 2001;438:451-6
31. Dib C, Faure S, Fizames C, et al. A comprehensive genetic map of the human genome based on 5,264 microsatellites. Nature 1996;380:152-4
32. Heilig R, Eckenberg R, Petit JL, et al. The DNA sequence and analysis of human chromosome 14. Nature 2003;421:601-7
33. International Human Genome Sequencing Consortium. Finishing the euchromatic sequence of the human genome. Nature 2004;431:931-46
34. Vogelstein B, Fearon ER, Kern SE, et al. Allelotype of colorectal carcinomas. Science 1989;244:207-11
35. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. Nature 1998;396:643-9
36. Lacy SE, Bönnemann CG, Buzney EA, et al. Identification of FLRT1, FLRT2, and FLRT3: a novel family of transmembrane leucine-rich repeat proteins. Genomics 1999;62:417-26
37. Böttcher RT, Pollet N, Delius H, et al. The transmembrane protein XFLRT3 forms a complex with FGF receptors and promotes FGF signaling. Nature Cell Biology 2004;6:38-44
38. Choi JW, Lee SY, Choi Y. Identification of a putative G protein-coupled receptor induced during activation-induced apoptosis of T cells. Cell Immunol 1996;168:78-84
39. Sin WC, Zhang Y, Zhong W, et al. G protein-coupled receptors GPR4 and TDAG8 are oncogenic and overexpressed in human cancers. Oncogene 2004;23:6299-303
40. Wang Z, Shen D, Parsons DW, et al. Mutational analysis of the tyrosine phosphatome in colorectal cancers. Science 2004;304:1164-6
41. Yanagi M, Keller G, Mueller J, et al. Comparison of loss of heterozygosity and microsatellite instability in adenocarcinomas of the distal esophagus and proximal stomach. Virchows Arch 2000;437:605-10
42. Gleeson CM, Sloan JM, McManus DT, et al. Comparison of p53 and DNA content abnormalities in adenocarcinoma of the oesophagus and gastric cardia. Br J Cancer 1998;77:277-86
43. Ireland AP, Shibata DK, Chandrasoma P, et al. Clinical significance of p53 mutations in adenocarcinoma of the esophagus and cardia. Ann Surg 2000;231:179-87

Chapter 6

Neuroendocrine cells in Barrett's mucosa and adenocarcinomas of the gastro-esophageal junction



International Journal of Surgical Pathology, 2004 Apr;12(2):117-25

Linetta B Koppert
Bas PL Wijnhoven
Hugo W Tilanus
Theo Stijnen
Herman van Dekken
Winand NM Dinjens

ABSTRACT

We estimated the prevalence and prognostic significance of neuroendocrine (NE) cells in a series of 208 resection specimens containing gastro-esophageal junction (GEJ) adenocarcinomas, with 56 specimens containing Barrett's mucosa. Immunohistochemically, chromogranin A (CGA) was positive in 49% (102/208) of GEJ adenocarcinomas and in 68% (38/56) of Barrett's mucosas. CGA in GEJ tumors correlated with pTNM stage. CGA in Barrett correlated with pTNM stage and tumor grade of the adjacent carcinoma. Patients with CGA in Barrett had better survival than patients without CGA in Barrett, with 5-year survival percentages of 56% and 9% respectively. In multivariate analysis, CGA in Barrett was an independent prognostic factor for survival after surgery. Therefore CGA in Barrett adjacent to GEJ adenocarcinoma might be helpful in the assessment of patient outcome.

INTRODUCTION

Neuroendocrine (NE) cells are defined as argentaffin and argyrophil cells that produce peptides or amines. They belong to the Diffuse Neuroendocrine System (DNES) and were previously known as amino-precursor-uptake-decarboxylation (APUD) cells. The neuroendocrine system of the normal gastro-intestinal tract might regulate proliferation and growth of epithelial and mesenchymal cells and probably function in sensation of hunger during fasting and food-intake [1]. Chromogranin A (CGA), a specific matrix component of endocrine granules, participates in vesicle aggregation, granulogenesis, and hormone secretion and serves as a precursor for bioactive peptides (prohormone function) in endocrine and NE cells [2-4]. CGA is stored in secretory granules of NE cells and is regarded as a general endocrine marker [5-7]. The presence of NE cells in carcinomas of the gastrointestinal tract is well documented but their role remains speculative [8-21]. In a number of immunohistochemical studies a prognostic relevance of NE cells in adenocarcinomas, mostly colorectal adenocarcinomas, has been reported, however, several other studies failed to demonstrate a relation between NE differentiation and biological behavior of colorectal adenocarcinomas [9-11,14-22]. Reports on the prevalence and prognostic significance of NE cells in adenocarcinomas of the esophagus and its precursor lesion, the Barrett's mucosa, have been scarce. Hamilton et al. did not find a significant correlation between the presence of chromogranin A (CGA) immunoreactive tumor cells and survival in patients with esophageal adenocarcinoma [23]. We analyzed the presence of NE cells in 208 resection specimens with adenocarcinoma of the gastro-esophageal junction (GEJ), i.e. gastric cardia or distal esophagus, and premalignant Barrett's mucosa and correlated immunostaining with tumor characteristics and patient survival in order to evaluate the possible application of NE cells as prognostic marker.

MATERIALS AND METHODS

Tissues and Patients

Two hundred and eight patients (176 men; 32 women) with GEJ adenocarcinoma who underwent transhiatal resection of the tumor with restoration of continuity of the gastrointestinal tract by a gastric tube with cervical anastomosis were included in this study. Patients were operated between April 1987 and April 2002 at the Department of Surgery, Erasmus MC, Rotterdam. A standard dissection of the perigastric, left gastric and coeliac nodes was performed. Macroscopic tumor clearance was aimed at in all cases but no extended lymph node dissection was done. Seventeen patients received neoadjuvant chemotherapy and 2 patients received neoadjuvant chemoradiation. Patient's mean age was 63.6 year (range 39-84 years) at the time of diagnosis. All patients were followed up until April 2003 or until death if earlier. All 208 pathology records were reviewed. Barrett's mucosa was diagnosed by the GI-patholo-

gist and was defined as the presence of intestinal type epithelium with Goblet cells in the tubular esophagus [24]. In 73 patients Barrett's mucosa had been sampled prior to development of adenocarcinoma. Barrett's mucosa adjacent to tumor could be obtained in 56 out of these 73 resection specimens, whereas in 17 resection specimens the Barrett's mucosa could not be detected in the resection specimen. Barrett's mucosa showed no signs of dysplasia in 22, low grade dysplasia in 22 and high grade dysplasia in 12 resection specimens. A carcinoma was considered to arise from the distal esophagus when premalignant Barrett's mucosa was present and/or the epicenter of the mass was located in the tubular esophagus extending from the tracheal bifurcation to the gastro-esophageal junction including the intra-abdominal esophagus, according to the TNM classification (International Classification of Diseases for Oncology C15.5). The tumor was considered to be cardiac when the epicenter was immediately below the gastro-esophageal junction, extending approximately 2 cm downwards. The tumor was classified as a junction carcinoma when the epicenter was just at the GEJ, without predominance for distal esophagus or gastric cardia and no Barrett's mucosa was present. Tumors arising from the fundus or corpus of the stomach and infiltrating the gastric cardia or distal esophagus were excluded. Of the adenocarcinomas in our patient group, 112 arose from the distal esophagus and 73 arose from the cardia. The exact location of 23 GEJ adenocarcinomas could not be specified as either distal esophagus or gastric cardia and these were classified as junction carcinomas.

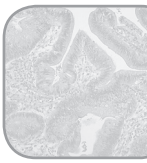
Immunohistochemical Analyses

From formalin-fixed paraffin-embedded tissue blocks of the primary tumor 4 μm thick sections were mounted on 3-aminopropyl-triethoxysilane (APES)-coated glass slides. For immunostaining a monoclonal antibody against CGA (Hybritech, San Diego, USA, at a dilution of 1:1250) was used. Staining was carried out by a standard avidin biotin immunoperoxidase technique, using a commercially available kit (Labvision, Fremont, USA). Deparaffinized sections were treated with methanol containing 3% H_2O_2 for 20 minutes. After washing with phosphate-buffered saline (PBS), blocking serum was applied for 5 minutes. Then, primary CGA antibodies were allowed to react at room temperature for 1 hour. After washing in PBS, biotin-conjugated secondary antibody was applied for 10 minutes followed by peroxidase-marked streptavidin. After rinsing in PBS, peroxidase was visualized by diaminobenzidine hydrochloride (Fluka, Neu-Ulm, Germany) with 0.03% H_2O_2 solution for 10 minutes. The slides were counterstained with Mayer's Haematoxylin and dehydrated in alcohol before mounting. Expression of CGA was evaluated by high power microscopic examination (400 X) of the entire tissue section. As negative controls normal mouse immunoglobulins and normal rabbit serum were applied on duplicate sections. Positive controls using normal colonic epithelium were also run with each batch, in addition to using non-involved normal gastric mucosa in the resection specimens, if present, as an internal positive control. Scoring of cytoplasmic CGA expression in adenocarcinomas was based on the percentage of positive cells: >20% of

cells with cytoplasmic staining (2+), 1-20% of cells with cytoplasmic staining (1+), no cells staining (0).

Statistical Analysis

Correlations between CGA immunoreactivity and patient and tumor characteristics were assessed by T-test and (a trend version of) χ^2 test. Survival rates were calculated according to the Kaplan-Meier method and differences in survival were assessed using the log rank test. $P<0.05$ was considered statistically significant. The Cox regression model was used to analyze the independent prognostic value of CGA expression after correction for possible confounding factors.



RESULTS

CGA expression was detected in 102/208 (49%) adenocarcinomas of the GEJ (Table 6.1). The CGA positive cells mostly presented diffusely scattered throughout the tissue or multifocally located in small nests, with just eight tumors having >20% (2+) CGA positive cells (Figure 6.1). For statistical comparison we hence combined the groups with 1+ and 2+ staining. Negative controls did not show staining and positive controls were positive. In 56 of the 208 resection specimens Barrett's mucosa adjacent to adenocarcinoma was detected. Positive staining for CGA was seen in 38/56 cases (68%). CGA immunoreactivity was absent in 18/38 (47%) tumors with CGA positive Barrett's mucosa (Table 6.1). There was no correlation between CGA immunoreactivity in Barrett's mucosas and CGA immunoreactivity in adenocarcinomas (P=0.57). In the patients with Barrett's mucosa adjacent to tumor, there was no correlation between CGA immunoreactivity in the Barrett and presence or degree of dysplasia (P=0.97 and P=0.65 respectively).

Table 6.1 CGA Expression in Adenocarcinomas of the GEJ and Barrett's Mucosa

	Non-Barrett	Barrett's mucosa		Total
		CGA positive	CGA negative	
Adenocarcinomas				
CGA positive	74	20	8	102
CGA negative	78	18	10	106
Total	152	38	18	208

CGA positive staining in GEJ tumors correlated with a more favorable pTNM stage (P=0.04, Table 6.2). CGA positive staining in Barrett's mucosas correlated with a more favorable pTNM stage and tumor grade (P=0.005 and P=0.024 respectively, Table 6.2). No difference in survival

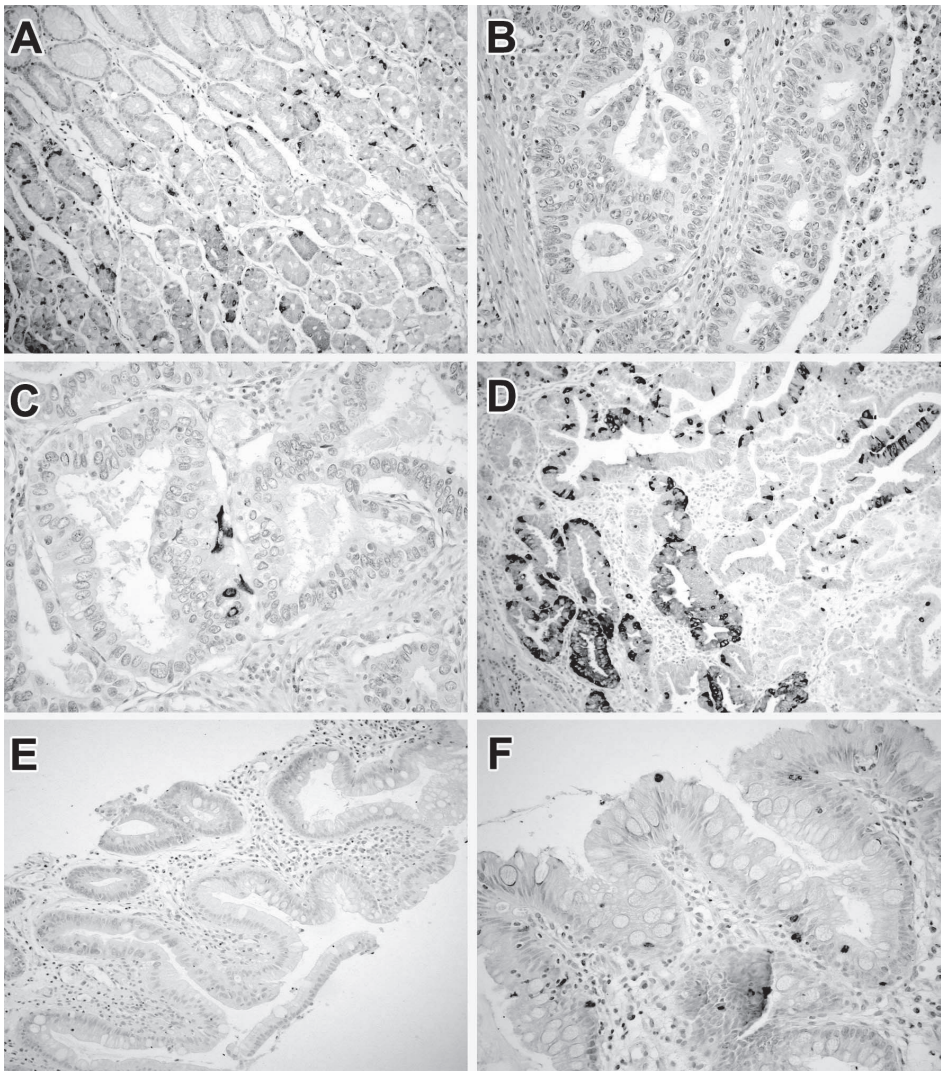
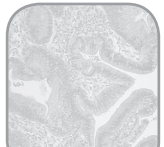


Fig. 6.1 CGA immunoreactivity in normal gastric epithelium (A), CGA negative adenocarcinoma (B). Scatters of individual tumor cells show CGA staining (1+) within a well-differentiated adenocarcinoma (C). Adenocarcinoma shows apparent cytoplasmic CGA staining in >20% (2+) of tumor cells (D). CGA negative Barrett's epithelium adjacent to adenocarcinoma (E), CGA positive cells in Barrett's epithelium most prominently located in the basal layer of the epithelium (F). *Also see color figures page 291.*

Table 6.2 Clinicopathological characteristics of 208 patients with GEJ adenocarcinomas and Barrett's mucosa adjacent to tumor (if present, 56 patients out of 208 patients).

	Chromogranin A in tumor				Chromogranin A in Barrett's mucosa			
Variable	***Number of tumors positive (%)***	***Number of tumors negative (%)***	***Total number of tumors (%)***	***P value*** [a]	***Number of Barrett's positive(%)***	***Number of Barrett's negative (%)***	***Total number of Barrett's (%)***	***P value*** [a]
Gender								
· male	85 (83)	91 (86)	176 (85)		30 (79)	17 (94)	47 (84)	
· female	17 (17)	15 (14)	32 (15)	0.76	8 (21)	1 (6)	9 (16)	0.25
Mean age (years) ± SD	63.6 ± 10.1	63.6 ± 9.2	63.6 ± 9.7	0.94 [b]	64.8 ± 10.0	64.2 ± 10.9	64.6 ± 10.2	0.99 [b]
pTNM-stage								
· I	11 (11)	5 (5)	16 (8)		10 (26)	-	10 (18)	
· II	34 (33)	33 (31)	67 (32)		16 (42)	4 (22)	20 (36)	
· III	48 (47)	50 (47)	98 (47)		7 (18)	11 (61)	18 (32)	
· IV	9 (9)	18 (17)	27 (13)	0.04	5 (13)	3 (17)	8 (14)	0.005
Tumor grade								
· well	5 (5)	4 (4)	9 (4)		4 (11)	-	4 (7)	
· moderate	47 (46)	41 (39)	88 (42)		18 (47)	5 (28)	23 (41)	
· poor	50 (49)	61 (58)	111 (53)	0.23	16 (42)	13 (72)	29 (52)	0.024
Radicality								
· R0	71 (70)	76 (72)	147 (71)		29 (76)	11 (61)	40 (71)	
· R1, R2	31 (30)	30 (28)	61 (29)	0.86	9 (24)	7 (39)	16 (29)	0.39
Tumor location								
· esophag.	58 (57)	54 (51)	112 (54)		38	18	56	
· GEJ	8 (8)	15 (14)	23 (11)		-	-	-	
· cardia	36 (35)	37 (35)	73 (35)	0.67	-	-	-	-
Dysplasia								
· absent					15 (40)	7 (39)	22 (39)	
· low grade					16 (42)	6 (33)	22 (39)	
· high grade					7 (18)	5 (28)	12 (21)	0.65

[a] P value by (a trend version of) χ^2 test; [b] P value by T-test.



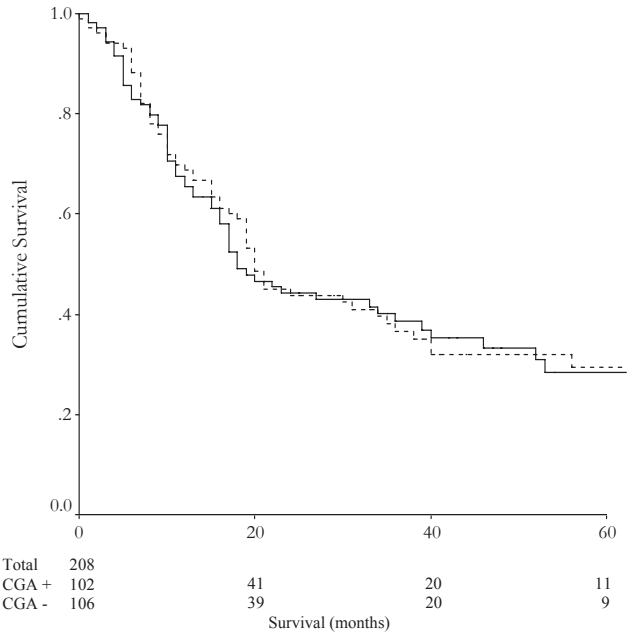


Fig. 6.2 Cumulative survival of patients with (n=102) and without (n=106) CGA immunoreactivity in adenocarcinomas of the GEJ (P=.69). Broken line represents CGA positive tumors, uninterrupted line represents CGA negative tumors.

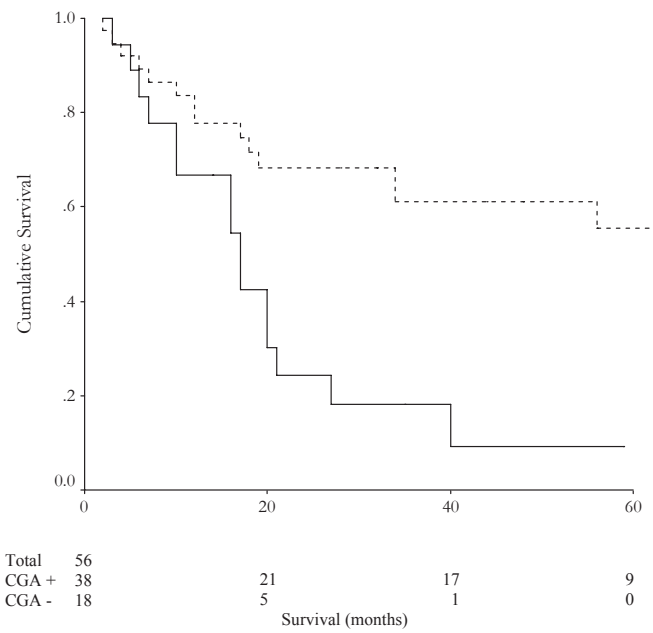


Fig. 6.3 Cumulative survival of patients with (n=38) and without (n=18) CGA immunoreactivity in Barrett's mucosa adjacent to adenocarcinoma (P=.0015). Broken line represents CGA positive Barrett's mucosas, uninterrupted line represents CGA negative Barrett's mucosas.

between patients with CGA positive and CGA negative adenocarcinomas was found (P=0.69, Figure 6.2). Five year survival percentages were 30% and 28% respectively. However, patients with CGA positive cells in Barrett's mucosa adjacent to the tumor had a better survival than patients without CGA positive cells in Barrett's mucosa (P=0.0015, Figure 6.3). Five year survival percentages were 56% and 9% for patients with and without CGA expression in Barrett respectively. Univariate analysis to identify prognostic variables in the total group showed pTNM stage, tumor grade and radicality of resection to be prognostic factors for survival (P=<0.001, P=0.012 and P=<0.001 respectively). In multivariate Cox regression analysis only pTNM-stage and radicality of resection turned out to be independent prognostic factors for survival in the total patient group (P=0.003 and P=0.006, Table 6.3A). However, in the group of patients with Barrett's mucosa, univariate analysis showed age, radicality of resection and CGA immunoreactivity in the Barrett's mucosa to be prognostic for survival (P=0.035, P=0.008 and P=0.003 respectively, Table 6.3B), which was substantiated by multivariate analysis (P=0.03, P=0.037 and P=0.003 respectively, Table 6.3B).

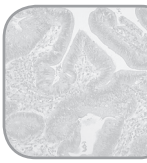


Table 6.3. Results of the Cox Regression Analyses in (A.) 208 GEJ Adenocarcinomas and in (B.) 56 Barrett's mucosas (adjacent to tumor), as part of the series of 208 GEJ adenocarcinomas

A.

Confounding Variable	Univariate RR[a] (CI[b])	P value	Multivariate-Adjusted RR[c] (CI)	P value
Age	1.01 (1.0-1.03)	0.15	1.02 (1.0-1.04)	0.11
Gender (M, F)	0.94 (0.59-1.52)	0.81	0.75 (0.45-1.25)	0.27
pTNM-stage I	-	<0.001	-	0.003
II	3.76 (1.16-12.22)	-	2.88 (0.88-9.48)	-
III	8.43 (2.63-27.04)	-	5.35 (1.63-17.56)	-
IV	8.08 (2.38-27.40)	-	5.22 (1.50-18.20)	-
Tumor grade well	-	0.012	-	0.118
moderate	6.84 (0.94-49.51)	-	4.95 (0.68-36.04)	-
Poor	9.98 (1.38-71.98)	-	6.24 (0.85-45.68)	-
Radicality of resection (R0 vs R1, R2)	2.43 (1.69-3.49)	<0.001	1.73 (1.17-2.57)	0.006
CGA tumor (positive vs negative)	1.09 (0.77-1.55)	0.63	1.12 (0.78-1.61)	0.56

B.

Confounding Variable	Univariate RR[a] (CI[b])	P value	Multivariate-Adjusted RR[c] (CI)	P value
Age	1.04 (1.0-1.08)	0.035	1.04 (1.0-1.10)	0.030
pTNM-stage (I, II vs III, IV)	1.92 (0.91-4.03)	0.086	0.63 (0.22-1.83)	0.395
Tumor grade (well, moderate vs poor)	1.33 (0.65-2.71)	0.435	0.93 (0.35-2.45)	0.883
Radicality of resection (R0 vs R1, R2)	2.63 (1.28-5.40)	0.008	2.91 (1.07-7.95)	0.037
CGA Barrett mucosa (positive vs negative)	3.12 (1.48-6.58)	0.003	4.21 (1.61-11.0)	0.003

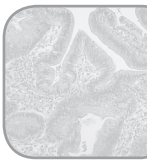
[a] Relative Risk; [b] 95% Confidence Interval. [c] In multivariate analysis correction was carried out for the confounding variables age, gender, pTNM-stage, tumor grade, radicality of resection and CGA immunoreactivity in the tumors (A.), age, pTNM-stage, tumor grade, radicality resection and CGA immunoreactivity in Barrett's mucosa (B.), variables are mentioned in the column 'Confounding Variables'.

DISCUSSION

Our study, which showed that the presence of NE cells in GEJ tumors did not correlate with 5-year survival rate, is in concordance with the results reported by Hamilton et al. They investigated the expression of CGA in 52 patients with adenocarcinomas of the esophagus and did not find a correlation with survival [23]. We also confirm their findings of CGA positive Barrett's mucosas with adjacent CGA negative tumors (namely in 38% of their tumors and in 47% of our tumors). Obviously neuroendocrine differentiation common disappears in invasive adenocarcinomas. We likewise observed NE cells more often in Barrett's mucosa without dysplasia or with low grade dysplasia than in high grade dysplastic Barrett's mucosas, although this difference lacked statistical significance. Hamilton et al. found expression of CGA in 62% (21/34) of the Barrett's mucosas, as compared to our finding of 68% (38/56). In our study patients with CGA positive Barrett's mucosa had a better survival rate than patients with CGA negative Barrett's mucosa. Our study differs from the study of Hamilton et al. in several ways. The monoclonal CGA antibody we used differs from the antibody used by Hamilton et al. Our study encompasses 208 patients versus 52 patients in the study of Hamilton et al. Finally, their population contained 37 of 52 patients who underwent preoperative therapy, compared to 19 of our 208 patients, and this might influence CGA staining. However, when the 19 patients that received preoperative therapy were left out from the analyses, our results did not change. Moreover, Shia et al. suggested that the increased endocrine differentiation shown in rectal adenocarcinomas treated by chemo(radio)therapy could be related to therapy induced cytotoxity which is in contrast with the lower percentage of CGA positive patients although higher percentage of pretreated patients in the study of Hamilton et al. as compared to our study [25]. Since adenocarcinomas of the distal esophagus and the gastric cardia are regarded

as one clinical entity by some authors [26,27], we investigated the expression of NE markers in adenocarcinomas of the GEJ whereas in the study of Hamilton et al. only esophageal adenocarcinomas were included. Because the prevalence of NE differentiation in our series was about the same in esophageal and cardia adenocarcinomas, the different results cannot be ascribed to the fact that in our study cardia adenocarcinomas were included.

Several studies concerning colorectal carcinomas showed that the presence of CGA positive cells does not influence prognosis [14-19], whereas other studies indicate that CGA expression in tumor cells might distinguish a subgroup of colorectal carcinomas with poorer prognosis [10-12,21]. Swatek and Chibowski reported that endocrine cells were significantly more frequent in less advanced and better differentiated colorectal carcinomas, using immunostaining for CGA [13]. Two other studies showed a significantly better survival in patients with NE expression in pancreatic cancer and non small cell lung cancer [28,29].



In the current study we demonstrated that CGA expression in Barrett's mucosa adjacent to the tumor is an independent predictor of improved survival after surgery for GEJ adenocarcinomas. To our knowledge this is the first example that in the concurrent presence of a premalignant lesion and a carcinoma a characteristic of the premalignant lesion has prognostic significance. An explanation for this finding remains obscure. The presence of NE cells in Barrett's epithelium has been described by several authors [30-34], suggesting that it arises from a multipotent gastrointestinal stem cell probably responsible for the risk of adenocarcinoma [31,34]. Smith and Haggitt have described 4 explanations for the presence of NE cells in non-carcinoid adenocarcinomas of the gastrointestinal tract [14]: 1) entrapment of normal NE cells within the malignant tumor; 2) benign proliferation of NE cells within a malignant population of intestinal cells; 3) malignant transformation of 2 distinct stem cell lines (1 neuroendocrine and 1 endodermal); and 4) malignant transformation of 1 stem cell line capable of both endodermal and neuroendocrine differentiation. The last hypothesis has found support from several studies [20,35], including that by De Bruïne et al., who demonstrated mucin and CGA expression in the colorectal cell line H716 [36].

Since NE cells comprise an integral part of the intestinal epithelium, the presence of NE cells in Barrett's mucosa can be the mere result of the intestinal type differentiation. However, additional factors in NE differentiation can be involved. Duodeno-gastro-esophageal reflux is known to be a risk factor for the development of Barrett's mucosa. Reflux disease is often treated by acid-suppressive therapy. Sanduleanu et al. found that serum CGA increases during profound gastric acid inhibition [37]. Furthermore, Helicobacter pylori infection was associated with higher serum CGA levels [37]. It should therefore be addressed that both long-term acid-suppressive therapy and Helicobacter pylori status might play a role in CGA expression in Barrett's mucosa and adenocarcinomas of the GEJ. Unfortunately, we were not able to obtain reliable information on previous acid-suppressive therapy or Helicobacter pylori status in our patient population.

Colombo et al. investigated the effect of CGA on neoplastic growth and morphogenesis by use of mouse models [38]. They found slower progression of mouse mammary adenocarcinoma after transfection of CGA cDNA and suggested that CGA may contribute to regulate tumor growth in a negative manner. Given the fact that distal esophageal adenocarcinomas often develop from a precursor lesion, i.e. Barrett's mucosa, being present for years already prior to tumor formation, we were able to investigate the significance of CGA on tumor growth in 56 patients with Barrett available adjacent to tumor [39]. CGA expression in these Barrett's mucosas indeed correlated with less advanced pTNM stage and tumor grade of adjacent tumors as compared to Barrett related carcinomas without CGA in the precursor lesion. Furthermore, CGA expression in the Barrett's mucosa came forward as an independent predictor of survival in Cox regression analysis. Future experiments focussing on transfection of CGA cDNA in Barrett's metaplasia cell lines could possibly gain more insight in the role of CGA in GEJ adenocarcinoma development.

In summary, the current study demonstrates NE differentiation in Barrett's epithelium to be correlated with survival in patients with Barrett's associated adenocarcinomas of the GEJ. CGA immunoreactivity in Barrett's mucosa adjacent to tumor is an independent prognostic factor for better survival after surgery. It appears from these data, obtained in a large patient group, that CGA expression in Barrett's mucosa might be helpful in the prognostic assessment of patient outcome.

ACKNOWLEDGMENTS

The authors would like to thank ECW den Adel and J van Velzen for technical assistance, CM Vollebregt-Uiterwijk for assistance in collecting the data and F van der Panne for photographic work.

REFERENCES

1. Hill P, Garbaczewski L, Koppeschaar H, Thijssen JH, de Waard F, Wynder EL: Peptide and steroid hormones in subjects at different risk for diet- related diseases. Am J Clin Nutr 48:782-6, 1988
2. Iacangelo AL, Eiden LE: Chromogranin A: current status as a precursor for bioactive peptides and a granulogenic/sorting factor in the regulated secretory pathway. Regul Pept 58:65-88, 1995
3. Winkler H, Fischer-Colbrie R: The chromogranins A and B: the first 25 years and future perspectives. Neuroscience 49:497-528, 1992
4. Kim T, Tao-Cheng JH, Eiden LE, Loh YP: Chromogranin A, an "on/off" switch controlling dense-core secretory granule biogenesis. Cell 106:499-509, 2001
5. Lloyd RV, Wilson BS: Specific endocrine tissue marker defined by a monoclonal antibody. Science 222:628-30, 1983
6. Chejfec G, Falkmer S, Askensten U, Grimelius L, Gould VE: Neuroendocrine tumors of the gastrointestinal tract. Pathol Res Pract 183:143-54, 1988
7. Wilson BS, Lloyd RV: Detection of chromogranin in neuroendocrine cells with a monoclonal antibody. Am J Pathol 115:458-68, 1984
8. Ulich TR, Cheng L, Glover H, Yang K, Lewin KJ: A colonic adenocarcinoma with argentaffin cells. An immunoperoxidase study demonstrating the presence of numerous neuroendocrine products. Cancer 51:1483-9, 1983
9. Arends JW, Wiggers T, Verstijnen K, Bosman FT: The occurrence and clinicopathological significance of serotonin immunoreactive cells in large bowel carcinoma. J Pathol 149:97-102, 1986
10. Hamada Y, Oishi A, Shoji T, Takada H, Yamamura M, Hioki K, Yamamoto M: Endocrine cells and prognosis in patients with colorectal carcinoma. Cancer 69:2641-6, 1992
11. de Bruine AP, Wiggers T, Beek C, Volovics A, von Meyenfeldt M, Arends JW, Bosman FT: Endocrine cells in colorectal adenocarcinomas: incidence, hormone profile and prognostic relevance. Int J Cancer 54:765-71, 1993
12. Grabowski P, Schindler I, Anagnostopoulos I, Foss HD, Riecken EO, Mansmann U, Stein H, Berger G, Buhr HJ, Scherubl H: Neuroendocrine differentiation is a relevant prognostic factor in stage III-IV colorectal cancer. Eur J Gastroenterol Hepatol 13:405-11, 2001
13. Swatek J, Chibowski D: Endocrine cells in colorectal carcinomas. Immunohistochemical study. Pol J Pathol 51:127-36, 2000
14. Smith DM, Jr, Haggitt RC: The prevalence and prognostic significance of argyrophil cells in colorectal carcinomas. Am J Surg Pathol 8:123-8, 1984
15. Foley EF, Gaffey MJ, Frierson HF, Jr.: The frequency and clinical significance of neuroendocrine cells within stage III adenocarcinomas of the colon. Arch Pathol Lab Med 122:912-4, 1998
16. Secco GB, Campora E, Fardelli R, Lapertosa G, De Lucchi F, Gianquinto D, Bonfante P: Chromogranin-A expression in neoplastic neuroendocrine cells and prognosis in colorectal cancer. Tumori 82:390-3, 1996
17. Mori M, Mimori K, Kamakura T, Adachi Y, Ikeda Y, Sugimachi K: Chromogranin positive cells in colorectal carcinoma and transitional mucosa. J Clin Pathol 48:754-8, 1995
18. Pagani A, Papotti M, Abbona GC, Bussolati G: Chromogranin gene expressions in colorectal adenocarcinomas. Mod Pathol 8:626-32, 1995
19. Ferrero S, Buffa R, Pruneri G, Siccardi AG, Pelagi M, Lee AK, Coggi G, Bosari S: The prevalence and clinical significance of chromogranin A and secretogranin II immunoreactivity in colorectal adenocarcinomas. Virchows Arch 426:587-92, 1995
20. Park JG, Choe GY, Helman LJ, Gazdar AF, Yang HK, Kim JP, Park SH, Kim YI: Chromogranin-A expression in gastric and colon cancer tissues. Int J Cancer 51:189-94, 1992
21. Indinnimeo M, Cicchini C, Memeo L, Stazi A, Provenza C, Ricci F, Mingazzini PL: Correlation between chromogranin-A expression and pathological variables in human colon carcinoma. Anticancer Res 22:395-8, 2002
22. Yao GY, Zhou JL, Lai MD, Chen XQ, Chen PH: Neuroendocrine markers in adenocarcinomas: an investigation of 356 cases. World J Gastroenterol 9:858-61, 2003
23. Hamilton K, Chiappori A, Olson S, Sawyers J, Johnson D, Washington K: Prevalence and prognostic significance of neuroendocrine cells in esophageal adenocarcinoma. Mod Pathol 13:475-81, 2000

24. Spechler SJ, Goyal RK: Barrett's esophagus. N Engl J Med 315:362-71, 1986
25. Shia J, Tickoo SK, Guillem JG, Qin J, Nissan A, Hoos A, Stojadinovic A, Ruo L, Wong WD, Paty PB, Weiser MR, Minsky BD, Klimstra DS: Increased endocrine cells in treated rectal adenocarcinomas: a possible reflection of endocrine differentiation in tumor cells induced by chemotherapy and radiotherapy. Am J Surg Pathol 26:863-72, 2002
26. Wijnhoven BP, Siersema PD, Hop WC, van Dekken H, Tilanus HW: Adenocarcinomas of the distal oesophagus and gastric cardia are one clinical entity.Rotterdam Oesophageal Tumor Study Group. Br J Surg 86:529-35, 1999
27. Ruol A, Parenti A, Zaninotto G, Merigliano S, Costantini M, Cagol M, Alfieri R, Bonavina L, Peracchia A, Ancona E: Intestinal metaplasia is the probable common precursor of adenocarcinoma in barrett esophagus and adenocarcinoma of the gastric cardia. Cancer 88:2520-8, 2000
28. Tezel E, Nagasaka T, Nomoto S, Sugimoto H, Nakao A: Neuroendocrine-like differentiation in patients with pancreatic carcinoma. Cancer 89:2230-6, 2000
29. Schleusener JT, Tazelaar HD, Jung SH, Cha SS, Cera PJ, Myers JL, Creagan ET, Goldberg RM, Marschke RF, Jr.: Neuroendocrine differentiation is an independent prognostic factor in chemotherapy-treated nonsmall cell lung carcinoma. Cancer 77:1284-91, 1996
30. Griffin M, Sweeney EC: The relationship of endocrine cells, dysplasia and carcinoembryonic antigen in Barrett's mucosa to adenocarcinoma of the oesophagus. Histopathology 11:53-62, 1987
31. Rindi G, Bishop AE, Daly MJ, Isaacs P, Lee FI, Polak JM: A mixed pattern of endocrine cells in metaplastic Barrett's oesophagus. Evidence that the epithelium derives from a pluripotential stem cell. Histochemistry 87:377-83, 1987
32. Buchan AM, Grant S, Freeman HJ: Regulatory peptides in Barrett's oesophagus. J Pathol 146:227-34, 1985
33. Jaskiewicz K, Louw J, Anichkov N: Barrett's oesophagus: mucin composition, neuroendocrine cells, p53 protein, cellular proliferation and differentiation. Anticancer Res 14:1907-12, 1994
34. Feurle GE, Helmstaedter V, Buehring A, Bettendorf U, Eckardt VF: Distinct immunohistochemical findings in columnar epithelium of esophageal inlet patch and of Barrett's esophagus. Dig Dis Sci 35:86-92, 1990
35. Kirkland SC: Clonal origin of columnar, mucous, and endocrine cell lineages in human colorectal epithelium. Cancer 61:1359-63, 1988
36. de Bruine AP, Dinjens WN, Pijls MM, vd Linden EP, Rousch MJ, Moerkerk PT, de Goeij AF, Bosman FT: NCI-H716 cells as a model for endocrine differentiation in colorectal cancer. Virchows Arch B Cell Pathol Incl Mol Pathol 62:311-20, 1992
37. Sanduleanu S, De Bruine A, Stridsberg M, Jonkers D, Biemond I, Hameeteman W, Lundqvist G, Stockbrugger RW: Serum chromogranin A as a screening test for gastric enterochromaffin- like cell hyperplasia during acid-suppressive therapy. Eur J Clin Invest 31:802-11, 2001
38. Colombo B, Curnis F, Foglieni C, Monno A, Arrigoni G, Corti A: Chromogranin A expression in neoplastic cells affects tumor growth and morphogenesis in mouse models. Cancer Res 62:941-6, 2002
39. Haggitt RC, Tryzelaar J, Ellis FH, Colcher H: Adenocarcinoma complicating columnar epithelium-lined (Barrett's) esophagus. Am J Clin Pathol 70:1-5, 1978

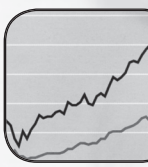
Part III

Epidemiology

Chapter 7

Comparison of comorbidity prevalence in esophageal and gastric carcinoma patients: a population-based study

European Journal of Gastroenterology and Hepatology, 2004 Jul;16(7):681-8



Linetta B Koppert
Maryska LG Janssen-Heijnen
Marieke WJ Louwman
Valery EPP Lemmens
Bas PL Wijnhoven
Hugo W Tilanus
Jan-Willem W Coebergh

ABSTRACT

We investigated the distribution of serious comorbidity in patients with newly diagnosed esophageal and gastric cancer between 1993 and 2001. Our special interest was comparing distal esophageal and gastric cardia adenocarcinoma patients since a common origin of these tumors has been suggested. Data on comorbidity (previous cancers, Chronic Obstructive Pulmonary Diseases (COPD), cardiovascular and cerebrovascular diseases, hypertension, ulcerative digestive tract diseases, liver diseases and diabetes) were derived from a population-based database in the Netherlands to compare risk factor profiles for 479 esophageal squamous cell carcinomas, 339 distal esophageal adenocarcinomas, 570 cardia adenocarcinomas and 1965 subcardia cancers. Comparable age and gender distribution was shown in distal esophageal and cardia adenocarcinoma patients. After adjustment for age and gender, only the prevalence of previous cancers differed between adenocarcinomas of distal esophagus and cardia (more frequent in distal esophageal adenocarcinoma patients, odds ratio (OR)=1.84, p=0.01). Ulcerative and liver diseases were more prevalent in esophageal squamous cell carcinoma patients as compared to distal esophageal adenocarcinoma patients (OR=1.90, p=0.02; OR=8.82, p=0.04 respectively), whereas diabetes was more prevalent in the latter (OR=0.56, p=0.03). Cardia adenocarcinoma patients significantly more often had hypertension as compared to subcardia cancer patients (OR=1.53, p=0.001), whereas the latter more often suffered from previous cancers and ulcerative diseases (OR=0.54, p=0.0009; OR=0.25, p< 0.0001 respectively). In terms of comorbidity at diagnosis cardia adenocarcinoma patients resemble distal esophageal adenocarcinoma rather than gastric subcardia carcinoma patients with likewise equal age and gender distribution.

INTRODUCTION

Consistent with countries in Western Europe and the USA, the incidence of distal esophageal as well as gastric cardia, i.e. gastro-esophageal junction (GEJ), adenocarcinomas in the Netherlands has been increasing during the last decades, especially in males [1-5]. Causal factors underlying the upward trend in incidence in GEJ adenocarcinomas are not elucidated yet. In contrast, the incidence of squamous cell carcinoma of the esophagus has been stable or decreasing in males. Subcardia gastric cancer incidence has decreased since before the Second World War in industrialised countries, but remained the world's second leading cause of cancer mortality [6].

Although clear differences exist in risk factor profiles and epidemiological features between adenocarcinomas of the distal esophagus and cardia [7-11], similarities in epidemiological and histomorphological features as well as parallel, rising incidence rates have been shown by others [12-14]. Gastro-esophageal reflux disease is a known risk factor for distal esophageal adenocarcinomas, for which Barrett's esophagus, a columnar cell metaplasia of the native squamous cell epithelium, is a risk indicator [8,14,15]. A subgroup of cardia carcinomas may develop within short segments of intestinal metaplasia at the GEJ [14,16] although a causal role of gastro-esophageal reflux has been difficult to establish. Additionally, alcohol and/or tobacco use may confer risk for esophageal and for cardia adenocarcinomas [17-20] and they are also established risk factors for esophageal squamous cell carcinomas [18,21].

Esophageal squamous cell carcinomas and distal esophageal adenocarcinomas show marked differences in pathogenesis, tumor biology and patient characteristics [18,22].

Helicobacter Pylori infection plays an important role in the pathogenesis of not only persistent gastritis, but also of peptic ulcers and gastric subcardia carcinomas [23]. Diet rich in salted, smoked or poorly preserved food is another well-known risk factor for gastric cancer [23]. Gastric subcardia carcinomas are clearly distinct from cardia adenocarcinomas concerning epidemiology [13,19,24].

To gain more insight in variation of risk factor profiles of esophageal and gastric cancer, we investigated the prevalence of prognostically relevant comorbidity in newly diagnosed esophageal and gastric cancer patients in the South of the Netherlands.

METHODS

Study population

Data were derived from the population based Eindhoven Cancer Registry, which covers approximately 2 million inhabitants in the Southeast of the Netherlands [5]. They are served by 16 community hospitals and two large radiotherapy institutes that collaborate with the Cancer Registry within the framework of the Comprehensive Cancer Centre South, IKZ. Upon notifi-

cation by one of six pathological laboratories and the hospital medical records departments, registration clerks actively collect information (diagnosis, tumor stage, treatment, comorbidity) from the medical records. The Eindhoven Cancer Registry, at the request of clinicians, has been collecting data on clinically relevant comorbidity for new cancer patients since 1993 [25], according to a slightly adapted version of the list of Charlson et al. [26], Table 7.1. Information on comorbidity was extracted from previous admissions, letters of referral from and discharge to general practitioners, the medical history, current medication, and preoperative assessment. Cardiovascular, cerebrovascular and other vascular diseases were also included after a circulatory event or vascular surgery. Digestive tract diseases mainly consisted of major surgery for ulcerative diseases in the upper digestive tract. Previous cancers did not include basal cell skin cancer and cervix carcinoma in situ.

Table 7.1 Classification of comorbidity, according to an adapted list of Charlson et al. [26]

Comorbidity
Previous malignancies (except basal cell skin carcinoma and cervix carcinoma in situ)
Chronic Obstructive Pulmonary Diseases
Cardiovascular diseases:
Myocardial infarction
Heart failure
Angina pectoris
Intermittent claudication
Abdominal aneurysm
Cardiomyopathy
Valve prothesis (aorta or mitralis)
Cerebrovascular diseases:
Cerebrovascular accident
Hemiplegia
Hypertension
Digestive tract diseases:
Ulcerative disease (only registered since 1997)
Patients who underwent major surgery for ulcerative disease (Billroth I or II)
Chronic inflammatory diseases (M. Crohn, Colitis Ulcerosa except polyposis coli)
Liver disease (cirrhosis, hepatitis)
Diabetes Mellitus
Other:
Urinary tract diseases
Connective tissue diseases
Dementia
Chronic infections

Selection of tumors

All primary tumors of the esophagus (N=952) and stomach (N=2581) that were newly diagnosed between 1 January 1993 and 31 December 2001 were included in our analysis.

Endoscopic, radiological, surgical and pathological data (if available) were taken into consideration for subsite assignment. Cancer of the esophagus was categorised into four groups based on the last ICD-O-3 digit: upper (codes C15.0 and C15.3), middle (C15.4), distal (C15.5) esophagus and other (overlapping and not otherwise specified (NOS); C15.8/9). Cancer of the stomach was classified: cardia (C16.0), midstomach (fundus, body or curvatures; C16.3/4/5/6 and C16.8), antrum/pylorus (C16.1/2) and other (unspecified; C16.9). Tumors were considered to be cardiac when the epicentre was at the gastric cardia, defined as the area at and immediately below the gastro-esophageal junction, extending approximately 2cm downwards. Tumors centred on the gastro-esophageal junction were considered to be of esophageal origin when Barrett's epithelium was present and as cardiac when Barrett's epithelium was absent, according to the advice of the International Union Against Cancer (UICC) [27]. Tumors arising from the fundus or corpus of the stomach and infiltrating the gastric cardia or distal esophagus were considered to be subcardiac. Esophageal tumors were classified as squamous cell carcinoma (codes 8050-8052 and 8047), adenocarcinoma (codes 8480-8490, 8140-8473 and 8500-8550) and all other morphology including unspecified cases and cases without histological confirmation (NOS). Gastric carcinomas were classified as adenocarcinomas, carcinomas with other morphology and NOS. Esophageal carcinomas were subdivided in squamous cell carcinomas, adenocarcinomas of the distal esophagus and other carcinomas. Gastric carcinomas were subdivided in cardia adenocarcinomas and subcardia carcinomas.

Statistical analysis

Multivariate (logistic regression) analyses, adjusted for gender and age (<55, 55-69, 70-84, ≥85 years) were performed for risk estimation of comorbidity in the different tumor groups. Odds ratios (OR) and 90% confidence intervals (90% CI) were computed.

RESULTS

Between 1993 and 2001, 952 esophageal cancer patients and 2581 gastric cancer patients were diagnosed. General characteristics are shown in Table 7.2. The age and gender distribution of patients with adenocarcinomas of the distal esophagus resembled that of patients with gastric cardia adenocarcinomas. In general, cardiovascular diseases, hypertension and previous malignancies were the most common associated concomitant diseases. Five patients with digestive diseases suffered from Crohn's disease. Below 70 years of age, 51% of males and 57% of females did not suffer from any serious comorbid condition, whereas this was true for only 29% and 42%, respectively, in the group over 70 years of age.

Table 3 shows the distribution of the various comorbidities according to subsite, gender and age. A rank order according to subsite is depicted in Figure 7.1.

Table 7.2 Distribution of subsites and subtypes of esophageal and gastric carcinomas diagnosed from 1993 to 2001, according to gender and age .

	Squamous cell carcinomas esophagus	*Adeno-carcinomas distal esophagus*	*Other esophageal carcinomas*[a]	*Adeno-carcinomas gastric cardia*	*Subcardia gastric carcinomas*	*Other gastric carcinomas*[b]	*Total*
Total cases	479	339	134	570	1965	46	3533
(%)	(13.6)	(9.6)	(3.8)	(16.1)	(55.6)	(1.3)	(100)
gender							
M	307	264	78	434	1199	28	2310
F	172	75	56	136	766	18	1223
M/F ratio	1.8	3.5	1.4	3.2	1.6	1.5	1.9
age (yrs)							
mean	64.2	65.8	70.1	65.8	70.0	67.6	68.1
range	27-97	26-95	41-94	30-91	25-100	35-93	25-100

[a] Tumors with other morphology than squamous cell (n=82) and adenocarcinomas of the upper 1/3 and middle 1/3 of the esophagus (n=52).

[b] Tumors of the gastric cardia with other than adenocarcinoma morphoplogy.

Esophageal squamous cell carcinoma patients most often presented with cardiovascular comorbidity (mainly in males over 70 years of age) and previous cancers, which mainly comprised skin cancer (16 patients), head and neck cancer (13 patients), or lung cancer (13 patients). Furthermore, hypertension (mainly elderly females), COPD (mainly in males) and ulcerative diseases (mainly in males) were also more common. Most obvious male preponderance was shown for COPD, cardiovascular and ulcerative diseases.

Distal esophageal adenocarcinoma patients most often presented with cardiovascular comorbidity (mainly in older males), hypertension (in older females and males), diabetes (mainly in older females and males), previous cancers, mainly comprising skin, prostate and breast cancer (11, 8, 5 patients) and COPD (mainly in older males). Evident male preponderance was shown for previous cancers, COPD, cardiovascular and ulcerative diseases.

Cardia adenocarcinoma patients presented with hypertension (mainly older females), cardiovascular disease (mainly in older males), diabetes (mainly in older females), COPD (mainly older males) and previous malignancies, mainly comprising colorectal cancer (9 patients), skin (6 patients), prostate (5 patients) and breast cancer (5 patients). Male preponderance was shown for COPD and cardiovascular diseases, whereas female preponderance was shown for hypertension and diabetes.

Gastric subcardia carcinoma patients presented with cardiovascular comorbidity (mainly older males), hypertension (mainly older females), ulcerative diseases (mainly older males), previous malignancies consisting of skin (55 patients), colorectal cancer (39 patients), prostate (33 patients) and breast cancer (31 patients) and diabetes (mainly older females). Obvious male preponderance in both cardia adenocarcinoma and subcardia cancer patients concerned COPD and cardiovascular comorbidity, whereas female preponderance was shown for

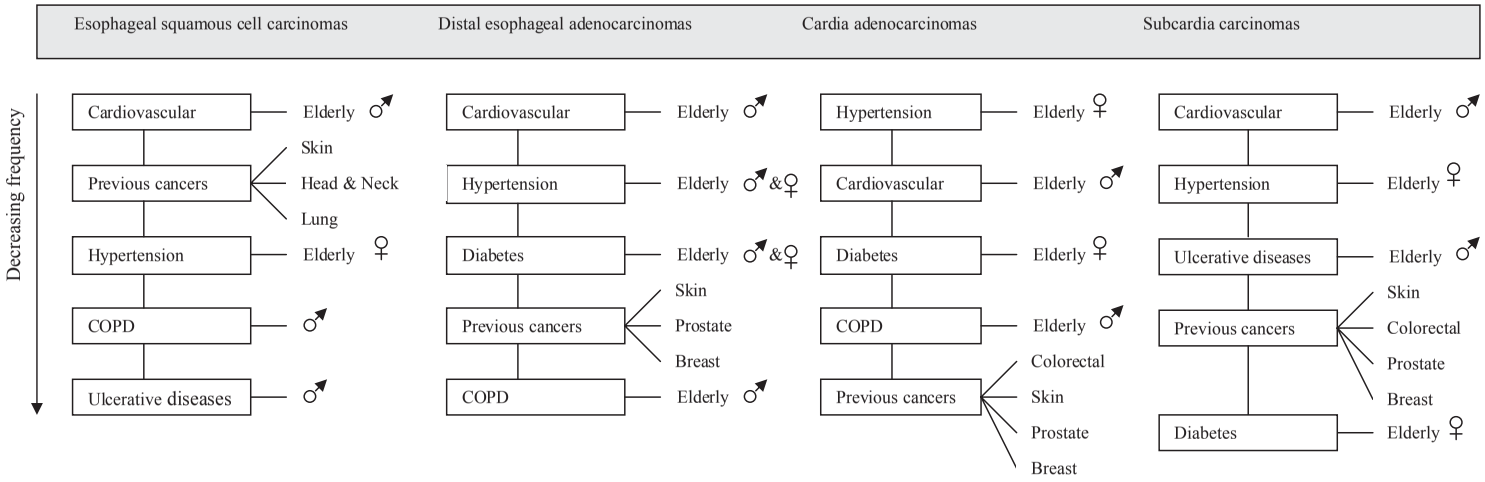


Figure 7.1 Rank order of comorbidity in esophageal and gastric cancer patients according to subsite (see table 7.3 for frequencies)

Table 7.3 Prevalence of comorbidity (%), according to tumor subsite, gender and age.

	Squamous cell carcinomas esophagus				**Adenocarcinomas distal esophagus**				**Adenocarcinomas gastric cardia**				**Subcardia gastric carcinomas**			
	Male		Female		Male		Female		Male		Female		Male		Female	
Male: (n=2310) Female: (n=1223)	<70 yrs (%)	*≥70 yrs (%)*	<70 yrs (%)	*≥70 yrs (%)*	<70 yrs (%)	*≥70 yrs (%)*	<70 yrs (%)	*≥70 yrs (%)*	<70 yrs (%)	*≥70 yrs (%)*	<70 yrs (%)	*≥70 yrs (%)*	<70 yrs (%)	*≥70 yrs (%)*	<70 yrs (%)	*≥70 yrs (%)*
Comorbidity [a]	n=220	n=87	n=99	n=73	n=168	n=96	n=33	n=42	n=279	n=155	n=57	n=79	n=579	n=620	n=280	n=486
Previous cancers	10	*22*	12	*16*	6	*22*	9	*10*	4	*10*	11	*6*	9	*15*	9	*15*
COPD	12	*14*	10	*5*	11	*18*	6	*10*	5	*19*	4	*9*	8	*15*	6	*8*
Cardiovascular	16	*32*	4	*18*	15	*35*	9	*5*	18	*28*	2	*20*	19	*30*	8	*20*
Cerebrovascular	2	*10*	4	*5*	8	*3*	-	*2*	1	*9*	2	*8*	3	*6*	1	*9*
Hypertension	16	*14*	8	*21*	13	*19*	9	*21*	15	*20*	21	*28*	10	*13*	14	*26*
Ulcerative diseases	12	*16*	-	*3*	7	*8*	-	*2*	3	*6*	5	*5*	15	*19*	9	*6*
Liver diseases	3	*2*	4	-	-	*1*	-	-	1	*1*	2	-	1	*0.5*	-	-
Diabetes	5	*10*	2	*8*	7	*15*	12	*17*	5	*9*	12	*23*	7	*9*	9	*18*
No comorbidity	**49**	***23***	**60**	***41***	**57**	***34***	**45**	***50***	**62**	***37***	**61**	***44***	**45**	***27***	**57**	***41***

[a] More than one comorbid condition per patient possible.

hypertension and diabetes.

Patients with 'other esophageal carcinomas' consisted of 52 patients with adenocarcinomas in the upper or middle 1/3 of the esophagus and of 82 patients with 'mixed type epithelial carcinomas' or 'epithelial carcinomas, not otherwise specified', mainly due to advanced disease at diagnosis with, consequently, abandoned resection. Patients with 'other esophageal carcinomas' did not clearly resemble either squamous cell carcinoma or distal esophageal adenocarcinoma patients concerning comorbidity (results not shown) and were excluded for further analysis. Also cardia carcinomas with other morphology than adenocarcinomas were excluded for further analysis.

In age and gender adjusted multivariate analysis, only previous cancers were more prevalent among patients with distal esophageal adenocarcinoma patients than among cardia adenocarcinoma patients (OR=1.84, 90% CI 1.22-1.75, Table 7.4a).

Esophageal squamous cell cancer patients exhibited a lower male/female ratio and had a younger mean age in comparison with distal esophageal adenocarcinoma patients (Table 7.2). Ulcerative diseases and liver diseases were more prevalent among patients with esophageal squamous cell carcinomas than among patients with distant esophageal adenocarcinomas (OR=1.90, 90% CI 1.18-3.03; OR=8.82 90% CI 1.57-49.7 respectively, Table 7.4b), whereas patients with distal esophageal adenocarcinoma were more often diagnosed with diabetes (OR=0.56, 90% CI 0.36-0.87, Table 7.4b).

Cardia adenocarcinoma patients exhibited a higher male/female ratio and had a younger mean age in comparison with subcardia cancer patients (Table 7.2). Previous cancers and ulcerative diseases were less common among patients with cardia adenocarcinomas than among those with gastric subcardia tumors (OR=0.54, 90% CI 0.40 -0.73; OR=0.25, 90% CI 0.17-0.36, Table 7.4c), contrasting hypertension (OR=1.53, 90% CI 1.23-1.89, Table 7.4c).

The prevalence of patients who suffered from both COPD and cardiovascular comorbidity, as an indicator of tobacco use, was equally distributed between esophageal squamous cell cancer patients and distal esophageal adenocarcinoma patients, between distal esophageal adenocarcinoma and cardia adenocarcinoma patients, and between cardia adenocarcinoma and gastric subcardia patients in multivariate analysis.

DISCUSSION

We performed a population-based study of comorbidity prevalence in order to assess risk factor profiles of esophageal and gastric cancer. Patients with distal esophageal adenocarcinoma and gastric cardia adenocarcinoma had the same age and gender distribution as well as almost comparable comorbidity patterns. A common origin of these tumors is therefore likely [12-14], contrasting reports that described distal esophageal adenocarcinomas and gastric cardia carcinomas as separate entities [10,11]. Only previous cancers were more prevalent in dis-

Table 7.4 Comorbidity prevalence in esophageal and gastric cancer patients according to tumor subsite. Multivariate [a] comparisons of comorbidity prevalence between adenocarcinomas of the distal esophagus and gastric cardia, between esophageal squamous cell carcinomas and distal esophageal adenocarcinomas, and between cardia adenocarcinomas and subcardia carcinomas.

	A.				**B.**				**C.**			
Comorbidity	*Adenocarcinomas distal esophagus % (n)*	*Adenocarcinomas gastric cardia % (n)*	*p-value*	*Odds ratio (90% CI)[b]*	*Squamous cell carcinomas esophagus % (n)*	*Adenocarcinomas distal esophagus % (n)*	*p-value*	*Odds ratio (90% CI)[b]*	*Adenocarcinomas gastric cardia % (n)*	*Subcardia Carcinomas % (n)*	*p-value*	*Odds ratio (90% CI)[b]*
Previous cancers	11 (38)	4 (37)	.01	1.84 (1.22-2.75)	13 (64)	11 (38)	.14	1.40 (0.96-2.05)	4 (37)	12 (242)	.0009	0.54 (0.40-0.73)
COPD	11 (53)	9 (51)	.14	1.40 (0.96-2.03)	11 (53)	11 (53)	.84	0.96 (0.66-1.39)	9 (51)	10 (195)	.62	0.92 (0.70-1.22)
Cardiovascular	19 (65)	19 (110)	.77	0.95 (0.71-1.28)	17 (80)	19 (65)	.7	1.08 (0.78-1.49)	19 (110)	21 (419)	.34	0.89 (0.72-1.09)
Cerebrovascular	4 (12)	4 (25)	.5	0.78 (0.44-1.43)	5 (22)	4 (12)	.31	1.46 (0.79-2.71)	4 (25)	5 (103)	.88	1.03 (0.7-1.52)
Hypertension	15 (52)	19 (107)	.19	0.78 (0.44-1.43)	15 (70)	15 (52)	.97	0.99 (0.71-1.39)	19 (107)	15 (302)	.001	1.53 (1.23-1.89)
Ulcerative diseases	6 (21)	4 (23)	.14	1.58 (0.94-2.64)	9 (43)	6 (21)	.02	1.90 (1.18-3.03)	4 (23)	13 (259)	< .0001	0.25 (0.17-0.36)
Liver diseases	0.3 (1)	1 (5)	.28	0.31 (0.05-1.89)	3 (12)	0.3 (1)	.04	8.82 (1.57-49.7)	1 (5)	0.3 (6)	.17	2.35 (0.85-6.49)
Diabetes	11 (37)	9 (52)	.33	1.26 (0.86-1.84)	6 (28)	11 (37)	.03	0.56 (0.36-0.87)	9 (52)	11 (208)	.86	1.03 (0.78-1.36)

[a] Adjustment for gender and age (<55 years, 55-69 years, 70-84 years and >84 years).

[b] 90% CI: 90 percent confidence intervals.

tal esophageal adenocarcinoma patients as compared to cardia adenocarcinoma patients. These previous malignancies mainly comprised skin cancer, prostate cancer and breast cancer in both patients groups and colorectal cancer in cardia adenocarcinoma patients. Two previous studies have analysed a possible common aetiology of colorectal and esophageal cancer and showed conflicting results [28,29]. We could not establish a common aetiology between specific previous cancers and esophageal or gastric cardia cancer, since they form a mixed subset with consequently small patient numbers.

Adenocarcinomas of the distal esophagus versus adenocarcinomas of the gastric cardia

Ulcerative diseases were comparable prevalent in subjects with adenocarcinoma of the distal esophagus and subjects with gastric cardia adenocarcinoma. Lagergren et al. found a probable causal relationship between gastro-esophageal reflux and esophageal adenocarcinoma, and a relatively weak relation with adenocarcinoma of the gastric cardia [8]. Nevertheless, digestive tract diseases were even more strongly related to esophageal squamous cell carcinomas than to distal esophageal adenocarcinomas in our patient cohort, whatever the mechanism for this.

Case-control studies report discrepant findings about nicotine and alcohol consumption as risk factors for the development of esophageal and cardia adenocarcinomas [17-20]. COPD, largely resulting from nicotine abuse, may confer risk to esophageal or gastric cardia adenocarcinoma [30,31] through its contribution to gastro-esophageal reflux by an increased thoracoabdominal pressure gradient during COPD exacerbation, high hiatal hernia prevalence and altered crural diaphragm function [31-33]. Furthermore, asthma medication can worsen or cause reflux by relaxing the lower esophageal sphincter. However, in our study COPD prevalence did not differ between distal esophageal and cardia adenocarcinoma patients.

For registration and hence comparison of GEJ adenocarcinomas careful designation of cancer subsite is of vital importance. The necessity to distinctly determine gastro-esophageal junction tumor subsite led, as a consequence, to the introduction of a separate code for cardia cancer in the Netherlands as early as with the 8th revision of the ICD system (1978). However, since no separate code for gastro-esophageal junction tumors exists, the decision whether a tumor originates from the distal esophagus or the true gastric cardia is difficult. In concordance with Wayman et al. we feel that recognition of cancers at the gastro-esophageal junction as distinct from other esophageal and gastric subsites might improve the use of cancer registration data [34].

Esophageal squamous cell carcinomas versus adenocarcinomas of the distal esophagus

In our study, esophageal squamous cell carcinoma patients exhibited a lower male/female ratio and were younger than distal esophageal adenocarcinoma patients. The prevalence of the various comorbid conditions between these 2 patient groups differed somewhat less than expected, if one takes into account the marked contrasts in pathogenesis, tumor

biology, and patient characteristics [18,22]. The presence of concomitant ulcerative diseases provided an increased risk for esophageal squamous cell cancer patients as compared to distal esophageal adenocarcinoma patients. Patients aged 70-84 years mainly accounted for this difference (results not shown). Also liver diseases were more prevalent in squamous cell carcinoma patients, albeit small patient numbers. Impaired hepatic function in squamous cell cancer patients may result from increased alcohol consumption, which is known to be an important aetiological factor for squamous cell cancer of the esophagus [18,21]. In a preoperative risk analysis esophageal squamous cell cancer patients had considerably impaired liver function as well as impaired pulmonary function as compared to esophageal adenocarcinoma patients [35]. Pulmonary comorbidity largely results from nicotine abuse, which is another important risk factor for squamous cell cancer [18,21]. In our study COPD and also cardiovascular comorbidity prevalence did not differ between squamous cell carcinoma and distal esophageal adenocarcinoma patients. The higher prevalence of lung as well as head and neck cancer in esophageal squamous cell cancer patients nevertheless affirmed the role of alcohol and nicotine abuse in these patients. Diabetes was more prevalent in distal esophageal adenocarcinoma patients as compared to esophageal squamous cell carcinoma patients, for which the reason is not understood. One could speculate that this higher diabetes prevalence might be related to a higher obesity prevalence, which is described to be a risk factor for distal esophageal (and cardia) adenocarcinomas [18,36].

Adenocarcinomas of the gastric cardia versus subcardia carcinomas

In previous studies, risk factors as well as epidemiological features from cardia cancers have been reported to differ from subcardia cancers, which is also reflected in largely different comorbidity patterns in our data [13,19,24]. Cardia adenocarcinoma patients presented more often with hypertension as compared to subcardia cancer patients, especially in the age group of 70-84 years (results not shown). This is consistent with Zhang et al., who compared risk factors between esophageal plus cardia adenocarcinoma patients and distal stomach adenocarcinoma patients, speculating on a common cause [19]. Obesity, possibly related to hypertension, has emerged as a major risk factor for gastro-esophageal reflux disease [37] and (subsequent) esophageal and gastric cardia adenocarcinomas, by increasing intra-abdominal pressure [7,18,36]. The mounting evidence that obesity is associated with increased (distal esophageal as well as) cardia adenocarcinoma risk is supported by our finding of a higher hypertension prevalence in cardia adenocarcinoma as compared to subcardia adenocarcinoma patients. Data on body weight were unfortunately not available in our study. Moreover, nicotine and alcohol consumption might contribute to the development of both hypertension as to the development of (esophageal and) cardia cancer. Case-control studies report discrepant findings about nicotine and alcohol consumption as a risk factor for the development of cardia adenocarcinomas [18-20]. Previous ulcerative diseases occurred more frequently in gastric subcardia cancer patients as compared to cardia cancer patients, mainly in the age group of

55-84 years (results not shown). The higher frequency of previous ulcerative diseases in subcardia cancer patients might be explained by the probable carcinogenic potential of (gastric) ulcers and Helicobacter Pylori infections as possible underlying cause of ulcer development. Unfortunately, information on Helicobacter Pylori infection status in the patients was not available. Neither ulcer origin, whether duodenal or gastric, was recorded. In our data previous malignancies, mainly comprising skin cancers, colorectal cancers, prostate and breast cancers, were more common in gastric subcardia cancer as compared to cardia adenocarcinoma patients. Patients in the age of 55-69 years mainly accounted for this difference (results not shown). The mechanism how these previous malignancies could confer risk for subcardia cancer is unclear. Moreover, as these previous malignancies form a mixed subset, it is difficult to speculate on a probable common origin of these cancers. As mentioned earlier, 2 previous studies have analysed a possible association between colorectal cancer and esophageal, but not gastric carcinomas [28,29].

In conclusion, only the prevalence of previous malignancies differed between distal esophageal adenocarcinoma and cardia adenocarcinoma patients, with predominance in the first mentioned. Esophageal squamous cell cancer patients had more ulcerative as well as liver diseases in comparison with distal esophageal adenocarcinoma patients, whereas diabetes was more common in the latter patients. Cardia adenocarcinoma patients were apparently different from gastric subcardia cancer patients concerning age and gender distribution as well as concerning previous malignancies, hypertension and ulcerative diseases. Hence, cardia adenocarcinoma patients resembled distal esophageal adenocarcinoma patients considerably more than subcardia carcinoma patients concerning comorbidity, with likewise equal age and gender distribution.

ACKNOWLEDGMENTS

The authors would like to thank the registration team of the Comprehensive Centre South for their dedicated data collection.

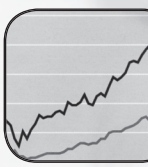
REFERENCES

1. Devesa SS, Blot WJ, Fraumeni JF, Jr.: Changing patterns in the incidence of esophageal and gastric carcinoma in the United States. Cancer 83:2049-53, 1998
2. Powell J, McConkey CC, Gillison EW, Spychal RT: Continuing rising trend in oesophageal adenocarcinoma. Int J Cancer 102:422-7, 2002
3. Wijnhoven BP, Louwman MW, Tilanus HW, Coebergh JW: Increased incidence of adenocarcinomas at the gastro-oesophageal junction in Dutch males since the 1990s. Eur J Gastroenterol Hepatol 14:115-22, 2002
4. Funkhouser EM, Sharp GB: Aspirin and reduced risk of esophageal carcinoma. Cancer 76:1116-9, 1995
5. Coebergh JW, Janssen M, Louwman M, Voogd A. Cancer, incidence, care and survival in the South of the Netherlands 1955-1999. Eindhoven: Eindhoven Cancer Registry (IKZ): 2001, pp 23-27
6. Alberts SR, Cervantes A, Van De Velde CJ: Gastric cancer: epidemiology, pathology and treatment. Ann Oncol 14 Suppl 2:II31-II36, 2003
7. Lagergren J, Bergstrom R, Nyren O: Association between body mass and adenocarcinoma of the esophagus and gastric cardia. Ann Intern Med 130:883-90, 1999
8. Lagergren J, Bergstrom R, Lindgren A, Nyren O: Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma. N Engl J Med 340:825-31, 1999
9. Lagergren J, Bergstrom R, Lindgren A, Nyren O: The role of tobacco, snuff and alcohol use in the aetiology of cancer of the oesophagus and gastric cardia. Int J Cancer 85:340-6, 2000
10. El-Serag HB, Mason AC, Petersen N, Key CR: Epidemiological differences between adenocarcinoma of the oesophagus and adenocarcinoma of the gastric cardia in the USA. Gut 50:368-72, 2002
11. Tajima Y, Nakanishi Y, Yoshino T, Kokawa A, Kusano M, Shimoda T: Clinicopathological study of early adenocarcinoma of the gastric cardia: comparison with early adenocarcinoma of the distal stomach and esophagus. Oncology 61:1-9, 2001
12. Wijnhoven BP, Siersema PD, Hop WC, van Dekken H, Tilanus HW: Adenocarcinomas of the distal oesophagus and gastric cardia are one clinical entity. Rotterdam Oesophageal Tumor Study Group. Br J Surg 86:529-35, 1999
13. Heidl G, Langhans P, Mellin W, Bunte H, Grundmann E: Adenocarcinomas of esophagus and cardia in comparison with gastric carcinoma. J Cancer Res Clin Oncol 120:95-9, 1993
14. Ruol A, Parenti A, Zaninotto G, Merigliano S, Costantini M, Cagol M, Alfieri R, Bonavina L, Peracchia A, Ancona E: Intestinal metaplasia is the probable common precursor of adenocarcinoma in barrett esophagus and adenocarcinoma of the gastric cardia. Cancer 88:2520-8, 2000
15. Ye W, Chow WH, Lagergren J, Yin L, Nyren O: Risk of adenocarcinomas of the esophagus and gastric cardia in patients with gastroesophageal reflux diseases and after antireflux surgery. Gastroenterology 121:1286-93, 2001
16. Mendes de Almeida JC, Chaves P, Pereira AD, Altorki NK: Is Barrett's esophagus the precursor of most adenocarcinomas of the esophagus and cardia? A biochemical study. Ann Surg 226:725-33; discussion 733-5, 1997
17. Menke-Pluymers MB, Hop WC, Dees J, van Blankenstein M, Tilanus HW: Risk factors for the development of an adenocarcinoma in columnar-lined (Barrett) esophagus. The Rotterdam Esophageal Tumor Study Group. Cancer 72:1155-8, 1993
18. Vaughan TL, Davis S, Kristal A, Thomas DB: Obesity, alcohol, and tobacco as risk factors for cancers of the esophagus and gastric cardia: adenocarcinoma versus squamous cell carcinoma. Cancer Epidemiol Biomarkers Prev 4:85-92, 1995
19. Zhang ZF, Kurtz RC, Sun M, Karpeh M, Jr., Yu GP, Gargon N, Fein JS, Georgopoulos SK, Harlap S: Adenocarcinomas of the esophagus and gastric cardia: medical conditions, tobacco, alcohol, and socioeconomic factors. Cancer Epidemiol Biomarkers Prev 5:761-8, 1996
20. Gammon MD, Schoenberg JB, Ahsan H, Risch HA, Vaughan TL, Chow WH, Rotterdam H, West AB, Dubrow R, Stanford JL, Mayne ST, Farrow DC, Niwa S, Blot WJ, Fraumeni JF, Jr.: Tobacco, alcohol, and socioeconomic status and adenocarcinomas of the esophagus and gastric cardia. J Natl Cancer Inst 89:1277-84, 1997

21. Brown LM, Hoover R, Silverman D, Baris D, Hayes R, Swanson GM, Schoenberg J, Greenberg R, Liff J, Schwartz A, Dosemeci M, Pottern L, Fraumeni JF, Jr.: Excess incidence of squamous cell esophageal cancer among US Black men: role of social class and other risk factors. Am J Epidemiol 153:114-22, 2001
22. Stein HJ, Brucher BL, Sendler A, Siewert JR: Esophageal cancer: patient evaluation and pre-treatment staging. Surg Oncol 10:103-11, 2001
23. Fuchs CS, Mayer RJ: Gastric carcinoma. N Engl J Med 333:32-41, 1995
24. Locke GR, 3rd, Talley NJ, Carpenter HA, Harmsen WS, Zinsmeister AR, Melton LJ, 3rd: Changes in the site- and histology-specific incidence of gastric cancer during a 50-year period. Gastroenterology 109:1750-6, 1995
25. Coebergh JW, Janssen-Heijnen ML, Post PN, Razenberg PP: Serious co-morbidity among unselected cancer patients newly diagnosed in the southeastern part of The Netherlands in 1993-1996. J Clin Epidemiol 52:1131-6, 1999
26. Charlson ME, Pompei P, Ales KL, MacKenzie CR: A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. J Chronic Dis 40:373-83, 1987
27. van Nieuwkerk CM, Kuipers EJ: Liver cirrhosis and peptic ulcer disease; a correlation with Helicobacter pylori? Neth J Med 56:203-5, 2000
28. Vaughan TL, Kiemeney LA, McKnight B: Colorectal cancer in patients with esophageal adenocarcinoma. Cancer Epidemiol Biomarkers Prev 4:93-7, 1995
29. Lagergren J, Nyren O: No association between colon cancer and adenocarcinoma of the oesophagus in a population based cohort study in Sweden. Gut 44:819-21, 1999
30. Lagergren J, Bergstrom R, Adami HO, Nyren O: Association between medications that relax the lower esophageal sphincter and risk for esophageal adenocarcinoma. Ann Intern Med 133:165-75, 2000
31. Ye W, Chow WH, Lagergren J, Boffetta P, Boman G, Adami HO, Nyren O: Risk of adenocarcinomas of the oesophagus and gastric cardia in patients hospitalized for asthma. Br J Cancer 85:1317-21, 2001
32. Harding SM: Gastroesophageal reflux, asthma, and mechanisms of interaction. Am J Med 111 Suppl 8A:8S-12S, 2001
33. Canning BJ: Role of nerves in asthmatic inflammation and potential influence of gastroesophageal reflux disease. Am J Med 111 Suppl 8A:13S-17S, 2001
34. Wayman J, Forman D, Griffin SM: Monitoring the changing pattern of esophago-gastric cancer: data from a UK regional cancer registry. Cancer Causes Control 12:943-9, 2001
35. Bollschweiler E, Schroder W, Holscher AH, Siewert JR: Preoperative risk analysis in patients with adenocarcinoma or squamous cell carcinoma of the oesophagus. Br J Surg 87:1106-10, 2000
36. Chow WH, Blot WJ, Vaughan TL, Risch HA, Gammon MD, Stanford JL, Dubrow R, Schoenberg JB, Mayne ST, Farrow DC, Ahsan H, West AB, Rotterdam H, Niwa S, Fraumeni JF, Jr.: Body mass index and risk of adenocarcinomas of the esophagus and gastric cardia. J Natl Cancer Inst 90:150-5, 1998
37. Locke GR, 3rd, Talley NJ, Fett SL, Zinsmeister AR, Melton LJ, 3rd: Risk factors associated with symptoms of gastroesophageal reflux. Am J Med 106:642-9, 1999

Chapter 8

Presence of substantial comorbidity in esophageal or gastric cancer patients affected treatment choice and survival: population based study



Submitted

Linetta B Koppert
Valery EPP Lemmens
Jan-Willem W Coebergh
Bas PL Wijnhoven
Hugo W Tilanus
Maryska LG Janssen-Heijnen

ABSTRACT

We analysed the influence of comorbidity on treatment choice and survival in patients with newly diagnosed esophageal and gastric cancer between 1995 and 2002. Data on comorbidity (previous cancers, Chronic Obstructive Pulmonary Diseases (COPD), cardiovascular and cerebrovascular diseases, hypertension, ulcerative digestive tract diseases, liver diseases and diabetes) were derived from the population-based Eindhoven Cancer Registry for 430 esophageal squamous cell carcinomas, 392 distal esophageal adenocarcinomas, 494 cardia adenocarcinomas and 1708 subcardia cancers. Surgical resection with or without (neo)adjuvant therapy was applied in 76% of TNM stage I-III patients: in 49% of esophageal squamous cell carcinoma patients, 75% of distal esophageal adenocarcinoma, 83% of cardia adenocarcinoma and 83% of subcardia carcinoma patients. Surgery was less often applied in patients aged 70 years or older and in patients with ≥ 2 comorbidities at diagnosis. After adjustment for age, gender, tumor differentiation grade, TNM stage (I vs. II-III), tumor type, socio-economic status and type of hospital (non-teaching vs. teaching), the presence of ≥ 2 comorbidities independently lessened the chance of receiving surgery (p=0.006, OR 0.6) and formed a negative prognostic factor in multivariable survival analysis (p=0.02, HR 1.2). Since there was an adjustment for TNM stage and applied therapy (surgical resection or palliation) within multivariable survival analysis, the dismal prognosis for patients with ≥ 2 comorbidities at cancer diagnosis is likely to be a direct effect of the poor condition of the patient. Further refinement in perioperative care seems especially important in patients with ≥ 2 comorbidities. The exact role of comorbidity in therapy decision making and prognosis has to be addressed in prospective studies.

INTRODUCTION

Patients with esophageal or gastric carcinoma are often diagnosed at an advanced state due to late presentation of symptoms [1]. Surgery forms the only treatment with curative intent and is currently the preferred treatment if the tumor is considered to be resectable without evidence of distant metastases. Moreover, a patient needs to be fit enough to undergo surgery. Five-year overall survival after surgery is approximately 25% in esophageal and gastric cardia carcinoma patients and 30% in gastric carcinoma patients [2,3]. Unfortunately, around 40% of patients present with locally advanced or metastatic disease and receive palliative instead of curative therapy [3,4].

In the Netherlands, annually around 900 patients are newly diagnosed with esophageal cancer and 2050 with gastric cancer [5]. Patient's age and fitness might influence treatment outcome and, moreover, prior treatment decision making especially if comorbidity concerning vital organs is present.

The aim of this study was to analyse the influence of age and comorbidity on treatment choice in patients with esophageal or gastric cancer using the population-based database of the Eindhoven Cancer Registry. Moreover we analysed the influence of age and comorbidity on survival.

PATIENTS AND METHODS

All patients newly diagnosed with esophageal or gastric cancer between 1st of January 1995 and 31st of December 2002 in the registration area of the population-based Eindhoven Cancer Registry were included. This registry covers approximately 2.4 million inhabitants in the Southeast of the Netherlands [6]. There are no university hospitals in the registry area. Information on diagnosis, staging, treatment and comorbidity was extracted from the medical records by trained registrars [7]. Comorbidity was recorded, according to a slightly adapted version of the list of Charlson et al., Table 8.1 [8].

Only patients aged 40 years or older were included (872 patients with esophageal cancer and 2345 patients with gastric cancer).

Endoscopic, radiological, surgical and pathological data (if available) were taken into consideration for subsite assignment [9]. Cancer of the esophagus was categorized into four groups based on the last ICD-O-3 digit: upper (codes C15.0 and C15.3), middle (C15.4), distal (C15.5) esophagus and other (overlapping and not otherwise specified (NOS); C15.8/9). Cancer of the stomach was classified as: cardia (C16.0), midstomach (fundus, body or curvatures; C16.3/4/5/6 and C16.8), antrum/pylorus (C16.1/2) and other (unspecified; C16.9). Tumors were considered to be cardiac when the epicenter was at the gastric cardia, defined as the area at and immediately below the gastro-esophageal junction, extending approximately 2

Table 8.1. Classification of comorbidity, according to an adapted version of the list of Charlson et al. [8].

Comorbidity
Previous malignancies (except basal cell skin carcinoma and cervix carcinoma in situ)
Chronic Obstructive Pulmonary Diseases
Cardiovascular diseases:
Myocardial infarction
Heart failure
Angina pectoris
Intermittent claudication
Abdominal aneurysm
Cardiomyopathy
Valve prothesis (aorta or mitralis)
Cerebrovascular diseases:
Cerebrovascular accident
Hemiplegia
Hypertension
Digestive tract diseases:
Ulcerative disease (only registered since 1997)
Patients who underwent major surgery for ulcerative disease (Billroth I or II)
Chronic inflammatory diseases (M. Crohn, Colitis Ulcerosa except polyposis coli)
Liver disease (cirrhosis, hepatitis)
Diabetes Mellitus
Other:
Urinary tract diseases
Connective tissue diseases
Dementia
Chronic infections

cm downwards. Tumors centered on the gastro-esophageal junction were considered to be of esophageal origin when Barrett's epithelium was present and as cardiac when Barrett's epithelium was absent, according to the advice of the International Union Against Cancer (UICC) [10]. Tumors arising from the fundus or corpus of the stomach and infiltrating the gastric cardia or distal esophagus were considered to be subcardiac. Esophageal carcinomas were subdivided in squamous cell carcinomas (N=430), adenocarcinomas of the distal esophagus (N=392) and other carcinomas (N=50). Gastric carcinomas were subdivided in cardia adenocarcinomas (N=494), subcardia carcinomas (N=1708) and other carcinomas (N=143). Both esophageal and gastric 'other carcinomas' were left out for further analysis (N=193). Primary treatment of esophageal and gastric cancer patients was classified as surgery alone; surgery in combination with chemotherapy, radiotherapy, or in combination with chemotherapy and radiotherapy; chemotherapy alone; radiotherapy alone; chemotherapy in combination with radiotherapy; palliative therapy (i.e. flexible wallstent placement mostly); other therapy; and unknown therapy. Using the database, no discrimination could be made between neoadjuvant or adjuvant treatment within the group of surgically treated patients. For analysis, surgically treated patients (with or without (neo)adjuvant therapy) were regarded as treated with curative intent (potentially curative treatment) and were compared to patients that did not

receive therapy or received other therapy than surgery (palliative treatment). Importantly, in our retrospective study, surgery in patients with TNM stage IV or unknown stage could well have been palliative instead of potentially curative. To overcome this problem, patients with TNM stage IV or unknown were excluded from the analyses regarding application of potentially curative treatment.

TNM stage was calculated using pathological TNM data or, in case of unresected patients, using clinical TNM data.

Socio-economic status (SES) of the patients was defined at neighbourhood level (based on postal code of residence area, 17 households on average) combining mean household income (in 1998) and mean value of the house/apartment (in 2000), derived from individual fiscal data made available at an aggregated level. Postal codes were assigned to 3 SES categories: low (1st-3rd decile), intermediate (4th-7th decile), and high (8th-10th decile). Postal codes of institutions, such as nursing homes, were assigned to a separate category and left out of the analysis (198 patients, mentioned as unknown in Table 8.2). Another 39 patients had missing SES data and were left out of the analysis.

Type of hospital was defined as non-teaching hospital or teaching hospital.

For each tumor type the applied treatment was analysed, according to number of comorbid conditions and age (<70, 70+ years). Differences between groups were determined using the chi-square test. Logistic regression was applied to determine the independent effects of age, gender, tumor type, tumor differentiation grade, TNM-stage, SES, type of hospital and number of comorbid conditions on the application of curative treatment. Survival time was defined as the time from diagnosis until death (any cause). Vital status of all patients on January 1st, 2005 was assessed through merging with the database of the Central Bureau for Genealogy, that collects data on all deceased Dutch citizens via the civil municipal registries. In this way, information on patients who moved outside the registry area was also obtained. Crude survival rates were calculated according to age, gender, tumor type, tumor differentiation grade, TNM-stage, SES, applied treatment, type of hospital and comorbidity. In a multivariable Cox's proportional hazard regression analysis, independent hazard ratios (HR) were estimated for the variables mentioned, with either number of comorbid conditions, type of comorbid conditions or combinations of comorbidities (separate models). Patients with TNM stage IV or unknown were excluded from the analysis (N=1707). In order to analyse whether 'applied treatment' influenced differences in survival within age categories or within comorbidity categories, the model was first run without this variable. The SAS computer package (version 8.2) was used for all statistical analyses (SAS Institute Inc., Cary, North Carolina, USA, 1999).

Table 8.2. Distribution of gender, age, comorbidity, TNM-stage and tumor differentiation grade according to subsites and subtypes of esophageal and gastric carcinomas diagnosed from 1995 to 2002.

	Squamous cell carcinomas	Adenocarcinomas distal esophagus	Adenocarcinomas gastric cardia	Subcardia gastric carcinomas
Total cases (%)	430 (14)	392 (13)	494 (16)	1708 (57)
Age (yrs)				
median	65	67	66	71
range	40-97	40-95	40-91	40-100
Gender M	275	317	375	1040
F	155	75	119	668
M/F ratio	1.8	4.2	3.2	1.6
Concomitant disease (% within type)[a]				
No comorbidity	152 (35)	138 (35)	202 (40)	522 (30)
Previous cancers	59 (14)	48 (12)	32 (6)	236 (14)
COPD	56 (13)	56 (14)	53 (11)	174 (10)
Cardiovascular	77 (18)	80 (20)	104 (20)	404 (24)
Cerebrovascular	19 (4)	15 (4)	21 (4)	92 (5)
Hypertension	69 (16)	67 (17)	94 (19)	300 (18)
Ulcerative digestive diseases	36 (8)	30 (8)	26 (5)	265 (16)
Liver diseases	10 (2)	1 (0.3)	3 (0.6)	7 (0.4)
Diabetes	25 (6)	44 (11)	46 (9)	189 (11)
Unknown	40 (9)	29 (7)	40 (8)	160 (9)
Number of comorbidities				
0	152 (35)	138 (35)	202 (40)	522 (30)
1	126 (29)	121 (31)	132 (26)	527 (30)
≥2	111 (26)	104 (26)	126 (25)	522 (30)
Unknown	40 (9)	29 (7)	40 (8)	160 (9)
TNM-stage				
I	23 (5)	23 (6)	54 (11)	325 (19)
II	67 (16)	58 (15)	75 (15)	267 (16)
III	89 (21)	60 (15)	76 (15)	200 (12)
IV	99 (23)	131 (33)	171(35)	540 (32)
Unknown	152 (35)	120 (31)	118 (24)	376 (22)
Differentiation grade				
Well	27 (6)	22 (6)	23 (5)	55 (3)
Moderate	180(42)	113 (29)	161 (33)	412 (24)
Poor	140 (33)	165 (42)	240 (49)	940 (55)
Unknown	83 (19)	92 (23)	70 (14)	301 (18)
Social Economic Status				
Low	136 (32)	101 (26)	135 (27)	568 (33)
Intermediate	148 (34)	150(38)	200 (40)	590 (35)
High	110 (26)	111 (28)	131 (27)	384 (22)
Unknown	33 (8)	30 (8)	28 (5)	146 (10)
Therapy				
Surgery[b]	97 (23)	116 (30)	185 (37)	885 (52)
No surgery[c]	327 (76)	271 (69)	301 (61)	799 (47)
Unknown	6 (1)	5 (1)	8 (2)	24 (1)
Hospital				
Non-teaching	305 (71)	281 (72)	375 (76)	1266 (74)
Teaching	125 (29)	111 (128)	119 (24)	442 (26)

[a] More concomitant diseases per patient possible. [b] Surgery with or without neoadjuvant or adjuvant therapy; [c] palliative therapy included chemotherapy and or radiotherapy, stent placement or no therapy.

RESULTS

General characteristics of the patients are shown in Table 8.2. Median age was 69 years (range 40-100 years). The most common comorbid conditions were cardiovascular (22%), hypertension (18%), upper digestive tract ulcerative diseases (12%) and COPD (11%). Comorbidity was absent in 41% of patients <70 years of age and in 24% of patients 70+ years of age. Potentially curative treatment (i.e. surgery with or without (neo)adjuvant therapy) within patients stage I-III was offered to 76% of patients: 49% of squamous cell carcinoma patients, 75% of distal esophageal adenocarcinoma patients, 83% of cardia adenocarcinoma patients and 83% of subcardia gastric carcinoma patients. Surgery was applied less often in patients aged 70+ as compared to patients aged <70 concerning squamous cell and distal esophageal adenocarcinoma patients (Table 8.3). Within distal esophageal adenocarcinoma patients aged 70+ years the application of surgery was significantly decreased with increasing number of comorbidities (Table 8.3).

Surgical therapy was applied to 5% of squamous cell carcinoma patients, 4% of distal esophageal adenocarcinoma patients, 6% of cardia adenocarcinoma patients and 16% of subcardia gastric carcinoma patients with TNM stage IV tumors, which can explained by the possible application of palliative instead of curative surgery (bypass to ensure food passage) or to the fact that pathological TNM data instead of clinical data were analysed ('upstaging'). Upstaging was however seen in only 48 patients out of the total group. In logistic regression analysis with adjustment for age, gender, tumor type, tumor differentiation grade, TNM stage, SES and type of hospital the presence of 2 or more comorbid conditions was significantly associated with a lower probability of receiving curative treatment (p=0.006, OR 0.6, Table 8.4). When the different types of comorbid conditions (i.e. previous cancer, COPD, cardiovascular disease, cerebrovascular disease, hypertension, ulcerative disease, liver disease, diabetes) were entered into the multivariable models (instead of the number of comorbid conditions; a separate model for each condition), no specific comorbid condition came forward that conferred a lower chance for receiving curative treatment. Concerning combinations of comorbidity, three most common combinations were analysed in separate multivariate models (hypertension and diabetes n=19, cardiovascular and hypertension n=42, cardiovascular and COPD n=19). The combination hypertension and diabetes conferred a lower chance for receiving curative treatment (p=0.049, HR 0.3, data not shown). The combination cardiovascular and hypertension as well as cardiovascular and COPD did not show an effect. Apart from comorbidity also age, TNM-stage, tumor differentiation grade and tumor type were independently correlated with administration of curative treatment with lowest chance for squamous cell carcinoma patients and increasing chances for distal oesophageal adenocarcinoma, cardia adenocarcinoma and subcardia cancer patients (as compared to squamous cell carcinoma patients, Table 8.4).

Table 8.3. Application of surgical therapy according to tumor type, age group and number of comorbidities among patients with stage I, II or III esophageal or gastric carcinoma.

Application of surgery[a]	<70 years (%)[b]	70+ years (%)[b]	p-value[c]
Squamous cell carcinomas esophagus			
Number of comorbidities			
0	36 (30)	4 (15)	0.096
1	23 (32)	5 (9)	0.003
≥2	14 (24)	5 (9)	0.04
p-value[c]	0.6	0.8	
Adenocarcinomas distal esophagus			
Number of comorbidities			
0	37 (39)	8 (20)	0.02
1	30 (45)	15 (27)	0.03
≥2	13 (33)	3 (5)	0.0002
p-value[c]	0.4	0.005	
Adenocarcinomas gastric cardia			
Number of comorbidities			
0	56 (88)	15 (79)	0.4
1	34 (83)	13 (76)	0.6
≥2	18 (75)	21 (72)	0.8
p-value[c]	0.4	0.9	
Subcardia gastric carcinomas			
Number of comorbidities			
0	126 (93)	86 (91)	0.7
1	110 (91)	111 (89)	0.6
≥2	81 (91)	140 (87)	0.3
p-value[c]	0.9	0.5	

[a] Surgery with or without neoadjuvant or adjuvant therapy. [b] Number of patients surgically treated out of total number of patients with specific number of comorbidities in specific age group; patients within categories 'unknown' in either group were excluded from analysis. [c] Linear-by-linear association.

One-month, 6-month, 1-year and 5-year survival rates are denoted for separate patient groups (Table 8.5). In multivariable Cox proportional-hazard analysis within TNM stage I-III patients, the presence of ≥ 2 comorbidities came forward as an independent prognostic factor for overall survival (Table 8.5). When the different types of comorbid conditions (i.e. previous cancer, COPD, cardiovascular disease, cerebrovascular disease, hypertension, ulcerative disease, liver disease, diabetes) were entered into the multivariable model instead of the number of comorbid conditions (a separate model for each condition), no specific condition came forward to confer an independent prognostic role. Three most common combinations of comorbidities were analysed in separate multivariable models but did not come forward

Table 8.4. Chance for administration of surgical treatment among patients with stage I, II or III esophageal or gastric carcinoma; logistic regression model including all listed variables.

	Surgery[a]	
	OR (95% CI)[b]	**p-value**
Age		
<70 years	1.0	-
70+ years	0.7 (0.5-0.98)	0.04
Gender		
Male[c]	1.0	-
Female	0.9 (0.6-1.3)	0.5
Tumor type		
Squamous cell ca esophagus[c]	1.0	-
Distal esophageal adenoca	2.6 (1.5-4.6)	0.0006
Cardia adenocarcinoma	3.8 (2.2-6.4)	<0.0001
Subcardia carcinoma	7.4 (4.6-11.8)	<0.0001
TNM-stage		
I[c]	1.0	
II, III	0.3 (0.2-0.4)	<0.0001
Tumor differentiation grade		
Well/moderate[c]	1.0	-
Poor	1.3 (0.9-1.9)	0.2
Unknown	0.3 (0.2-0.5)	<0.0001
No. of comorbid conditions		
0[c]	1.0	-
1	0.9 (0.6-1.3)	0.5
≥2	0.6 (0.4-0.8)	0.006
Social Economic Status		
Low[c]	1.0	-
Intermediate	0.8 (0.5-1.2)	0.3
High	0.8 (0.5-1.2)	0.3
Hospital		
Non-teaching[c]	1.0	-
Teaching	1.5 (0.99-2.2)	0.06

[a] Surgery with or without neoadjuvant or adjuvant therapy (whereas non-surgical therapy included: chemotherapy and or radiotherapy, stent placement or no therapy). [b] Odds Ratio with 95% Confidence Interval. [c] Reference category.

to confer an independent prognostic role. When the model was run without 'applied therapy' as an independent variable, the presence of ≥ 2 comorbidities conferred an even stronger independent significant effect on overall survival (p=0.005, HR 1.3, data not shown). Apart from TNM-stage and tumor differentiation grade, also age 70+ and tumor type subcardia carcinoma came forward as independent prognostic factors (p=0.04, HR 1.2; p=0.003, HR 0.7 respectively, data not shown).

Table 8.5. Uni- and multivariable analyses for overall survival of stage I, II and III esophageal and gastric cancer patients, according to age.

	<70 years univariable			70+ years univariable			multivariable	
	1- and 6-month survival (%)	1- and 5-year survival (%)	P-value	1- and 6-month survival (%)	1- and 5-year survival (%)	P-value	HR[a]	P-value
Age								
<70 years[b]	93 64	41 15		- -	- -		1.0	-
70+ years	- -	- -		87 50	32 10		1.1	0.07
Gender								
Male[b]	94 63	40 14		86 49	31 9		1.0	-
Female	93 67	44 18	0.03	89 51	34 12	0.2	0.9	0.3
Tumor type								
Squamous cell ca esophagus[b]	95 62	32 10		88 50	21 6		1.0	-
Distal esophageal adenoca	96 67	44 12		90 51	32 5		0.9	0.4
Cardia adenocarcinoma	94 63	39 11		89 52	32 10		1.0	0.8
Subcardia carcinoma	93 66	46 20	<0.0001	86 49	35 13	0.07	0.9	0.3
TNM-stage								
I[b]	99 91	85 63		94 80	72 43		1.0	-
II, III	98 78	34 7	<0.0001	95 66	26 5	<0.0001	2.2	<0.0001
Tumor differentiation grade								
Well/moderate[b]	97 70	50 20		89 54	37 13		1.0	-
Poor	93 62	38 13		87 47	29 10		1.2	0.009
Unknown	90 59	35 12	<0.0001	82 48	32 9	0.001	1.1	0.3
No. of comorbid conditions								
0[b]	95 67	42 16		91 52	34 13		1.0	-
1	93 62	40 16		86 51	33 11		1.1	0.3
≥2	93 65	43 14	0.9	86 48	32 9	0.1	1.2	0.02
Social Economic Status								
Low[b]	93 63	39 14		85 48	31 11		1.0	-
Intermediate	94 67	42 17		89 52	36 11		1.0	0.8
High	94 62	42 14	0.6	89 55	35 11	0.5	1.0	0.7
Treatment								
Surgery[b]	98 84	67 30		94 71	58 26		1.0	-
No surgery	89 47	19 2	<0.0001	83 38	17 2	<0.0001	1.8	<0.0001
Hospital								
Non-teaching[b]	93 64	41 15		86 48	31 10		1.0	-
Teaching	94 64	40 14	0.9	90 53	36 13	0.03	1.1	0.4

[a] Hazard Ratio. [b] Reference category.

DISCUSSION

A surgical resection is currently the preferred treatment for esophageal and gastric cancer if the tumor is considered to be resectable without evidence of distant metastases (TNM stage I-III). Moreover a patient needs to be fit enough to undergo surgery. Intuitively, presence of comorbidities as well as increasing age might be associated with worse outcome of esophagectomy as well as gastrectomy. Curative therapy might be applied less often because of a subjective perception about life expectancy and ability to tolerate treatment. In this retrospective series from the area of the Eindhoven Cancer Registry 76% of patients with TNM stage I-III carcinomas received surgery. Within logistic regression analysis restricted to TNM stage I-III patients, age 70+ years as compared to age ≤ 70 significantly lowered the chance for application of curative treatment. Also the presence of ≥ 2 comorbidities clearly altered the chance for receiving curative therapy in multivariable analysis.

In retrospect, intuitions on possible worse outcome after surgery in elderly patients and in patients suffering from comorbidity seemed to be followed indeed. Only 49% of TNM stage I-III squamous cell carcinoma patients received surgery. Percentages for distal esophageal adenocarcinoma, cardia adenocarcinoma and subcardia carcinoma patients were 75%, 83% and 83% respectively. The question is whether this is good clinical practice, i.e. whether a higher percentage of patients should have received surgery instead of palliation. To answer this question, one might wonder whether decreased prognosis in palliatively treated patients is due to comorbidity or due to less aggressive treatment. In multivariable Cox proportional-hazard analysis restricted to TNM stage I-III patients, the presence of ≥ 2 comorbidities came forward as an independent prognostic factor. Since there was an adjustment for stage and applied therapy the dismal prognosis of patients with ≥ 2 comorbidities is likely an effect of the poor condition of the patient. Given the increasing age of the population and parallel increasing comorbidity and given the rising incidence of distal esophageal as well as cardia adenocarcinoma, weighing of risk becomes more and more important in clinical decision-making. Application of a tailor made therapy approach might be indicated to augment the patient's prognosis.

In the literature, a composite scoring system has been described with data on cardiovascular, pulmonary, hepatic and renal function as well as alcohol drinking habits and willingness to undergo major surgery [11,12]. Strict application of this scoring system led to a marked reduction of postoperative mortality after esophagectomy [11,13]. Data on comorbidity might add to data on detailed organ function in preoperative 'fitness' assessment.

Within a retrospective study in a tertiary medical center, comorbidity, as defined by the Charlson comorbidity index score, was univariately associated with increased mortality after esophagectomy, which effect disappeared in multivariable analysis [14]. In two recently performed studies concerning gastric cancer surgery in elderly patients, comorbidity not only was an independent prognostic factor for survival (Charlson index) but also formed a fre-

quent cause of death during follow-up [15,16]. Studies from the surgical literature on outcomes from colorectal carcinoma and from primary non-small cell lung cancer suggested that comorbidity, more than age, was a significant predictor for mortality [17,18].

Our data showed that the presence of ≥ 2 comorbidities had not only an effect on treatment choice, but also had an independent prognostic effect on overall survival. No specific type of comorbidity came forward (i.e. previous cancers, COPD, cardiovascular or cerebrovascular diseases, hypertension, ulcerative digestive tract diseases, liver diseases or diabetes) to confer a significant independent effect. Although patients with the combination of hypertension and diabetes at diagnosis had a independently lower chance for receiving surgery, this combination was not associated with worse survival in Cox proportional hazard analyses. Two other investigated combinations of comorbidity (cardiovascular and hypertension, cardiovascular and COPD) also did not confer significancy concerning survival. Noteworthy, patient numbers were very small. Multiple comorbidities in gastric cancer patients aged 80+ years involved much higher operative mortality in a retrospective study from Italy, however the presence of postoperative complications nor the number of comorbidities significantly influenced 5-year survival rate of curatively resected patients [19].

Concerning age, previous single-institution retrospective studies demonstrated that esophagectomy can be performed as safely in elderly patients as in younger patients with comparable long term survival [16,20-23]. Also surgery for gastric carcinoma has been reported to be relatively safe in patients aged 70+ years with survival that is comparable to younger patients [24]. In our multivariable survival analysis age 70+ years showed a trend to worse survival although not significant.

Limitations of our study exist in its retrospective nature. Some desirable information was not available, for instance data on performance status, scheme of (neo)adjuvant therapy and details about applied surgical technique. The Charlson's list was used to score prognostic comorbidity, without subdivision according to severity, because this was too complex for the registrars [25]. Misclassification of comorbidity is limited, because the comorbid diseases are recorded routinely by trained registry personnel and data are collected directly from the medical records of the patients, which is considered to be the most reliable source for comorbidity [26].

The fact that only 49% of squamous cell carcinoma patients received surgery as compared to 75% of distal esophageal adenocarcinomas, 83% of cardia carcinoma patients and 83% of subcardia carcinoma patients is reflected in univariable survival analysis with large differences in survival between tumor types. Within the multivariable analysis tumor type was not shown to be an independent prognostic factor which is in accordance with studies comparing squamous cell and adenocarcinomas of the esophagus [27,28]. This observation is in contrast with studies that show a markedly better prognosis for resected esophageal adenocarcinoma as compared to squamous cell carcinoma [29,30]. In the studies mentioned no adjustment was however applied for comorbidity.

We found a limited effect of socioeconomic status on choice of treatment or on overall survival in our population-based database, which did not change when a division was made between patients aged >70 and 70+ years. This is in contrast with Trivers and coworkers who found low income to be associated with shorter survival in esophageal and gastric cancer patients with exception of subcardia cancers [31]. The impact of socioeconomic status is most pronounced for cancers with relatively good survival, probably reflecting variations in access to quality health care [32,33].

In summary, this population-based study confirmed that surgery was applied less often in esophageal and gastric cancer patients (TNM stage I-III) than would be expected based on their TNM stage. This was most pronounced for esophageal squamous cell carcinoma patients and, to less extent, for distal esophageal adenocarcinoma patients. In retrospect, age 70+ years as well as presence of ≥ 2 comorbidities independently influenced chances for receiving surgery. No specific type of comorbidity came forward to confer this effect, unless the combination of hypertension and diabetes which independently lowered the chance for receiving surgery although patient numbers were small. The presence of ≥ 2 comorbidities was independently associated with worse survival, after adjustment for therapy. The balance between outcome of surgery on the one hand and the risk of withholding higher-risk patients any prospect of cure by not performing surgery on the other hand is a difficult dilemma for upper gastrointestinal surgeons. Further refinement in perioperative care seems especially important in patients with ≥ 2 comorbidities. The exact role of comorbidity in therapy decision making and prognosis has to be addressed in future prospective studies.

ACKNOWLEDGMENTS

We thank the registration team of the Eindhoven Cancer Registry for their dedicated data collection.

REFERENCES

1. Hundahl SA, Menck HR, Mansour EG, Winchester DP: The National Cancer Data Base report on gastric carcinoma. Cancer 80:2333-41, 1997
2. Wijnhoven BP, Siersema PD, Hop WC, van Dekken H, Tilanus HW: Adenocarcinomas of the distal oesophagus and gastric cardia are one clinical entity. Rotterdam Oesophageal Tumor Study Group. Br J Surg 86:529-35, 1999
3. Dickson JL, Cunningham D: Systemic treatment of gastric cancer. Eur J Gastroenterol Hepatol 16:255-63, 2004
4. Enzinger PC, Mayer RJ: Esophageal cancer. N Engl J Med 349:2241-52, 2003
5. Visser O, Siesling SS, Dijck van JAAM (eds.). Incidence of cancer in the Netherlands 1999/2000. Netherlands Cancer Registry, 2003.
6. Janssen-Heijnen MLG, Louwman WJ, van de Poll-Franse LV, Coebergh JWW (eds.). Results of 50 years cancer registry in the South of the Netherlands: 1955-2004 (in Dutch). Eindhoven: Eindhoven Cancer Registry, 2005.
7. Janssen-Heijnen ML, Houterman S, Lemmens VE, Louwman MW, Maas HA, Coebergh JW: Prognostic impact of increasing age and co-morbidity in cancer patients: a population-based approach. Crit Rev Oncol Hematol 55:231-40, 2005
8. Charlson ME, Pompei P, Ales KL, MacKenzie CR: A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. J Chronic Dis 40:373-83, 1987
9. Koppert LB, Janssen-Heijnen ML, Louwman MW, Lemmens VE, Wijnhoven BP, Tilanus HW, Coebergh JW: Comparison of comorbidity prevalence in oesophageal and gastric carcinoma patients: a population-based study. Eur J Gastroenterol Hepatol 16:681-688, 2004
10. Wittekind C HD, Hutter RVP, Sobin LH. TNM Supplement. A Commentary on Uniform Use, 2nd ed. 2001.
11. Bartels H, Stein HJ, Siewert JR: Preoperative risk analysis and postoperative mortality of oesophagectomy for resectable oesophageal cancer. Br J Surg 85:840-4, 1998
12. Bartels H, Stein HJ, Siewert JR: Risk analysis in esophageal surgery. Recent Results Cancer Res 155:89-96, 2000
13. Stein HJ, Brucher BL, Sendler A, Siewert JR: Esophageal cancer: patient evaluation and pre-treatment staging. Surg Oncol 10:103-11, 2001
14. Atkins BZ, Shah AS, Hutcheson KA, Mangum JH, Pappas TN, Harpole DH, Jr., D'Amico TA: Reducing hospital morbidity and mortality following esophagectomy. Ann Thorac Surg 78:1170-6; discussion 1170-6, 2004
15. Gasparini G, Inelmen EM, Enzi G, Santoro C, Sergi G, Cardin F, Terranova O: Clinical and prognostic aspects of gastric carcinoma in the elderly. J Gastrointest Surg 10:395-401, 2006
16. Kinugasa S, Tachibana M, Yoshimura H, Dhar DK, Shibakita M, Ohno S, Kubota H, Masunaga R, Nagasue N: Esophageal resection in elderly esophageal carcinoma patients: improvement in postoperative complications. Ann Thorac Surg 71:414-8, 2001
17. Birim O, Maat AP, Kappetein AP, van Meerbeeck JP, Damhuis RA, Bogers AJ: Validation of the Charlson comorbidity index in patients with operated primary non-small cell lung cancer. Eur J Cardiothorac Surg 23:30-4, 2003
18. Lemmens VE, Janssen-Heijnen ML, Verheij CD, Houterman S, Repelaer van Driel OJ, Coebergh JW: Co-morbidity leads to altered treatment and worse survival of elderly patients with colorectal cancer. Br J Surg 92:615-23, 2005
19. Roviello F, Marrelli D, De Stefano A, Messano A, Pinto E, Carli A: Complications after surgery for gastric cancer in patients aged 80 years and over. Jpn J Clin Oncol 28:116-22, 1998
20. Poon RT, Law SY, Chu KM, Branicki FJ, Wong J: Esophagectomy for carcinoma of the esophagus in the elderly: results of current surgical management. Ann Surg 227:357-64, 1998
21. Ellis FH, Jr., Williamson WA, Heatley GJ: Cancer of the esophagus and cardia: does age influence treatment selection and surgical outcomes? J Am Coll Surg 187:345-51, 1998
22. Jougon JB, Ballester M, Duffy J, Dubrez J, Delaisement C, Velly JF, Couraud L: Esophagectomy for cancer in the patient aged 70 years and older. Ann Thorac Surg 63:1423-7, 1997
23. Sabel MS, Smith JL, Nava HR, Mollen K, Douglass HO, Gibbs JF: Esophageal resection for carcinoma in patients older than 70 years. Ann Surg Oncol 9:210-4, 2002

24. Schwarz RE, Karpeh MS, Brennan MF: Factors predicting hospitalization after operative treatment for gastric carcinoma in patients older than 70 years. J Am Coll Surg 184:9-15, 1997
25. Louwman WJ, Janssen-Heijnen ML, Houterman S, Voogd AC, van der Sangen MJ, Nieuwenhuijzen GA, Coebergh JW: Less extensive treatment and inferior prognosis for breast cancer patient with comorbidity: a population-based study. Eur J Cancer 41:779-85, 2005
26. Kieszak SM, Flanders WD, Kosinski AS, Shipp CC, Karp H: A comparison of the Charlson comorbidity index derived from medical record data and administrative billing data. J Clin Epidemiol 52:137-42, 1999
27. Lerut T, De Leyn P, Coosemans W, Van Raemdonck D, Scheys I, LeSaffre E: Surgical strategies in esophageal carcinoma with emphasis on radical lymphadenectomy. Ann Surg 216:583-90, 1992
28. Lieberman MD, Shriver CD, Bleckner S, Burt M: Carcinoma of the esophagus. Prognostic significance of histologic type. J Thorac Cardiovasc Surg 109:130-8; discussion 139, 1995
29. Stein HJ, Siewert JR: Improved prognosis of resected esophageal cancer. World J Surg 28:520-5, 2004
30. Siewert JR, Stein HJ, Feith M, Bruecher BL, Bartels H, Fink U: Histologic tumor type is an independent prognostic parameter in esophageal cancer: lessons from more than 1,000 consecutive resections at a single center in the Western world. Ann Surg 234:360-7; discussion 368-9, 2001
31. Trivers KF, De Roos AJ, Gammon MD, Vaughan TL, Risch HA, Olshan AF, Schoenberg JB, Mayne ST, Dubrow R, Stanford JL, Abrahamson P, Rotterdam H, West AB, Fraumeni JF, Chow WH: Demographic and lifestyle predictors of survival in patients with esophageal or gastric cancers. Clin Gastroenterol Hepatol 3:225-30, 2005
32. Kogevinas M, Porta M: Socioeconomic differences in cancer survival: a review of the evidence. IARC Sci Publ:177-206, 1997
33. Auvinen A, Karjalainen S: Possible explanations for social class differences in cancer patient survival. IARC Sci Publ:377-97, 1997

Part IV

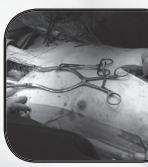
Therapy

Chapter 9

Outcome of surgical treatment for early adenocarcinoma of the esophagus or gastro-esophageal junction

Virchows Archives, 2005 May;446(5):497-504

Marinke Westerterp
Linetta B Koppert
Christianne J Buskens
Hugo W Tilanus
Fiebo JW ten Kate
Jacques JHGM Bergman
Peter D Siersema
Herman van Dekken
Jan JB van Lanschot



ABSTRACT

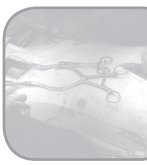
To contribute to therapeutic decision making we retrospectively analysed the outcome of transhiatal esophagectomy in 120 patients with pathologically proven high grade dysplasia (HGD; n=13) or T1-adenocarcinoma (n=107) of the distal esophagus or gastro-esophageal junction (GEJ). Adenocarcinoma of the esophagus or GEJ has a poor prognosis. Early lesions (*i.e.* HGD or T1-carcinoma) are pre-eminently the tumors which are potentially curable. Local endoscopic therapies are promising treatment options for superficial lesions, however for deeper lesions surgical resection is considered to be the treatment of choice. Tumors were subdivided into six different depths of invasion ('T1-mucosal' m1- m3, 'T1-submucosal' sm1-sm3), and the frequency of lymphatic dissemination and time to locoregional and/or distant recurrence were analysed. Only one of the 79 T1m1-3/sm1 tumors (1%) showed lymph node metastases as compared to 18 out of 41 T1sm2-3 tumors (44%). There was a significant difference in recurrence free period between T1m1-m3/sm1 versus T1sm2-sm3 tumor patients (p log rank < 0.0001), with 5-year recurrence free percentages of 97% and 57%, respectively. In multivariate analysis including age, gender, tumor differentiation grade and depth of invasion, only depth of invasion (T1sm2-3 versus T1m1-m3/sm1) was an independent prognostic factor for recurrence free period (hazard rate=7.5, 95% CI 2.0-27.7). These data indicate that T1m1-m3/sm1 adenocarcinomas of esophagus or GEJ show a very low risk of lymphatic dissemination and are therefore eligible for local endoscopic therapy. After transhiatal surgical resection almost half of the patients with T1sm2-sm3 lesions develop recurrent disease within 5 years, and therefore need additional therapy to improve survival.

INTRODUCTION

The incidence of adenocarcinomas of the esophagus or gastro-esophageal junction is rising [1-3]. As a result of the increased awareness of the malignant potential of Barrett's esophagus, the more generally applied endoscopic surveillance programmes, and the availability of more accurate endoscopic techniques (*e.g.* methylene blue staining, high resolution endoscopy, fluorescence endoscopy, optical coherence tomography and narrow band imaging),[4,5] high grade dysplasia (HGD) and early esophageal adenocarcinoma are being diagnosed with increasing frequency in the Western world [6-8] . Early carcinoma is defined as a tumor limited to the mucosa or submucosa irrespective of the presence of lymph node metastases [9].

Many institutions favor radical esophagectomy with lymphadenectomy as standard therapy for esophageal carcinoma. As this procedure is associated with substantial morbidity and mortality,[10] the question rises if this extensive resection is also necessary in patients with early malignancies of the esophagus [11,12] . Presently, various techniques have been applied for the treatment of early esophageal carcinoma, *i.e.* local endoscopic, organ-preserving treatment modalities (*e.g.* endoscopic mucosal resection (EMR) or photodynamic therapy (PDT)),[13-15] limited surgical resection (*e.g.* Merendino operation or vagal-sparing esophagectomy)[16,17] and radical esophagectomy with or without systematic lymphadenectomy (transthoracic or transhiatal).

In this study we retrospectively evaluated the outcome of transhiatal esophagectomy in 120 patients with early adenocarcinoma of the esophagus or gastro-esophageal junction. In order to precisely subclassify this series of early cancers we subdivided the mucosa and submucosa into six successive layers (m1, m2, m3, sm1, sm2, sm3), as previously has been described for early squamous cell carcinoma of the esophagus by the Japanese Society for Esophageal Diseases (1999) [18]. Subsequently, we analysed whether a possible correlation between the depth of tumor infiltration on the one hand, and lymphatic dissemination and recurrence free period on the other hand, was present.



MATERIALS AND METHODS

Patient materials

Data from all patients operated for carcinoma of the esophagus or gastro-esophageal junction (GEJ) in the Erasmus University Medical Center in Rotterdam and the Academic Medical Center at the University of Amsterdam, The Netherlands, are continuously collected in prospective databases since 1980 and 1993, respectively. A cancer was termed esophageal adenocarcinoma, if the centre was clearly situated in the distal esophagus with or without specialised Barrett's epithelium. Also cancers at the GEJ in the presence of Barrett's epithelium were considered esophageal adenocarcinoma. A tumor was designated GEJ adenocar-

cinoma, if the centre was located at the junction in the absence of Barrett's mucosa, or if the centre of the tumor was seen in the proximal stomach. Between January 1980 and July 2002, 702 patients underwent subtotal esophageal resection with proximal gastrectomy for HGD or adenocarcinoma in Rotterdam; between January 1993 and July 2002, 398 patients underwent this same procedure in Amsterdam. All patients with a pathologically proven HGD (n=13) or pT1 invasive adenocarcinoma (n=107), who underwent a transhiatal resection, were included in the present study. All 13 HGD lesions were situated in Barrett's mucosa. The 107 pT1 carcinomas included 94 early cancers of the distal esophagus, 13 were classified as GEJ adenocarcinomas. None of the patients received (neo)adjuvant chemo- and/or radiotherapy.

Histopathologic assessment

The resection specimens of all included patients were reviewed by two experienced pathologists (FJWtK, HvD) to determine size and location of the primary tumor, the number of resected and involved lymph nodes, and the radicality of resection. All tumors were graded, staging was performed according to the 2002 UICC TNM classification [9]. The depth of tumor invasion was measured and subclassified based on the criteria proposed by the Japanese Society for Esophageal Diseases [18]. High-grade dysplasia (carcinoma *in situ*) arising in a Barrett segment was defined by the lack of obvious invasion through the basement membrane (HGD, m1). Intramucosal carcinoma was defined as tumor cells extending beyond the basement membrane into the lamina propria (m2), or as carcinomas with deepest invasion into the muscularis mucosae (m3). In the presence of a double muscularis mucosae (*i.e.* a superficial and a deep one in Barrett's mucosa), we considered the deep one as the pre-existing muscularis mucosae and the superficial one as newly formed [19]. Consequently, a carcinoma invading into the superficial, *i.e.* newly formed, muscularis mucosae was defined as m2 and invasion into the deeper (pre-existing) muscularis mucosae was defined as m3. Carcinomas infiltrating into the submucosa without invasion of the muscularis propria were further subclassified as invasion limited to the upper third (sm1), intermediate third (sm2), or lower third part of the submucosal layer (sm3).

Follow-up

Patients were followed until death or until April 2003 with a median follow-up time of 44 months (range, 1 day to 13.6 years). They were seen on a regular basis for 5 years in the outpatient clinic (at 3- to 4-month intervals for the first 2 years and at 6-month intervals thereafter). For the present study, patients and/or their family practitioners were contacted by telephone to assess their current status when the patient had been discharged after 5 years. No patients were lost to follow-up.

Statistical analysis

All statistical analyses were performed using the Statistical Software Package version 11.5 (SPSS INC., Chicago, IL, USA). Recurrence free periods were calculated according to the Kaplan-Meier method and differences in recurrence free period were assessed using the log-rank test. P-values of <0.05 were considered statistically significant. The Cox regression model was used to analyse the independent prognostic value of different variables.

RESULTS

Pathological findings in the esophagectomy specimens

Of the 1100 patients operated upon for HGD or adenocarcinoma in both institutions, 120 patients were found to have an early lesion and underwent a transhiatal resection. There were 103 men (85%) and 17 women (15%), with a median age of 65.4 years (range 31-83 years). Hundred and seven patients had an early lesion of the distal esophagus (94 adenocarcinomas, 13 HGD's), in all but 2 cases associated with specialised intestinal metaplasia (Barrett's epithelium). Thirteen patients had a GEJ carcinoma (including 9 gastric cardia adenocarcinomas). All the 13 HGD cases were associated with Barrett's mucosa. Of the 107 invasive adenocarcinomas 26 were classified as well differentiated, 58 as moderately differentiated, whereas the remaining 23 tumors showed a poor differentiation grade.

Thirteen patients (11%) had a T1m1 tumor without clear invasion into the deeper layers (HGD, carcinoma in situ, Table 9.1). In 18 patients invasion was limited to the lamina propria (T1m2, 15%), 23 patients had the deepest tumor infiltration into the (deeper, pre-existent) muscularis mucosae (T1m3, 19%). In 25 patients the tumor extended into the upper third of the submucosal layer (T1sm1, 21%), 23 of the patients had a cancer infiltrating into the intermediate one-third (T1sm2, 19%), and in 18 patients the carcinoma infiltrated into the lower third part of the submucosal layer (T1sm3, 15%). Examples of each T1-substage are depicted in Figure 9.1. Of the 79 patients with T1m1-m3 or sm1 cancers, only one patient (T1m3) showed lymph node metastasis (1%), whereas 18 out of 41 patients with T1sm2-sm3 tumors (44%) had lymph node metastases (Table 9.1). The 19 cases with lymph node involvement included 18 distal esophageal adenocarcinomas and 1 GEJ cancer. The mean number of resected lymph nodes was 8.6 (range 1-40). The ratio of infiltrated to removed lymph nodes in these 19 pN+ cases was 0.21, the mean number of resected nodes was 10.0 (range 3-20). In 16 patients only regional lymph node metastases were present (paraesophageal, paracardial and/or along the lesser curvature), in 3 patients lymph nodes at the origin of the left gastric artery contained metastatic tumor (M1a).

Table 9.1 Histopathological features in 120 transhiatal esophagectomy specimens with high grade dysplasia (m1) or early invasive adenocarcinoma of the esophagus or gastro-esophageal junction.

	T1m1	T1m2	T1m3	T1sm1	T1sm2	T1sm3	Total
Tumor characteristics	**(n=13)**	**(n=18)**	**(n=23)**	**(n=25)**	**(n=23)**	**(n=18)**	**(n=120)**
Lymph node involvement							
Negative	13	18	22	25	17	6	101
Positive	0	0	1	0	6	12	19
Locoregional recurrence							
Absent	13	17	23	25	19	14	111
Present	0	1	0	0	4	4	9
Distant recurrence							
Absent	13	18	22	24	19	13	109
Present	0	0	1	1	4	5	11
Mortality							
No	13	12	18	21	12	7	83
Yes	0	6	5	4	11	11	37

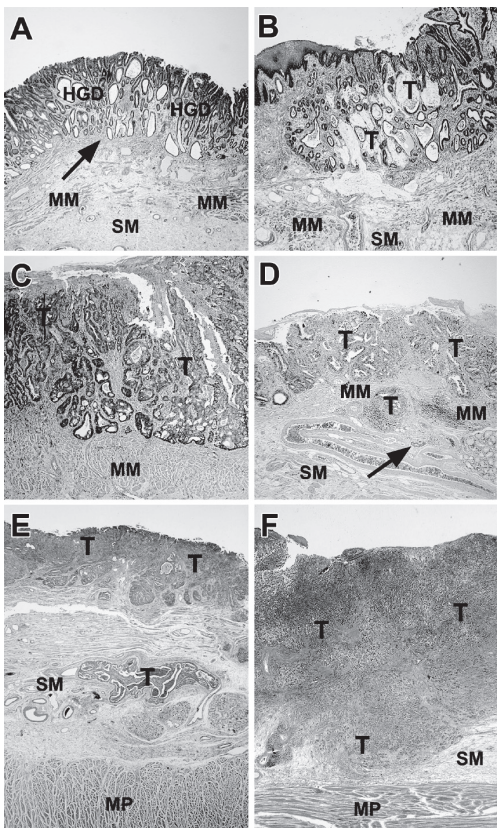
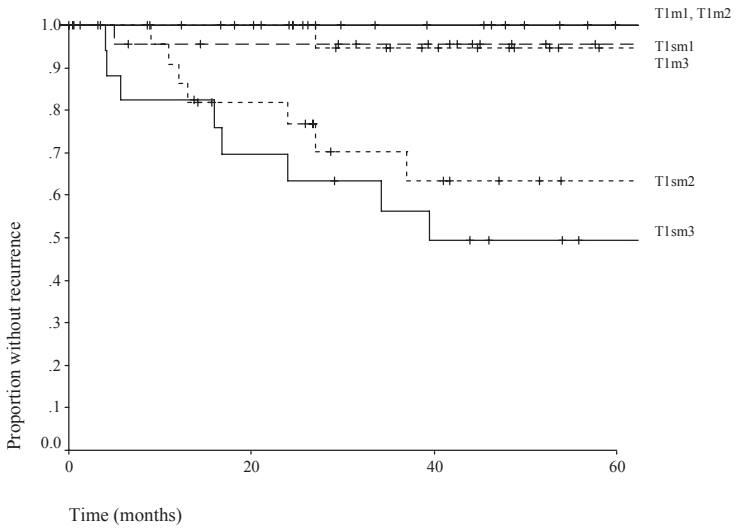


Figure 9.1. Hematoxylin-eosin stained histological sections of examples of the six different subgroups for depth of tumor invasion. A. T1m1, HGD; note the newly formed Barrett-associated muscularis mucosae (arrow) in relation to the pre-existent muscularis mucosae. B. T1m2, a mucinous, well differentiated, intra-mucosal adenocarcinoma. C. T1m3, adenocarcinoma reaches into the (pre-existent) muscularis mucosae. D. T1sm1, a small group of tumor cells is present in the superficial submucosa (arrow). E. T1sm2, carcinoma can be seen in the middle part of the submucosa, surrounded by vessels and esophageal salivary glands. F. T1sm3, a poorly differentiated adenocarcinoma infiltrates the deep submucosa. A-C, 4X objective, D-F, 2X objective. Abbreviations: HGD, high grade dysplasia; MP, muscularis propria; MM, muscularis mucosae; SM, submucosa; T, tumor.

Outcome after surgical resection

In all patients microscopically complete resection (R0) of the tumor was achieved. Five patients died in the hospital (mortality = 4%). Overall, the 120 patients with early lesions had a 5-year disease free survival of 68%. During follow-up 37 patients died. The proportions of patients without recurrence, per subgroup of depth of tumor invasion, are shown in Figure 9.2. Recurrence free period of patients with T1sm1 tumors did not differ from that of patients with T1m1, nor from that of patients with T1m2, or from that of patients with T1m3 tumors. However, recurrence free period did significantly differ from that of patients with T1sm2 and T1sm3 tumors (Figure 9.2). We, therefore, redefined depth of invasion into two subclasses, *i.e.* group I (T1m1, T1m2, T1m3 and T1sm1) and group II (T1sm2 and T1sm3), and subsequently compared recurrence free periods in both groups. A significant difference in recurrence free period was found between group I and group II (p logrank < 0.0001, Figure 9.3), with



No at risk

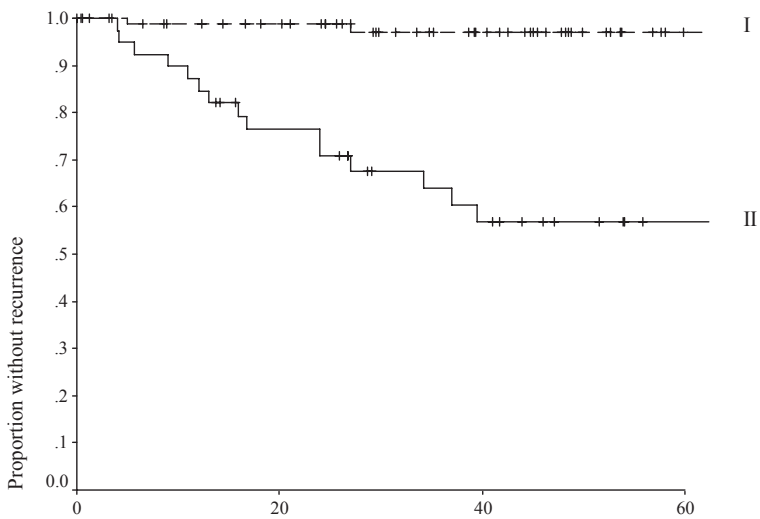
Total	120	94	64	34
T1m1	13	11	9	4
T1m2	18	15	8	6
T1m3	23	21	14	7
T1sm1	25	20	17	10
T1sm2	23	16	9	4
T1sm3	18	11	7	3

Figure 9.2. Proportion of patients without recurrence for depth of tumor invasion subgroups T1m1, T1m2, T1m3, T1sm1, T1sm2 and T1sm3. Patients with T1sm1 did not differ from patients with T1m1, T1m2 or T1m3 (p logrank=0.5, 0.6 and 0.9, respectively), but differed from patients with T1sm2 or T1sm3 (p logrank=0.02 and 0.002, respectively). Tick marks represent censored patients, i.e. end of follow-up.

Table 9.2 Results of the Cox regression analysis, concerning recurrence free period, in the total group of 120 early carcinomas.

Prognostic factors	Univariate 5 yrs recurrence free survival %	p-value	Multivariate[a] HR[b] (CI)[c]	p-value
Age[d]			1.0 (0.9-1.1)	0.9
Gender				
M[e]	81		1.0	
F	100	.3	0.5 (0.06-3.8)	0.5
Tumor grade				
Well/Moderate[e]	87		1.0	
Poor	59	.001	2.0 (0.7-5.2)	0.2
Depth of tumor invasion				
T1m1, T1m2, T1m3, T1sm1[e]	97		1.0	
T1sm2, T1sm3	57	< .0001	7.5 (2.0-27.7)	0.002

[a] In multivariable analysis correction was carried out for the prognostic variables age, gender, tumor differentiation grade and depth of tumor invasion; variables are mentioned in the column 'Prognostic factors'. [b] Hazard Rate. [c] CI: 95% Confidence Interval. [d] In the multivariable analysis, age is included as a continuous variable. [e] Reference category.



No at risk

Total	120	94	64	34
Group I	79	67	48	27
Group II	41	27	16	7

Figure 9.3. Proportion of patients without recurrence, T1m1, T1m2, T1m3, T1sm1 (group I, broken line, n=79) versus T1sm2 or T1sm3 (group II, uninterrupted line, n=41). A clear difference can be seen between the two groups (p logrank < 0.0001). Five-year recurrence free percentages for group I and group II were 97% (95% CI 93%-100%) and 57% (95% CI 40%-74%), respectively. Tick marks represent censored patients, i.e. end of follow-up.

5- year recurrence free percentages of 97% (95% CI 93%-100%) and 57% (95% CI 40%-74%), respectively. Importantly, when patients with HGD were left out of analysis, the difference in recurrence free period remained significant between T1m2, T1m3, T1sm1 (group I minus T1m1) and T1sm2, T1sm3 (group II) patients (p logrank < 0.0001), with 5-year recurrence free percentages of 96% (95% CI 92%-100%) and 57% (95% CI 40%-74%), respectively. Univariate Cox regression analysis to identify prognostic variables in the total group showed tumor differentiation grade and depth of tumor invasion to be prognostic factors for recurrence free period (p = 0.001 and p < 0.0001, respectively; Table 9.2). In multivariate analysis only depth of tumor invasion was an independent prognostic factor (HR 7.5, 95% CI=2.0-27.7, p=0.002). Alternatively, when recurrence free survival instead of recurrence free period was analysed, the significant difference between group I (T1m1, T1m2, T1m3, T1sm1) and group II (T1sm2, T1sm3) remained intact (p logrank=0.0001) with 5-year recurrence free survival percentages of 83 % (95% CI 65%-92%) and 42% (95% CI 25%-59%), respectively. Again, when patients with HGD were left out of this analysis a significant difference in recurrence free survival remained (p logrank=0.0008) with 5-year recurrence free survival percentages of 80% (95% CI 68%-91%) and 42% (95% CI 25%-59%), respectively.

Table 9.3 Tumor recurrence; of the 18 patients, some patients had recurrence at more than one site.

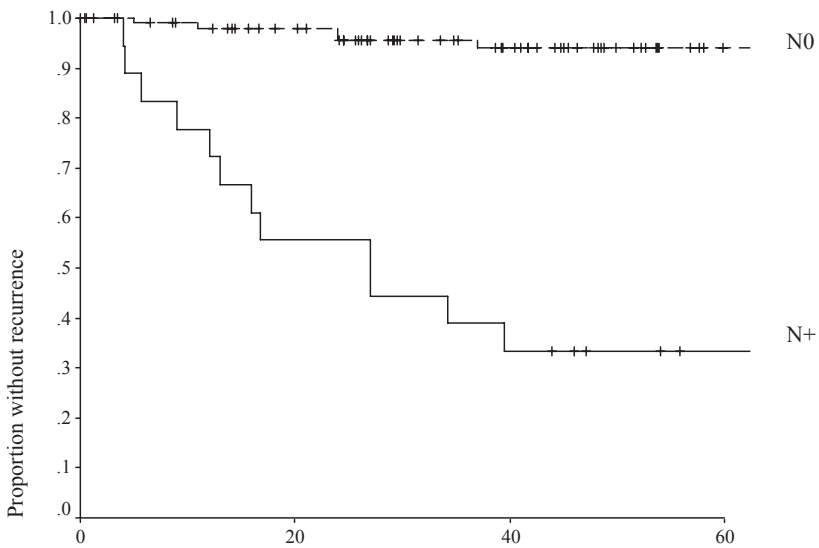
Recurrence	Number of patients
Locoregional	7
Distant	8
Bone	2
Pleural	3
Peritoneal	1
Liver	3
Truncal region	1
Both locoregional and distant	3
Truncal region	1
Bone	1
Supraclav lymph node	1

During the follow-up period 18 patients developed tumor recurrence (Table 9.3). The mean interval between operation and recurrence was 16 months (range 4-85 months). Seven patients died because of locoregional tumor recurrence without evidence of distant disease, eight patients developed only distant recurrence (of whom one patient was still alive at the end of follow-up), and three patients died because of both locoregional and distant recurrence. One patient died because of a second primary carcinoma in the lung (Table 9.4). Twelve out of eighteen patients, who died of locoregional recurrence and/or distant metastases, already had lymph node metastases at the time of resection. Recurrence free period differed significantly between patients with N0 (n=101) and N+ (n=19) tumors (p logrank < 0.0001,

Figure 9.4) with 5-year recurrence free percentages of 94% (95% CI 89%-99%) and 33% (95% CI 12%-55%), respectively.

Table 9.4 Distribution of patients at the end of follow-up.

Distribution	Number of patients
In-hospital mortality	5
Intercurrent death without disease	14
Death with locoregional recurrence only	7
Death with locoregional and distant recurrence	3
Death with distant recurrence only	7
Death due to second primary cancer	1
Alive with distant recurrence	1
Alive without evidence of disease	82
TOTAL	120



No at risk

Total	120	94	64	34
N0	101	84	58	33
N+	19	10	6	1

Figure 9.4. Proportion of patients without recurrence, N0 (broken line, n=101) versus N+ (uninterrupted line, n=19). Patients with positive lymph nodes display a strikingly higher frequency of recurrence (p logrank < 0.0001). Five-year recurrence free percentages for N0 and N+ patients were 94% (95% CI 89%-99%) and 33% (95% CI 12%-55%), respectively. Tick marks represent censored patients, i.e. end of follow-up.

DISCUSSION

We have created a detailed map of T1-substages of early Barrett's and GEJ cancers in relation to lymphatic dissemination and tumor recurrence. A favorable outcome was characterised by vertical tumor growth into, but not deeper than the upper layer of the submucosa. In contrast, cancers proliferating into the middle and deeper layers of the submucosa showed an unfavorable outcome with a high rate of recurrence after surgical resection. In our study the overall 5-year disease free survival was 68%, which is comparable to the sparse literature on this subject [20-23] . In the literature 5-year overall survival rates of 100% have been reported for T1 tumors limited to the mucosa, and rates declining to 63-100% for T1 tumors invading into the submucosa.

The group of T1m1-m3/sm1 tumors had lymph node metastases in only 1% as compared to 44% in the group of T1sm2-sm3 carcinomas. The very low frequency of lymphatic dissemination in the first group could not be attributed to the inclusion of 13 HGDs, as the recurrence free period did not alter by excluding them from analysis. In a previous study of our group the correlation between vertical cancer growth, angio-lymphatic permeation and the presence of lymph node metastasis has already been investigated. However, this concerns only a subset of the present cohort that was operated on with various procedures.

The histopathological examination of endoscopically removed T1 adenocarcinomas in Barrett's esophagus can be difficult due to the presence of a double muscularis mucosae [19]. In Barrett's esophagus the mucosa consists of columnar epithelium, a superficial lamina propria, a superficial (newly formed) muscularis mucosae, a deep (pre-existent) lamina propria and a deep (pre-existent) muscularis mucosae. Precise classification of T1m2, T1m3 or T1sm1 cancer can be hampered, if there is doubt concerning the presence of the deep muscularis mucosae in the endomucosal resection specimen. Another, likely more important issue, is the correct classification of superficial tumor invasion of the submucosa in EMR specimens, because the deep (pre-existent) muscularis mucosae is not always a continuous structure [24]. However, our data indicate that this probably has no therapeutic consequences, since there is no difference in prognosis between T1m3 and T1sm1.

The 5-year recurrence free percentage of the patients with early tumors with lymph node invasion after transhiatal resection is disappointing (33%) and comparable to the prognosis of tumors with a more advanced T-stage and lymph node invasion [25]. The potential, particularly of the T1sm2-sm3 tumors, for both lymphatic and hematogenous dissemination already in an early stage is an indication of their aggressiveness. This might justify a more radical treatment, *i.e.* transthoracic esophagectomy with extended lymphadenectomy of the posterior mediastinum, upper abdomen and perhaps even the neck [26]. Moreover, the unordered pattern of lymphatic dissemination in esophageal cancer, resulting in the frequent presence of tumor cells at a relatively large distance from the primary lesion, [27] is once more a reason for extensive procedures. Extended resection is believed to reduce the rate of locoregional re-

currence, thereby increasing the quality of life and prolonging the recurrence free period and perhaps even survival. However, a more extensive resection for patients with early carcinomas could only be legitimated if it leads to a higher chance for cure. The possible advantages should be weighed against the increased risk of postoperative morbidity and mortality, since these patients are exposed to the higher risk of surgical complications [28].

To improve long term outcome, many institutes apply neoadjuvant chemoradiotherapy for advanced esophageal carcinoma, especially after the recent publication of favorable long term results of a randomized MRC-trial comparing neoadjuvant chemotherapy followed by surgery versus surgery alone [29]. However, the poor prognosis of submucosal T1 tumors, and the high frequency of lymphatic dissemination indicate that these patients could also benefit from neoadjuvant chemoradiotherapy.

In conclusion, based on these results we suggest that the combined group of mucosal (T1m1-m3) and superficial submucosal (T1sm1) adenocarcinomas are eligible for local endoscopic treatment, since they carry only a 1% risk of lymphatic dissemination in combination with a 5-year recurrence free period of 97%. For the T1sm2-sm3 adenocarcinoma group, which showed lymph node metastases in 44% and a recurrence free period of 57%, surgical resection should be the treatment of choice. The substantial locoregional recurrence with or without distant dissemination after limited transhiatal resection is an argument in favor of more extensive surgery, and/or neoadjuvant chemoradiation.

ACKNOWLEDGMENTS

The authors are grateful to WCJ Hop, PhD (Department of Epidemiology and Biostatistics, Erasmus University Medical Center, Rotterdam) for statistical advice.

REFERENCES

1. Jenkins GJ, Doak SH, Parry JM, D'Souza FR, Griffiths AP, Baxter JN: Genetic pathways involved in the progression of Barrett's metaplasia to adenocarcinoma. Br J Surg 89:824-37, 2002
2. Wijnhoven BP, Tilanus HW, Dinjens WN: Molecular biology of Barrett's adenocarcinoma. Ann Surg 233:322-37, 2001
3. Falk GW: Barrett's esophagus. Gastroenterology 122:1569-91, 2002
4. Bruno MJ. Manification endoscopy, high resolution endoscopy, and chromoscopy; towards a better optical diagnosis. Gut 52 Suppl 4:iv12-11, 2003
5. Van Dam J. Novel methods of enhanced endoscopic imaging. Gut 52 Suppl 4:iv12-16, 2003
6. Froelicher P, Miller G: The European experience with esophageal cancer limited to the mucosa and submucosa. Gastrointest Endosc 32:88-90, 1986
7. van Sandick JW, van Lanschot JJ, Kuiken BW, Tytgat GN, Offerhaus GJ, Obertop H: Impact of endoscopic biopsy surveillance of Barrett's oesophagus on pathological stage and clinical outcome of Barrett's carcinoma. Gut 43:216-22, 1998
8. Bytzer P, Christensen PB, Damkier P, Vinding K, Seersholm N: Adenocarcinoma of the esophagus and Barrett's esophagus: a population-based study. Am J Gastroenterol 94:86-91, 1999
9. Sobin LH, Wittekind C. TNM classification of malignant tumors: International UnionAgainst Cancer (UICC). Sixth ed. New York: Wiley-Liss; 2002
10. van Lanschot JJ, Hulscher JB, Buskens CJ, Tilanus HW, ten Kate FJ, Obertop H: Hospital volume and hospital mortality for esophagectomy. Cancer 91:1574-8, 2001
11. Pera M, Trastek VF, Carpenter HA, Allen MS, Deschamps C, Pairolero PC: Barrett's esophagus with high-grade dysplasia: an indication for esophagectomy? Ann Thorac Surg 54:199-204, 1992
12. Zaninotto G, Parenti AR, Ruol A, Costantini M, Merigliano S, Ancona E: Oesophageal resection for high-grade dysplasia in Barrett's oesophagus. Br J Surg 87:1102-5, 2000
13. Ell C, May A, Gossner L, Pech O, Gunter E, Mayer G, Henrich R, Vieth M, Muller H, Seitz G, Stolte M: Endoscopic mucosal resection of early cancer and high-grade dysplasia in Barrett's esophagus. Gastroenterology 118:670-7, 2000
14. Gossner L, Stolte M, Sroka R, Rick K, May A, Hahn EG, Ell C: Photodynamic ablation of high-grade dysplasia and early cancer in Barrett's esophagus by means of 5-aminolevulinic acid. Gastroenterology 114:448-55, 1998
15. Hage M, Siersema PD, van Dekken H, Steyerberg EW, Haringsma J, van de Vrie W, Grool TE, van Veen RL, Sterenborg HJ, Kuipers EJ: 5-aminolevulinic acid photodynamic therapy versus argon plasma coagulation for ablation of Barrett's oesophagus: a randomised trial. Gut 53:785-90, 2004
16. Stein HJ, Feith M, Mueller J, Werner M, Siewert JR: Limited resection for early adenocarcinoma in Barrett's esophagus. Ann Surg 232:733-42, 2000
17. Akiyama H, Tsurumaru M, Ono Y, Udagawa H, Kajiyama Y: Esophagectomy without thoracotomy with vagal preservation. J Am Coll Surg 178:83-5, 1994
18. Japanese Society of Esophageal Diseases. Gidelines for the clinical and pathologic studies on carcinoma of the esophagus. 9th ed. Tokyo: Kanehara; 1999
19. Takubo K, Sasajima K, Yamashita K, Tanaka Y, Fujita K: Double muscularis mucosae in Barrett's esophagus. Hum Pathol 22:1158-61, 1991
20. Holscher AH, Bollschweiler E, Schneider PM, Siewert JR: Prognosis of early esophageal cancer. Comparison between adeno- and squamous cell carcinoma. Cancer 76:178-86, 1995
21. Streitz JM, Jr., Ellis FH, Jr., Gibb SP, Balogh K, Watkins E, Jr.: Adenocarcinoma in Barrett's esophagus. A clinicopathologic study of 65 cases. Ann Surg 213:122-5, 1991
22. Lerut T, Coosemans W, Van Raemdonck D, Dillemans B, De Leyn P, Marnette JM, Geboes K: Surgical treatment of Barrett's carcinoma. Correlations between morphologic findings and prognosis. J Thorac Cardiovasc Surg 107:1059-65; discussion 1065-6, 1994
23. Menke-Pluymers MB, Schoute NW, Mulder AH, Hop WC, van Blankenstein M, Tilanus HW: Outcome of surgical treatment of adenocarcinoma in Barrett's oesophagus. Gut 33:1454-8, 1992
24. Offerhaus GJ, Correa P, van Eeden S, Geboes K, Drillenburg P, Vieth M, van Velthuysen ML, Watanabe H, Sipponen P, ten Kate FJ, Bosman FT, Bosma A, Ristimaki A, van Dekken H, Riddell R, Tytgat GN: Report of an Amsterdam working group on Barrett esophagus. Virchows Arch 443:602-8, 2003

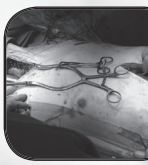
25. Millikan KW, Silverstein J, Hart V, Blair K, Bines S, Roberts J, Doolas A: A 15-year review of esophagectomy for carcinoma of the esophagus and cardia. Arch Surg 130:617-24, 1995
26. Lerut T, De Leyn P, Coosemans W, Van Raemdonck D, Scheys I, LeSaffre E: Surgical strategies in esophageal carcinoma with emphasis on radical lymphadenectomy. Ann Surg 216:583-90, 1992
27. Akiyama H, Tsurumaru M, Kawamura T, Ono Y: Principles of surgical treatment for carcinoma of the esophagus: analysis of lymph node involvement. Ann Surg 194:438-46, 1981
28. Hulscher JB, van Sandick JW, de Boer AG, Wijnhoven BP, Tijssen JG, Fockens P, Stalmeier PF, ten Kate FJ, van Dekken H, Obertop H, Tilanus HW, van Lanschot JJ: Extended transthoracic resection compared with limited transhiatal resection for adenocarcinoma of the esophagus. N Engl J Med 347:1662-9, 2002
29. Surgical resection with or without preoperative chemotherapy in oesophageal cancer: a randomised controlled trial. Lancet 359:1727-33, 2002

Chapter 10

Surgical mortality in patients with esophageal cancer: development and validation of a simple risk score

Journal of Clinical Oncology, in press

Ewout W Steyerberg
Bridget A Neville
Linetta B Koppert
Valery EPP Lemmens
Hugo W Tilanus
Jan-Willem W Coebergh
Jane C Weeks
Craig C Earle



ABSTRACT

Surgery has curative potential in a proportion of patients with esophageal cancer, but is associated with considerable peri-operative risks. We aimed to develop and validate a simple risk score for surgical mortality that could be applied to administrative data. We analyzed 3592 esophagectomy patient from 4 cohorts. We applied logistic regression analysis to predict mortality occurring within 30 days after esophagectomy for 1327 esophageal cancer patients over 65 years of age, diagnosed between 1991 and 1996 in the linked Surveillance, Epidemiology, and End Results (SEER) - Medicare database. A simple score chart for preoperative risk assessment of surgical mortality was developed and validated on 3 other cohorts, including 714 SEER-Medicare patients diagnosed between 1997 and 1999, 349 patients from a population-based registry in the Netherlands diagnosed between 1993 and 2001, and 1202 patients from a referral hospital in the Netherlands diagnosed between 1980 and 2002. Surgical mortality in the 4 cohorts was 11% (147/1327), 10% (74/714), 7% (25/349) and 4% (45/1202) respectively. Predictive patient characteristics included age, comorbidity (cardiac, pulmonary, renal, hepatic, and diabetes), preoperative radiotherapy or combined chemoradiotherapy, and a relatively low hospital volume. At validation, the simple score showed good agreement of predicted risks with observed mortality rates (calibration), but low discrimination (area under the ROC curve 0.58 to 0.66). A simple risk score combining clinical characteristics along with hospital volume to predict surgical mortality after esophagectomy from administrative data may form a basis for risk adjustment in quality of care assessment.

INTRODUCTION

Surgical resection offers a chance of long-term survival in patients with esophageal cancer [1]. However, even after careful staging, survival remains disappointing with less than 25% of patients surviving at 5 years after esophagectomy [2]. Better results may be achieved with the concomitant use of preoperative (neoadjuvant) chemotherapy and radiotherapy, although the benefits may be small [3-5]. The surgical risk in the short-term and the potential loss in quality of life have to be weighed against the long-term benefit, such as a longer survival [6 7].

Accurate prediction of surgical mortality is important not only for appropriate selection of candidates for esophagectomy [8], but also for evaluation of quality of care and policy decisions. Risk-adjustment is particularly necessary when comparing surgical mortality rates between institutions [9-11]. It is well known that the short-term surgical risk of esophagectomy varies by clinical characteristics, such as age [12] and presence of concomitant diseases (comorbidity) [8, 13]. Further, esophagectomy is among the procedures where physician and hospital characteristics, especially volume, have been found to be strongly related to the surgical outcome [14-18]. Hence, patients at higher risk may most appropriately undergo surgery at high volume centers [18-20].

Patient characteristics have been combined in multivariable prediction models for short-term mortality after esophagectomy. However, these models were often based on selected patient groups in specialized centers [8, 21], thus limiting the generalizability of the results. Furthermore, validation on new patients was often not performed, or showed unsatisfactory results [22]. The aim of the present study was to develop a simple and robust prediction model for surgical mortality in esophageal cancer patients. We first analyzed several previously identified predictive characteristics in a large population-based cohort, then developed a simple risk score, and finally validated this score in 3 other cohorts.

METHODS

We analyzed 4 cohorts: two population-based series of 1327 and 714 patients from the linked Surveillance, Epidemiology, and End Results (SEER) - Medicare database [23], another population-based series of 349 patients from the Netherlands ('Eindhoven') [24], and 1202 patients from a referral hospital in the Netherlands ('Rotterdam') [25, 26]. The larger SEER–Medicare cohort served as the model development set and the other three as validation sets.

Patients: SEER-Medicare

The SEER database is made up of 11 tumor registries covering approximately 14% of the United States population. It has been linked to the Centers for Medicare and Medicaid services Medicare database through the end of 2001. We identified 2 sets of patients diagnosed with

pathologically confirmed esophageal cancer. The first set ('SEER 91-96') included patients diagnosed between January 1, 1991 and December 31, 1996. It was used for development of the prediction model. The second set included patients diagnosed between January 1, 1997 and December 31, 1999 ('SEER 97-99'), for validation of the developed model. The selection criteria and definitions of variables were identical in both sets. We excluded patients for whom the date of death differed by more than three months between the SEER and Medicare database, patients who were diagnosed from death certificate or autopsy, and patients for whom the month of diagnosis was not available. We also excluded patients who were only eligible for Medicare on the basis of end-stage renal failure or disability; therefore, all patients were 65 years or older.

We considered combinations of surgery, radiation, and chemotherapy [27]. Surgery was identified from the Medicare database using the International Classification of Diseases, 9th revision (ICD-9 codes 42.0 to 43.99) [28]. Information on radiation use was based on SEER records and Medicare data [29]. Information on chemotherapy was based on Medicare data only [30].

Comorbidity was determined based on Medicare claims between 13 months and 1 month before diagnosis [31]. Missing values were assigned to patients without Medicare data from this time window if no comorbidity was registered. Missing values were statistically imputed to allow for analysis of the available information from other predictors [32]. Exclusion of these patients in a sensitivity analysis did not affect results (data not shown). ICD-9 codes of both inpatient and outpatient bills were analyzed [33, 34]. Comorbidities were grouped as cardiovascular (previous myocardial infarction, heart failure, peripheral arterial disease, cerebrovascular disease), diabetes (with or without complications), pulmonary (COPD), renal (mild to severe), and hepatic (mild to severe) [8, 35]. We created a simple comorbidity score based on the presence of cardiac, pulmonary, renal, or hepatic comorbidity, or diabetes. For simplicity, each comorbidity was counted as one point, based on similar regression coefficients [36]. Patients were classified as having surgery performed in a teaching hospital versus not in a teaching hospital. Further hospital characteristics included the hospital volume, based on the sum of esophagectomies per hospital using the unique hospital provider number in the Medicare data [37]. Low, intermediate and high volumes were defined by tertile of patients.

Patients: Eindhoven

As a second validation cohort, we considered 349 patients who underwent surgical resection for a primary tumor of the esophagus, diagnosed between January 1, 1993 and December 31, 2001 in the Southeast of the Netherlands [24]. The Eindhoven Cancer Registry covers approximately 2 million inhabitants who are served by 16 community hospitals and two large radiotherapy institutes (16% of the Netherlands). Upon notification by one of six pathological laboratories and the hospital medical records departments, registration clerks actively collect information on diagnosis, tumor stage, treatment, and comorbidities from the medical re-

cords. The Eindhoven Cancer Registry has been collecting detailed data on clinically relevant comorbidity for new cancer patients since 1993 [35].

Patients: Rotterdam

As a third validation cohort, we considered 1202 patients who underwent surgical resection for a primary tumor of the esophagus at the University Hospital Rotterdam, between January 1, 1980 and December 31, 2002. This hospital serves as a referral center for the Southwest of the Netherlands. A database system is maintained with detailed information on diagnosis, tumor stage, treatment, and comorbidity [25, 26]. Information on hepatic and renal disease was not available for these patients.

Statistical analysis

Descriptive statistics were used for univariate analyses, with cells with fewer than 5 patients in the SEER cohorts indicated as '<5'. We applied logistic regression analysis to relate patient and treatment characteristics to mortality within 30 days after surgery. Mortality was considered irrespective of the cause. Odds ratios (ORs) were calculated with 95% confidence intervals (95%CI). Potential predictive characteristics were chosen from the clinical literature and expert opinion [38]. The performance of the model was assessed with respect to calibration and discrimination [39]. Calibration refers to the agreement between observed outcomes and predicted probabilities and is the most important quality when trying to predict the expected mortality rate for a group of patients. Calibration was assessed graphically, and tested with the Hosmer-Lemeshow goodness of fit test [40]. Discrimination refers to the ability to distinguish patients who will die from those who will survive. Discrimination was quantified by the Area Under the Receiver Operating Characteristic Curve (AUC), which is identical to the concordance (c) statistic [39]. An AUC of 0.5 indicates no discriminative ability at all, i.e. a coin flip, while an AUC of 1 indicates perfect discrimination, i.e. a test with 100% sensitivity and 100% specificity. Prediction models with an AUC exceeding 0.8 have often been labeled as good to excellent, those with AUC between 0.7 and 0.8 as moderate, and those with AUC between 0.6 and 0.7 as providing low discrimination.

Multivariable models were internally and externally validated. Internal validation was performed with a standard bootstrap procedure [38, 39]. Bootstrap samples were drawn with replacement, of the same size as the original sample. Predictions from each bootstrap model were evaluated in the original sample. The difference in performance in the bootstrap sample and in the original sample quantifies the optimism that may be expected when the multivariable model is applied to new, but similar, patients. A score chart was derived from the multivariable regression coefficients. For simple application, the coefficients were multiplied by two and rounded.

For external validation, we constructed logistic regression models for each cohort, containing the same predictors as the multivariable model based on SEER 1991-96. We studied whether

the predictors had similar effects. Subsequently, we derived a combined model based on all patient data, with stratification for study.

RESULTS

The SEER-Medicare patients were on average 73 and 74 years of age (Table 10.1). The patients in the Eindhoven and Rotterdam series were approximately 10 years younger on average (64 and 62 years respectively), since these series included patients of all ages, not just over 65 or older. The majority of patients was male. Comorbidity was found in around 20% of the SEER-Medicare patients (19% and 23%, respectively, especially pulmonary (9% and 9%), cardiovascular (9% and 9%), and diabetes (8% and 10%)). Pulmonary and cardiovascular comorbidities were more often registered for the Rotterdam patients (15% and 16% respectively), while cardiovascular comorbidity was more common in the Eindhoven patients (18%). Most patients had adenocarcinoma, and pathologically confirmed locoregional disease. Neoadjuvant treatment was given in around 20% of the SEER-Medicare patients, 7% of the Eindhoven patients and 38% of the Rotterdam patients. Most SEER-Medicare patients were treated in teaching hospitals, though the annual volumes of esophagectomies were relatively small. This information was not reliably available for the Eindhoven patients. The Rotterdam center is a referral hospital with over 50 esophagectomies per year.
Of the 1317 and 714 SEER-Medicare patients undergoing surgery, 147 (11%) and 74 (10%) died within 30 days after surgery, respectively. Mortality was lower among the Eindhoven and Rotterdam patients (7% and 4% respectively).

Univariate analyses

In univariate analysis of the 1317 SEER 91-96 patients, characteristics that were statistically significantly associated with mortality included age and comorbidity (pulmonary, diabetes) (Table 10.2). Neoadjuvant treatment with radiotherapy was associated with a substantially higher surgical mortality risk (23%), as was neoadjuvant chemoradiotherapy (16%). In contrast, neoadjuvant chemotherapy alone was associated with a somewhat lower risk of mortality (6%). For robust further analyses we combined the patients without any neoadjuvant treatment with those with neoadjuvant chemotherapy. Finally, higher hospital volume was clearly associated with lower surgical mortality (p-value for trend 0.003).
Similar relationships were observed in the other 3 cohorts, with higher mortality among older patients, those with comorbidity, and those who had neoadjuvant radiotherapy or chemoradiotherapy (Table 10.2).

Table 10.1 Characteristics of patients with esophageal cancer undergoing cancer-directed surgery in 4 cohorts.

Characteristic	SEER 91-96 n=1327		SEER 97-99 n=714		Eindhoven n=349		Rotterdam n=1202	
Demographics	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
Age (years)								
<55	-		-		74	21%	294	24%
55-64.9	-		-		104	30%	389	32%
65-74.9	853	64%	457	64%	112	32%	406	34%
75-84.9	409	31%	218	31%	54	15%	110	9%
85+	65	5%	39	5%	5	1%	3	0%
Male gender	1002	76%	523	73%	251	72%	909	76%
Comorbidities								
Pulmonary disease	95/909	9%	58/668	9%	31	9%	185	15%
Cardiovascular disease	95/1002	9%	62/667	9%	64	18%	195	16%
Diabetes	84/998	8%	70/667	10%	28	8%	69	6%
Hepatic disease	2/987	0%	2/665	0%	2	1%	-	
Renal disease	5/987	1%	6/665	1%	0	0%	-	
Comorbidity count								
0	1073	81%	553	77%	239	68%	839	70%
1	210	16%	127	18%	95	27%	283	24%
2+	44	4%	34	5%	15	4%	80	7%
Cancer characteristics								
Histology								
Adenocarcinoma	700	53%	423	59%	258	74%	737	61%
Squamous cell	538	41%	255	36%	85	24%	429	36%
Mixed and other	89	7%	36	5%	6	2%	33	3%
Pathological stage								
Local/In situ	468	35%	270	38%	139	40%	592	49%
Regional	484	36%	289	40%	195	56%	461	38%
Distant	152	11%	87	12%	12	3%	138	11%
Unknown	223	17%	68	10%	3	1%	11	1%
Neoadjuvant treatment								
No neoadjuvant treatment	1069	81%	568	80%	326	93%	751	62%
Preoperative radiotherapy	80	6%	21	3%	11	3%	253	21%
Preoperative chemotherapy	49	4%	13	2%	0	0%	167	14%
Preoperative chemoradiotherapy	129	10%	112	16%	12	3%	31	3%
Hospital characteristics								
Teaching hospital	768	58%	443	62%	-		1202	100%
Volume of hospital					-			
<=1 esophagectomy /yr	477	36%	198	28%			-	
1.1–2.5 / yr	358	27%	169	24%			-	
>= 2.6 /yr	492	37%	347	49%			1202	100%
Outcome								
30-day mortality	147	11%	74	10%	25	7%	45	4%

Table 10.2 Relationships between patient characteristics and 30-day mortality after cancer- directed surgery for esophageal cancer in 4 cohorts (univariate analyses). Mortality is shown by absolute numbers and as a percentage of patients with a characteristic.

Characteristic	SEER 91-96 147/1327 11%		SEER 97-99 74/714 11%		Eindhoven 25/349 7%		Rotterdam 45/1202 4%	
Demographics	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
Age (years)								
<55	-		-		6	8%	4	1%
55-64.9	-		-		6	6%	16	4%
65-74.9	84	10%	39	9%	9	8%	20	5%
75-84.9	49	12%	31	14%				
85+	14	22%	4	10%	4	7%	5	5%
Male gender	108	11%	59	11%	16	6%	37	4%
Comorbidities								
Pulmonary disease	21	22%	3	5%	4	13%	12	6%
Cardiovascular disease	14	15%	12	19%	3	5%	10	5%
Diabetes	16	19%	14	20%	4	14%	5	7%
Hepatic disease	-		-		-		-	
Renal disease	-		-		-		-	
Comorbidity count								
0	107	10%	50	9%	14	6%	24	3%
1	28	13%	18	14%	10	11%	16	6%
2+	12	27%	6	18%	1	-	5	6%
Cancer characteristics								
Histology								
Adenocarcinoma	75	11%	36	9%	20	8%	23	3%
Squamous cell	66	12%	33	13%	4	5%	22	5%
Mixed and other	6	7%	5	14%	1	-	40	4%
Neoadjuvant treatment								
No neoadjuvant treatment	106	10%	54	10%	23	7%	22	3%
Preoperative radiotherapy	18	23%	4	19%	1	-	18	7%
Preoperative chemotherapy	3	6%	1	8%	-	-	3	2%
Preoperative chemoradiotherapy	20	16%	15	13%	1	-	2	6%
Hospital characteristics								
Teaching hospital	73	10%	50	11%	-		-	
Volume of hospital					-		-	
<=1 esophagectomy /yr	66	14%	27	14%				
1.1–2.5 / yr	42	12%	30	18%				
>= 2.6 /yr	39	8%	17	5%				

Multivariable analyses

Neoadjuvant treatment remained associated with an increased risk of surgical mortality in multivariable logistic regression analysis of the 1317 SEER 91-96 patients, with adjusted ORs of 2.5 and 1.9 for radiotherapy and chemoradiotherapy respectively (Table 10.3). Comorbi-

dity and age were also highly predictive, with an OR of 1.6 per comorbid condition, and an OR 1.6 per decade of age. Higher volume hospitals exhibited close to half the mortality of lower volume hospitals (OR 0.59).

The effects of age, comorbidity, and neoadjuvant therapy were very similar for the 714 SEER 97-99 patients. For the Eindhoven patients, comorbidity was associated with a higher mortality (OR 1.5 per condition), while age had no effect (OR close to 1). For the Rotterdam patients, predictive effects of age, comorbidity and neoadjuvant therapy were largely similar to those for the SEER-Medicare patients. When we combined all 4 cohorts (n=3592), the predictive effects were similar to those observed in the SEER 91-96 patients that were initially used for model development. For hospital volume, we found that mortality in 'high volume' centers was about half that in 'low volume' centers. For a 'very high' volume center such as Rotterdam the mortality was only a third of that in 'low volume' centers (Table 10.3).

Table 10.3 Multivariable logistic regression analyses in 4 cohorts. The combined data set contained 3592 patients, of whom 291 had died by 30 days.

Characteristic	SEER 91-96	SEER 97-99	Eindhoven	Rotterdam	Combined
Age per decade	1.6 [1.2-2.0]	1.5 [1.0-2.2]	0.94 [0.6-1.4]	1.4 [1.0-2.0]	1.4 [1.2-1.7]
Comorbidity count	1.6 [1.2-2.1]	1.3 [.91-1.9]	1.5 [0.8-2.9]	1.5 [1.0-2.3]	1.5 [1.2-1.8]
Neoadjuvant therapy					
No neoadjuvant treatment	1	1	-	1	1
Radiotherapy	2.5 [1.4-4.4]	2.5 [0.78-8.1]		3.1 [1.7-5.9]	2.6 [1.8-3.8]
Chemoradiotherapy	1.9 [1.1-3.3]	1.4 [0.74-2.7]		2.9 [0.7-13]	1.8 [1.3-2.7]
Volume of hospital			-	-	
<=1 /yr	1	1			1
1.1–2.5 / yr	0.80 [0.52-1.2]	1.5 [0.82-2.6]			0.88 [0.65-1.2]
>= 2.6 /yr	0.59 [0.39-0.90]	0.36 [0.19-0.69]			0.49 [0.35-0.70]
± 50/yr					0.30 [0.20-0.46]*
Performance					
Apparent ROC area	0.66 [0.61-0.70]	0.70 [0.64-0.76]	0.56 [0.45-0.68]	0.66 [0.58-0.75]	0.70 [0.67-0.73]
Score chart ROC area	0.65 [0.61-0.69]	0.66 [0.60-0.72]	0.58 [0.46-0.70]	0.66 [0.57-0.74]	0.70 [0.67-0.73]

* Estimate for Rotterdam, a very high volume referral center

Model performance and risk score

The multivariable model based on the 1317 SEER 91-96 patients showed low discrimination (AUC 0.66). Internal validation of this model indicated a slight decrease in discriminative ability (AUC 0.65). The multivariable model performed similarly in the 714 SEER 97-99 patients (AUC 0.70) and Rotterdam patients (AUC 0.66), but poor in the Eindhoven patients (AUC 0.56).

A simple chart assigned 1 point per 15 years of age, 1 point per comorbidity, 1 point for neoadjuvant chemoradiotherapy, and 1.5 points for radiotherapy (Table 10.4). Hospital volume was scored as 0, –0.5, –1.5 and –2 for low, intermediate, high and very high volume respectively, based on the multivariable effects from Table 10.3. A summary score corresponds to

Table 10.4 Score chart to estimate 30-day mortality after cancer-directed surgery for esophageal cancer.

Characteristic	Value	Score
Age (years)	50	-1
	65	0
	80	1
Comorbidity	Pulmonary	1
	Cardiovascular	1
	Diabetes	1
	Hepatic	1
	Renal	1
Neoadjuvant therapy	Radiotherapy	1.5
	Chemoradiotherapy	1
Hospital volume	Low (<1/ yr)	0
	Intermediate (1.1-2.5/ yr)	-0.5
	High (>2.6/yr)	-1.5
	Very high (±50/yr)	-2
Sum score (add scores)		

Intermediate scores for age can be approximated by linear interpolation, e.g. age 72 corresponds to a score of +0.5.
The formula to calculate the predicted probability of surgical mortality is
P(mort) = 1 / [1+exp(2.41 – 0.32 * score)].

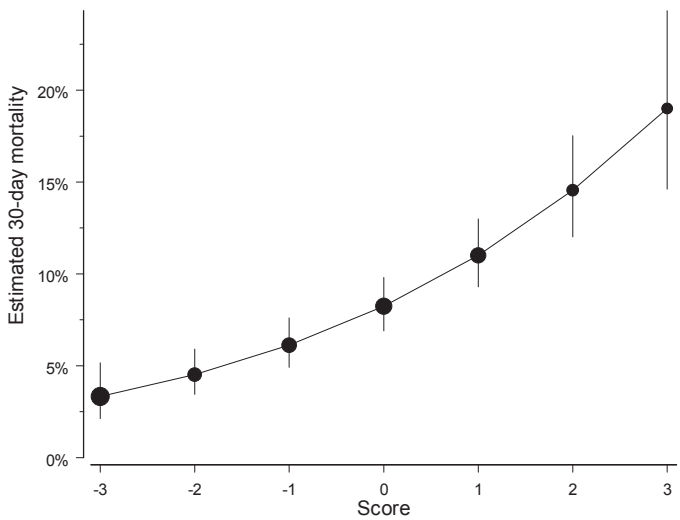


Figure 10.1 Estimated surgical mortality in relation to the sum score that can be obtained from Table 10. 4. The 95% confidence intervals are based on analysis of 4 cohorts, containing 3592 patients undergoing surgery for esophageal cancer. Dot size is proportional to number of patients.

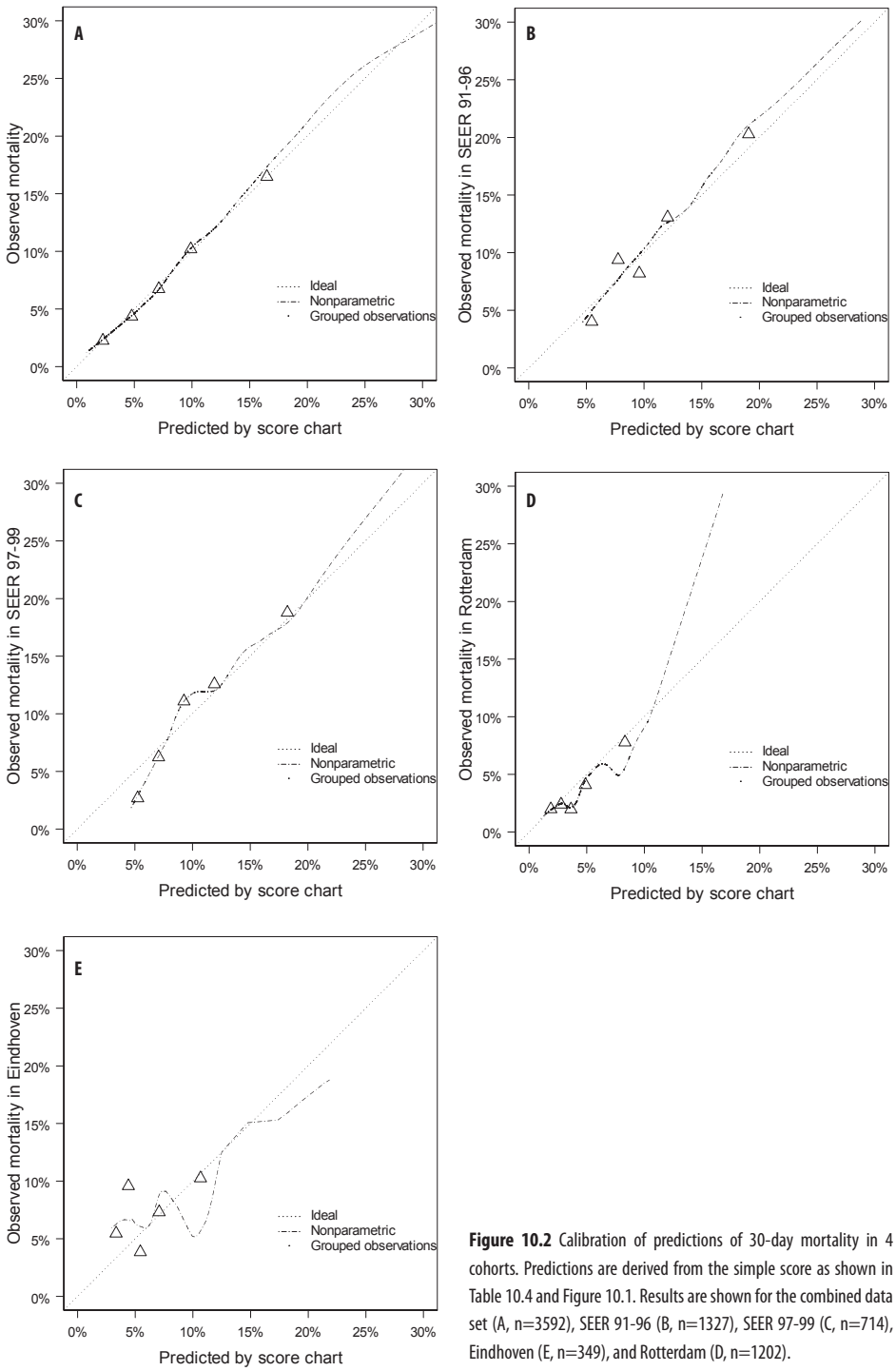


Figure 10.2 Calibration of predictions of 30-day mortality in 4 cohorts. Predictions are derived from the simple score as shown in Table 10.4 and Figure 10.1. Results are shown for the combined data set (A, n=3592), SEER 91-96 (B, n=1327), SEER 97-99 (C, n=714), Eindhoven (E, n=349), and Rotterdam (D, n=1202).

a predicted probability of 30-day mortality (Figure 10.1). For example, a 65-year-old patient, who has pulmonary and cardiovascular comorbidity, has not received neoadjuvant treatment, and undergoes surgery in a low volume hospital has a score of 0 + 2 + 0 + 0 = 2 points. This score corresponds to a predicted mortality of 17% [95%CI 14 – 21%]. If this patient were to be treated in a very high volume hospital (score –2, sum score=0), the predicted mortality would be 7% [5.7 – 8.5%]. The performance of the risk score was similar to the original model for each cohort (Table 10.3).

In Figure 10.2 we show the calibration of the predictions corresponding the risk scores. Predictions were above 19% for only 5% of the patients, consistent with the low discrimination. Calibration was excellent for all patients combined (Fig 10.2 A), and for the 2 series of SEER patients (Fig 10.2 B-C). Calibration was modest for Eindhoven and reasonable for Rotterdam (Figures 10.2 D-E).

DISCUSSION

Surgical mortality after esophagectomy is an important quality of care measure, but is only predictable to a certain extent with a limited set of patient, treatment and hospital characteristics. Using data from 3 different settings we found, as expected, that age and comorbidity were strongly related to outcome. Also, preoperative radiotherapy and chemoradiotherapy were clearly associated with 30-day mortality, as was a lower hospital volume. The discriminative ability of a simple risk score that combined these characteristics was however low.

Age predicts surgical risk for a wide range of procedures. For esophagectomy, one major study found that 30-day mortality increased from 10.7% for patients between 65 and 69 years of age to over 20% for those over 80 years [12]. We confirmed this trend in our data, especially in the SEER-Medicare cohorts, where we observed a relative increase in mortality of 40% per decade in adjusted analysis. The presence of comorbidity is known to affect outcome in many cancers. A number of scoring systems have been developed to measure it, including the Charlson score [41] and the ACE-27 [13]. We used a simple count of comorbid cardiovascular, pulmonary, renal, hepatic conditions, and diabetes, and found that each point was on average associated with a 50% increase in surgical risk (OR 1.5). Comorbidity scoring was claims-based in the SEER-Medicare data, and chart-based in the other 2 cohorts. Despite these and other differences in definitions, the comorbidity-mortality relationship was similar across the 4 study cohorts, in line with findings in a previous study [42].

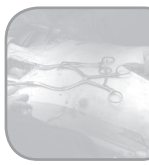
Preoperative unimodality radiotherapy was clearly associated with higher surgical mortality. This treatment strategy has largely been abandoned in recent years [43], so a more relevant finding is that neoadjuvant chemoradiotherapy nearly doubled mortality compared to no neoadjuvant treatment or neoadjuvant chemotherapy alone. It is possible that these results may be somewhat confounded by selection of patients with more advanced tumors for

neoadjuvant treatment. However, our findings are consistent with recent meta-analyses of randomized trials, showing that neoadjuvant chemoradiotherapy was associated with a 1.72 fold higher surgical mortality (95%CI 0.96 – 3.1, p = 0.07) [3], while neoadjuvant chemotherapy alone was not (OR 1.08 [95%CI 0.45 – 2.6]; p=0.87) [44]. This implies that part of the benefit of neoadjuvant chemoradiotherapy (e.g. on small metastases which would not be resected by surgery) may be offset by higher surgical mortality. This issue requires further detailed evaluation in randomized trials. Our results suggest that measures to reduce surgical risk should especially be considered for patients with neoadjuvant chemoradiotherapy.

Reported surgical risks vary widely in the literature. Much of this variation can be explained by differences in hospital volume [14-19, 45, 46]. Hospital volume remained important after adjustment for case-mix, which is generally an important methodological consideration in such analyses of observational data [9-11]. Many authors suggest that a policy of concentrating care in high-volume centers should be considered especially for esophagectomy, where outcome varies substantially between low-volume and high-volume providers [20, 47]. As illustrated, a patient could have a 17% or 7% predicted mortality risk depending on surgery in a low volume or in a high volume center.

Our study has some limitations. Our 4 cohorts were of considerable size, but the Eindhoven and Rotterdam series had only few events, which makes firm conclusions on external validity difficult [48]. We included all patients undergoing esophagectomy. Around 10% had pathologically distant disease, and we cannot exclude that a few patients had clinically known distant disease before surgery. The inclusion of these patients may have led us to overestimate risk for patients with true locoregional disease. On the other hand, we considered 30-day mortality, and in-hospital mortality can be substantially higher. We further note that the model was mainly based on patients over 65 years of age; validity may be best for this patient category. Next, the exact limits to define low, medium and high volume centers are hard to determine. We used rather low annual volumes (<1, 1-2.5, >=2.6 per year), based on tertiles of patients, while higher limits may be better defendable. Finally, we did not have information on physiologic variables such as performance status or ASA score which have been used in clinical models [45, 49]. Our risk model hence had only a low discriminative ability, and can therefore only be a relatively rough, though evidence-based, basis for the surgical risk of individual patients. Our score is easy to calculate from existing, readily available data, however, and so could well serve for case-mix adjustment when comparing surgical mortality rates between institutions.

In conclusion, we found substantial mortality after esophagectomy, which was related to patient, neoadjuvant therapy, and hospital characteristics. We developed and externally validated a simple risk score, which provides an admittedly rough estimate of surgical mortality with which to compare actual outcomes. Further validation and extension of this score is mandatory.



REFERENCES

1. Leonard GD, McCaffrey JA, Maher M: Optimal therapy for oesophageal cancer. Cancer Treat Rev 29:275-82, 2003
2. Wu PC, Posner MC: The role of surgery in the management of oesophageal cancer. Lancet Oncol 4:481-8, 2003
3. Urschel JD, Vasan H: A meta-analysis of randomized controlled trials that compared neoadjuvant chemoradiation and surgery to surgery alone for resectable esophageal cancer. Am J Surg 185:538-43, 2003
4. Kaklamanos IG, Walker GR, Ferry K, Franceschi D, Livingstone AS: Neoadjuvant treatment for resectable cancer of the esophagus and the gastroesophageal junction: a meta-analysis of randomized clinical trials. Ann Surg Oncol 10:754-61, 2003
5. Geh JI: The use of chemoradiotherapy in oesophageal cancer. Eur J Cancer 38:300-13, 2002
6. Weeks J: Overview of outcomes research and management and its role in oncology practice. Oncology (Williston Park) 12:11-3, 1998
7. Blazeby JM, Farndon JR, Donovan J, Alderson D: A prospective longitudinal study examining the quality of life of patients with esophageal carcinoma. Cancer 88:1781-7, 2000
8. Bartels H, Stein HJ, Siewert JR: Preoperative risk analysis and postoperative mortality of oesophagectomy for resectable oesophageal cancer. Br J Surg 85:840-4, 1998
9. DeLong ER, Peterson ED, DeLong DM, Muhlbaier LH, Hackett S, Mark DB: Comparing risk-adjustment methods for provider profiling. Stat Med 16:2645-64, 1997
10. Shahian DM, Blackstone EH, Edwards FH, Grover FL, Grunkemeier GL, Naftel DC, Nashef SA, Nugent WC, Peterson ED: Cardiac surgery risk models: a position article. Ann Thorac Surg 78:1868-77, 2004
11. Halm EA, Lee C, Chassin MR: Is volume related to outcome in health care? A systematic review and methodologic critique of the literature. Ann Intern Med 137:511-20, 2002
12. Finlayson EV, Birkmeyer JD: Operative mortality with elective surgery in older adults. Eff Clin Pract 4:172-7, 2001
13. Piccirillo JF, Tierney RM, Costas I, Grove L, Spitznagel EL, Jr.: Prognostic importance of comorbidity in a hospital-based cancer registry. Jama 291:2441-7, 2004
14. Begg CB, Cramer LD, Hoskins WJ, Brennan MF: Impact of hospital volume on operative mortality for major cancer surgery. Jama 280:1747-51, 1998
15. Swisher SG, Deford L, Merriman KW, Walsh GL, Smythe R, Vaporicyan A, Ajani JA, Brown T, Komaki R, Roth JA, Putnam JB: Effect of operative volume on morbidity, mortality, and hospital use after esophagectomy for cancer. J Thorac Cardiovasc Surg 119:1126-32, 2000
16. Dimick JB, Cattaneo SM, Lipsett PA, Pronovost PJ, Heitmiller RF: Hospital volume is related to clinical and economic outcomes of esophageal resection in Maryland. Ann Thorac Surg 72:334-9; discussion 339-41, 2001
17. Bachmann MO, Alderson D, Edwards D, Wotton S, Bedford C, Peters TJ, Harvey IM: Cohort study in South and West England of the influence of specialization on the management and outcome of patients with oesophageal and gastric cancers. Br J Surg 89:914-22, 2002
18. Finlayson EV, Goodney PP, Birkmeyer JD: Hospital volume and operative mortality in cancer surgery: a national study. Arch Surg 138:721-5; discussion 726, 2003
19. Birkmeyer JD, Siewers AE, Finlayson EV, Stukel TA, Lucas FL, Batista I, Welch HG, Wennberg DE: Hospital volume and surgical mortality in the United States. N Engl J Med 346:1128-37, 2002
20. Birkmeyer JD, Stukel TA, Siewers AE, Goodney PP, Wennberg DE, Lucas FL: Surgeon volume and operative mortality in the United States. N Engl J Med 349:2117-27, 2003
21. Saito T, Shimoda K, Kinoshita T, Shigemitsu Y, Miyahara M, Kobayashi M, Shimaoka A: Prediction of operative mortality based on impairment of host defense systems in patients with esophageal cancer. J Surg Oncol 52:1-8, 1993
22. Zafirellis KD, Fountoulakis A, Dolan K, Dexter SP, Martin IG, Sue-Ling HM: Evaluation of POSSUM in patients with oesophageal cancer undergoing resection. Br J Surg 89:1150-5, 2002
23. Potosky AL, Riley GF, Lubitz JD, Mentnech RM, Kessler LG: Potential for cancer related health services research using a linked Medicare-tumor registry database. Med Care 31:732-48, 1993

24. Koppert LB, Janssen-Heijnen ML, Louwman MW, Lemmens VE, Wijnhoven BP, Tilanus HW, Coebergh JW: Comparison of comorbidity prevalence in oesophageal and gastric carcinoma patients: a population-based study. Eur J Gastroenterol Hepatol 16:681-8, 2004
25. Tilanus HW, Hop WC, Langenhorst BL, van Lanschot JJ: Esophagectomy with or without thoracotomy. Is there any difference? J Thorac Cardiovasc Surg 105:898-903, 1993
26. Polee MB, Hop WC, Kok TC, Eskens FA, van der Burg ME, Splinter TA, Siersema PD, Tilanus HW, Stoter G, van der Gaast A: Prognostic factors for survival in patients with advanced oesophageal cancer treated with cisplatin-based combination chemotherapy. Br J Cancer 89:2045-50, 2003
27. Steyerberg EW, Earle CC, Neville BA, Weeks JC: Racial differences in surgical evaluation, treatment, and outcome of locoregional esophageal cancer: a population-based analysis of elderly patients. J Clin Oncol 23:510-7, 2005
28. Cooper GS, Virnig B, Klabunde CN, et al. Use of SEER-Medicare data for measuring cancer surgery. Med Care 40:IV43-48, 2002
29. Virnig BA, Warren JL, Cooper GS, et al. Studying radiation therapy using SEER-Medicare-linked data. Med Care 40:IV49-54, 2002
30. Warren JL, Harlan LC, Fahey A, et al. Utility of the SEER-Medicare data to identuft chemotherapy use. Med Care 40:IV55-61, 2002
31. Klabunde CN, Warren JL, Legner JM. Assessing comorbidity using claims data: an overview. Med Care 40:IV26-35, 2002
32. Arnold AM, Kronmal RA: Multiple imputation of baseline data in the cardiovascular health study. Am J Epidemiol 157:74-84, 2003
33. Deyo RA, Cherkin DC, Ciol MA: Adapting a clinical comorbidity index for use with ICD-9-CM administrative databases. J Clin Epidemiol 45:613-9, 1992
34. Klabunde CN, Potosky AL, Legler JM, Warren JL: Development of a comorbidity index using physician claims data. J Clin Epidemiol 53:1258-67, 2000
35. Coebergh JW, Janssen-Heijnen ML, Post PN, Razenberg PP: Serious co-morbidity among unselected cancer patients newly diagnosed in the southeastern part of The Netherlands in 1993-1996. J Clin Epidemiol 52:1131-6, 1999
36. Wang PS, Walker A, Tsuang M, Orav EJ, Levin R, Avorn J: Strategies for improving comorbidity measures based on Medicare and Medicaid claims data. J Clin Epidemiol 53:571-8, 2000
37. Schrag D, Bach PB, Dahlman C, et al. Identifying and measuring hospital characteristics using the SEER-Medicare data and other claims-based sources. Med Care 40:IV96-103, 2002
38. Steyerberg EW, Eijkemans MJ, Harrell FE, Jr., Habbema JD: Prognostic modelling with logistic regression analysis: a comparison of selection and estimation methods in small data sets. Stat Med 19:1059-79, 2000
39. Harrell FE, Jr., Lee KL, Mark DB: Multivariable prognostic models: issues in developing models, evaluating assumptions and adequacy, and measuring and reducing errors. Stat Med 15:361-87, 1996
40. Hosmer DW, Hosmer T, Le Cessie S, Lemeshow S: A comparison of goodness-of-fit tests for the logistic regression model. Stat Med 16:965-80, 1997
41. Charlson ME, Pompei P, Ales KL, MacKenzie CR: A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. J Chronic Dis 40:373-83, 1987
42. Newschaffer CJ, Bush TL, Penberthy LT: Comorbidity measurement in elderly female breast cancer patients with administrative and medical records data. J Clin Epidemiol 50:725-33, 1997
43. Arnott SJ, Duncan W, Gignoux M, et al. Preoperative radiotherapy for esophageal carcinoma. Cochrane Database Syst Rev: CD001799, 2000
44. Urschel JD, Vasan H, Blewett CJ: A meta-analysis of randomized controlled trials that compared neoadjuvant chemotherapy and surgery to surgery alone for resectable esophageal cancer. Am J Surg 183:274-9, 2002
45. McCulloch P, Ward J, Tekkis PP: Mortality and morbidity in gastro-oesophageal cancer surgery: initial results of ASCOT multicentre prospective cohort study. Bmj 327:1192-7, 2003
46. van Lanschot JJ, Hulscher JB, Buskens CJ, Tilanus HW, ten Kate FJ, Obertop H: Hospital volume and hospital mortality for esophagectomy. Cancer 91:1574-8, 2001
47. Shahian DM, Normand SL: The volume-outcome relationship: from Luft to Leapfrog. Ann Thorac Surg 75:1048-58, 2003

48. Vergouwe Y, Steyerberg EW, Eijkemans MJ, Habbema JD: Substantial effective sample sizes were required for external validation studies of predictive logistic regression models. J Clin Epidemiol 58:475-83, 2005
49. Sauvanet A, Mariette C, Thomas P, Lozac'h P, Segol P, Tiret E, Delpero JR, Collet D, Leborgne J, Pradere B, Bourgeon A, Triboulet JP: Mortality and morbidity after resection for adenocarcinoma of the gastroesophageal junction: predictive factors. J Am Coll Surg 201:253-62, 2005

Part V

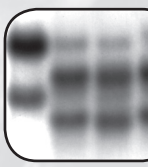
Identification of high-risk patients

Chapter 11

The CHEK2*1100delC mutation has no major contribution in esophageal carcinogenesis

British Journal of Cancer, 2004 Feb 23;90(4):888-891

Linetta B Koppert
Mieke Schutte
Mustaffa Abbou
Hugo W Tilanus
Winand NM Dinjens



ABSTRACT

In response to DNA damage, the cell cycle checkpoint kinase 2 (CHEK2) may phosphorylate p53, Cdc25A and Cdc25C, and regulate BRCA1 function, leading to cell cycle arrest and DNA repair. The truncating germline mutation CHEK2*1100delC abrogates kinase activity and confers low-penetrance susceptibility to breast cancer. We found CHEK2*1100delC in 0.5% of 190 esophageal squamous cell carcinomas and in 1.5% of 196 esophageal adenocarcinomas. In addition we observed the mutation in 3.0% of 99 Barrett's metaplasias and 1.5% of 66 dysplastic Barrett's epithelia, both known precursor lesions of esophageal adenocarcinoma. Since CHEK2*1100delC mutation frequencies did not significantly differ among esophageal squamous cell carcinomas, adenocarcinomas and (dysplastic) Barrett's epithelia as compared to healthy individuals, we conclude that the CHEK2*1100delC mutation has no major contribution in esophageal carcinogenesis.

INTRODUCTION

Esophageal carcinoma is the ninth most common tumor type worldwide. Despite surgical intervention, 5-year overall survival is less then 20%, mainly due to the fact that patients often present with an advanced tumor stage. Alcohol and tobacco use are established risk factors for the development of esophageal squamous cell carcinomas [1]. The presence of Barrett's esophagus is the main risk factor for adenocarcinoma formation, being 30-125 times higher in patients with Barrett's esophagus as compared to the general population. Barrett's esophagus is defined as a columnar cell metaplasia of the native distal esophageal squamous cell epithelium [2], accompanied by the presence of Goblet cells, as a result of chronic gastro-esophageal reflux. Barrett's metaplasia can progress to low and high grade dysplasia and ultimately to invasive and metastasising adenocarcinoma. Patients with Barrett's esophagus receive endoscopic surveillance to detect dysplasia and to diagnose carcinoma at an early and possibly treatable stage. The identification of genes that confer susceptibility for adenocarcinoma formation in Barrett's esophagus would imply improved manageability of patients with Barrett's esophagus. Familial cases of esophageal cancer are however rare, and susceptibility genes for esophageal cancer are thus unlikely to be found by linkage analysis. Consequently, screening of candidate susceptibility genes may be a more feasible approach for esophageal cancer.

CHEK2 (also known as CHK2) is the mammalian homologue of Saccharomyces cerevisiae Rad53 and Schizosaccharomyces pombe Cds1 genes [3,4]. The CHEK2 gene, located on human chromosome 22q12, encodes a cell cycle checkpoint kinase that is implicated in DNA damage responses. Phosphorylation of the p53, Cdc25A and Cdc25C protein results in arrests in various phases of the cell cycle [5,6]. In addition, CHEK2 has been implicated in the regulation of DNA repair by the BRCA1 protein [5,6]. CHEK2*1100delC is a truncating germline variant of CHEK2 that abrogates kinase activity [7,8] and has initially been reported in families suffering from the Li-Fraumeni syndrome without p53 mutations [9]. In familial gastric cancers, which are known to cluster in Li-Fraumeni families, germline CHEK2 mutations were absent [10]. In sporadic (osteo)sarcomas, lung cancers, breast cancers, ovarian cancers, colon cancers and hematopoietic neoplasms, CHEK2 was found to be rarely mutated [9,11-17]. From recent publications it appeared that the germline CHEK2*1100delC mutation in fact confers low-penetrance susceptibility to breast cancer [18,19]. An increased frequency of CHEK2*1100delC was found among breast carcinoma families without BRCA1 or BRCA2 mutations, associated with an approximately twofold increase of breast cancer risk in female carriers [18].

The p53 protein is one of the downstream targets of CHEK2 kinase. Mutations of the p53 gene result in a variety of disturbances in growth control involving DNA replication, DNA repair and apoptosis. Like in breast carcinoma, mutations of the p53 gene appear to play an important role in the development of esophageal squamous cell carcinoma, dysplastic Barrett's epithelium and the progression to esophageal adenocarcinoma [20-22]. Ample studies have re-

ported mutations in p53 in esophageal carcinomas, with mutation frequencies varying from 40-90% [23-26]. As CHEK2 and p53 are thought to be participants of the same biological pathway we aimed to establish whether CHEK2*1100delC confers susceptibility to esophageal cancer by determining the frequency of the mutation among an unselected series of esophageal cancers and precursor lesions.

MATERIALS AND METHODS

Tissue specimens and controls

We investigated a cohort of 190 esophageal squamous cell cancer patients, 196 esophageal adenocarcinoma patients, 99 patients with Barrett's metaplasia and 66 patients with dysplastic Barrett's epithelium. Tissue samples were obtained from resection specimens (carcinomas) or endoscopic biopsies (Barrett's metaplasia and dysplasia), all derived from different patients. We microscopically confirmed that the endoscopic biopsy specimens did not exhibit any tumor cell invasion. Tissue fragments were digested from routine formalin-fixed, paraffin-embedded tissue blocks, without deparaffinization, in 180μL of 50mM/L Tris/HCl (pH=8.0), and 20μL of Proteinase K (20mg/μL) was added. After overnight incubation at 56°C, the lysates were boiled for ten minutes and subsequently centrifuged. Two series of Dutch control individuals consisted of (A) 184 spouses of individuals heterozygous with respect to cystic fibrosis from the Southwest Netherlands, and (B) 460 individuals at ages 55 and older ascertained through the Erasmus Rotterdam Health and the Elderly Study (ERGO) [18].

Allele-specific oligonucleotide hybridisation assay

CHEK2 exon 10 was amplified using forward CHEK2 primer (5'-CAACATTATTCCCTTTTG-TACTG-3') and reverse CHEK2 primer (5'-GTTCCACATAAGGTTCTCATG-3'). DNA samples (1μL) were subjected to PCR analysis in a total volume of 50 μL containing 1x Promega buffer, 1.5 mM $MgCl_2$-solution, 4 μM dGTP, dTTP, dCTP and dATP, 3U Taq DNA polymerase (Promega, Madison, USA), and 0.2μg of forward and reverse CHEK2 primer. PCR amplification consisted of 35 cycles (95°C for 30 sec, 55°C for 45 sec and 72°C for 45 sec) followed by a final extension at 72°C for 10 minutes. We detected the CHEK2*1100delC mutation by application of diluted PCR products to nylon filters and hybridisation under high stringency of [^{32}P]-labelled oligonucleotides complementary to CHEK2*1100delC and the wild-type sequence (5'-TTAG-ATTATGATTTTGGG-3' and 5'-TTAGATTACTGATTTTGG-3' respectively).

Polymorphic marker analysis

DNA was radioactively amplified essentially as described above, using forward primer (5'-TAAGGTGGGAGGTTCACTTG-3') and reverse primer (5'-ACCCATCCTCCTGCCTTAG-3') for the D22S275 locus. PCR products were separated on a 6% polyacrylamide denaturing gel. After

electrophoresis, gels were dried on blotting paper and exposed to X-ray films. Films were evaluated by visual inspection.

Immunohistochemistry

From formalin-fixed paraffin-embedded tissue blocks, 4-μm thick sections were mounted on 3-aminopropyl-triethoxysilane (APES)-coated glass slides. The sections were incubated with a mouse monoclonal antibody DCS 270.1 against the human CHEK2 protein (Novocastra Laboratories, Newcastle, UK; at a dilution of 1:50). Immunoreactivity was visualised by a standard avidin biotin immunoperoxidase technique, using a commercially available kit (Labvision, Fremont, USA)[27,28].

Statistics

Differences of the CHEK2*1100delC mutation frequency between patients and controls were expressed in terms of odds ratios (OR) and 95% confidence intervals (95% CI), and tested with the χ^2-test.

RESULTS AND DISCUSSION

We analysed tumor and biopsy samples obtained from 551 Dutch patients by a CHEK2*1100delC allele-specific oligonucleotide hybridisation assay. CHEK2*1100delC mutations were detected in 0.5% of 190 squamous cell carcinomas, 1.5% of 196 adenocarcinomas, 3.0% of 99 Barrett's metaplasias and in 1.5% of 66 dysplasias (Table 11.1). Chi-square analysis revealed no significant differences between patient groups and the CHEK2*1100delC mutation frequency of 1.4% among 644 control individuals (p=.94) and the Odds Ratio of the total patient group compared to the controls was 1.04 (95%CI 0.35-3.06, Table 11.1). These results suggest that CHEK2*1100delC does not substantially contribute to the development of esophageal carcinoma. CHEK2*1100delC could still confer a three-fold risk, which is greater than the estimated two-fold risk associated with breast cancer and CHEK2*1100delC thus may still be a low-penetrance susceptibility gene to esophageal cancer. Given the low frequency of the mutation, however, even the maximal possible three-fold risk conferred by CHEK2*1100delC would only marginally contribute to the overall incidence of esophageal cancer.

Examples of the hybridisation assay are shown in Figure 11.1. Median ages of patient groups at diagnosis were 59.6, range 14-86 years (Barrett's metaplasias), 60.3, range 32-84 years (dysplasias), 63.9, range 36-84 years (adenocarcinomas) and 60.9, range 31-79 years (squamous cell carcinomas). Ages at diagnosis of CHEK2*1100delC mutation carriers were 48, 73 and 77 years (Barrett's metaplasias), 59 years (dysplasia), 44, 50 and 63 years (adenocarcinomas) and 73 years (squamous cell carcinoma), which was not different from non-carriers and again not supporting a major role of CHEK2*1100delC in esophageal cancer predisposition.

Table 11. 1 CHEK2*1100delC mutation frequencies.

CHEK2*1100delC mutation					
	number tested	carriers	percentage	OR (95%CI)[a]	P-value[a]
Controls					
Netherlands (A)[b]	184	3	1.6		
Netherlands (B-ERGO)[b]	460	6	1.3		
Total	644	9	1.4		
Barrett's metaplasias	99	3	3.0	2.20 (0.38-9.04)	.23
Dysplasias	66	1	1.5	1.09 (0.02-8.05)	.93
Adenocarcinomas	196	3	1.5	1.10 (0.19-4.45)	.89
Squamous cell carcinomas	190	1	0.5	0.37 (0.01-2.73)	.33
Total	551	8	1.5	1.04 (0.35-3.06)	.94

[a]OR: Odds Ratio, 95%CI: 95% Confidence Interval, and P-values are determined by χ^2-test, as compared to frequency in controls. [b]CHEK2*1100delC frequency in Dutch control cohorts A and B by Meijers-Heijboer et al., 2002.

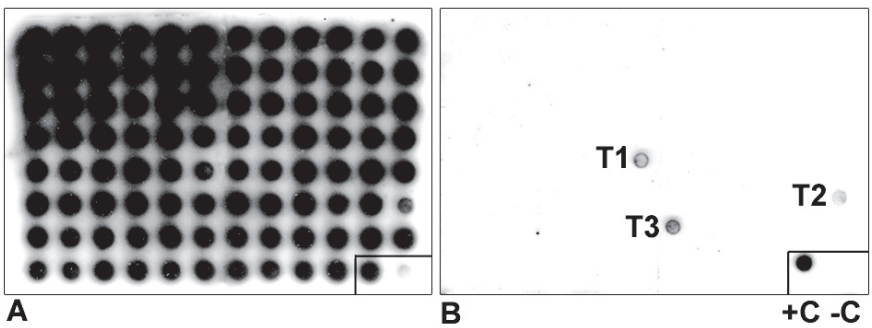


Figure 11.1 Allele-specific oligonucleotide hybridisation assay of 94 adenocarcinomas. Blot A, hybridisation with wild-type oligonucleotide. Blot B, hybridisation with mutant oligonucleotide. Adenocarcinoma samples T1, T2 and T3 are positive for the CHEK2*1100delC mutation, sample '+C' represents a control individual with CHEK2*1100delC, and sample '-C' represents a control individual negative for CHEK2*1100delC.

All mutations were confirmed and proven to be germline-derived by investigating patients' normal tissue. Only paraffin embedded samples of tumor negative lymph nodes were available, precluding confirmation of mutations by sequencing of long range PCR products [29]. The CHEK2*1100delC germline mutation has however been found to be linked to one specific allele of the D22S275 polymorphic marker, that is located in intron 4 of CHEK2, which is present in 13% of the Dutch population [18]. All eight mutation-positive cases were demonstrated to carry the D22S275 allele linked to the CHEK2 mutation, which supports the detected mutations (Figure 11.2). Comparison of allele patterns in mutated tumors with their normal tissues revealed LOH in only one of the three informative carcinomas (T1 without LOH in Figure 11.2B, T4 with LOH in Figure 11.2C). LOH was also observed in three out of 14 informative non-mutated tumor samples. Limited data are however available on LOH of CHEK2 in tumors and the possible tumor suppressing role of CHEK2 therefore awaits further studies.

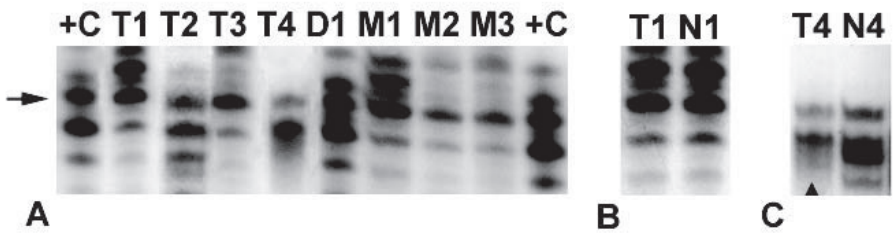


Figure 11.2 D22S275 polymorphic marker analysis in samples with CHEK2*1100delC. A, the allele (arrow), known to be present in all carriers of CHEK2*1100delC[18], is present in all samples with the CHEK2*1100delC mutation (T1-T3 mutated adenocarcinoma samples, T4 mutated squamous cell carcinoma, D1 mutated dysplastic tissue, M1-M3 mutated metaplastic tissue), '+C' represents a control individual with CHEK2*1100delC. B, C, LOH patterns from 2 mutated tumors (T1 adenocarcinoma and T4 squamous cell carcinoma) compared with corresponding normal tissues (N1 and N4) are shown. Arrowhead points to the deleted allele in T4.

Immunohistochemistry using monoclonal antibody DCS 270.1 on a series of mutated and non-mutated tumor tissues showed clear nuclear staining in all cases (Figure 11.3). Since the DCS 270.1 epitope lies within the N-terminus of CHEK2, staining of both wild type and mutant protein may be expected. We observed no differences in CHEK2 protein levels, i.e. no lower intensity nor a lower percentage of CHEK2-positive cells, between CHEK2*1100delC mutated and non-mutated cancers. This was also true for the single mutated tumor with LOH (T4 in Figure 11.3F), suggesting that a theoretical two-fold reduction in CHEK2 protein level can not be detected by the applied immunohistochemistry method. This appears to contrast results of Vahteristo et al. who reported loss of expression in three of the four CHEK2*1100delC tumors and reduction of CHEK2 protein expression in the fourth, using the same antibody [19]. Comparison of the two studies is however difficult, as they did not indicate the precise level of reduction in protein expression [19]. Since only few CHEK2*1100delC tumors have currently been reported, both the data of Vahteristo et al. and the present data should be interpreted with caution.
In summary, our study of a large and unselected series of Barrett's metaplasias and dysplasias, esophageal adenocarcinomas and squamous cell carcinomas suggests that the germline CHEK2*1100delC mutation has no major contribution in esophageal carcinogenesis.

ACKNOWLEDGMENTS

We are grateful to Dr. Anneke van der Wurff (Department of Pathology, Elisabeth Hospital Tilburg) for kindly providing biopsy samples and to Dr. Wim van Putten (Department of Statistics, Daniël den Hoed Cancer Clinic, Rotterdam) for statistical analyses. We wish to thank Marijke Wasielewski, Antoinette Hollestelle and Fons Elstrodt for technical assistance, Frieda van der Ham for performing immunohistochemistry and Frank van der Panne for photographic work.

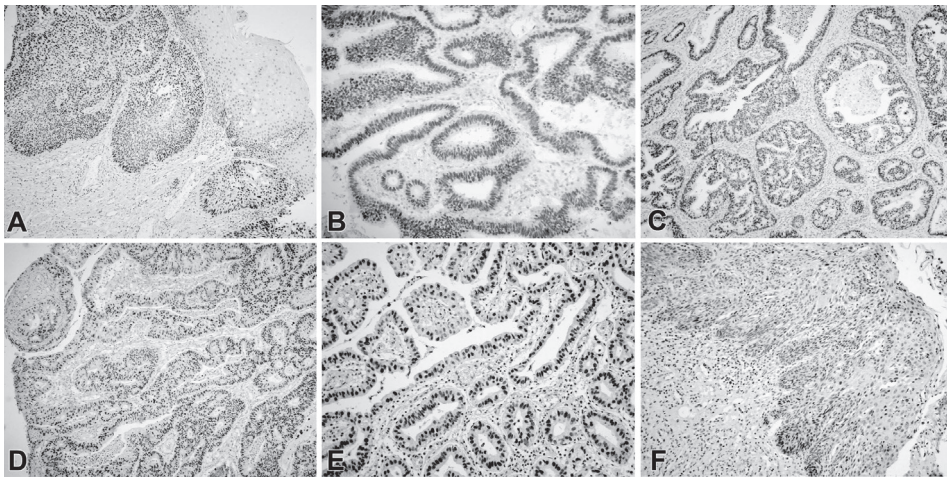


Figure 11.3 CHEK2 protein expression. Non-mutated squamous cell carcinoma in A, non-mutated adenocarcinoma in B. Remaining samples are from the 4 mutated tumors: adenocarcinomas T1, T2 and T3 shown in C, D, E, squamous cell carcinoma T4 shown in F. Magnification 100 x B, E 50 x A, C, D, F. Note: the strong nuclear CHEK2 immunoreactivity in the tumor cells. *Also see color figures page 291.*

REFERENCES

1. Muir CS, McKinney PA: Cancer of the oesophagus: a global overview. Eur J Cancer Prev 1:259-64, 1992
2. Spechler SJ, Goyal RK: Barrett's esophagus. N Engl J Med 315:362-71, 1986
3. Chaturvedi P, Eng WK, Zhu Y, Mattern MR, Mishra R, Hurle MR, Zhang X, Annan RS, Lu Q, Faucette LF, Scott GF, Li X, Carr SA, Johnson RK, Winkler JD, Zhou BB: Mammalian Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway. Oncogene 18:4047-54, 1999
4. Matsuoka S, Huang M, Elledge SJ: Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science 282:1893-7, 1998
5. Bartek J, Falck J, Lukas J: CHK2 kinase--a busy messenger. Nat Rev Mol Cell Biol 2:877-86, 2001
6. Zhou BB, Elledge SJ: The DNA damage response: putting checkpoints in perspective. Nature 408:433-9, 2000
7. Wu X, Webster SR, Chen J: Characterization of tumor-associated Chk2 mutations. J Biol Chem 276:2971-4, 2001
8. Lee SB, Kim SH, Bell DW, Wahrer DC, Schiripo TA, Jorczak MM, Sgroi DC, Garber JE, Li FP, Nichols KE, Varley JM, Godwin AK, Shannon KM, Harlow E, Haber DA: Destabilization of CHK2 by a missense mutation associated with Li-Fraumeni Syndrome. Cancer Res 61:8062-7, 2001
9. Bell DW, Varley JM, Szydlo TE, Kang DH, Wahrer DC, Shannon KE, Lubratovich M, Verselis SJ, Isselbacher KJ, Fraumeni JF, Birch JM, Li FP, Garber JE, Haber DA: Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. Science 286:2528-31, 1999
10. Kimura K, Shinmura K, Yoshimura K, Shimizu K, Katai H, Beppu Y, Moriya H, Yokota J: Absence of germline CHK2 mutations in familial gastric cancer. Jpn J Cancer Res 91:875-9, 2000
11. Miller CW, Ikezoe T, Krug U, Hofmann WK, Tavor S, Vegesna V, Tsukasaki K, Takeuchi S, Koeffler HP: Mutations of the CHK2 gene are found in some osteosarcomas, but are rare in breast, lung, and ovarian tumors. Genes Chromosomes Cancer 33:17-21, 2002
12. Ingvarsson S, Sigbjornsdottir BI, Huiping C, Hafsteinsdottir SH, Ragnarsson G, Barkardottir RB, Arason A, Egilsson V, Bergthorsson JT: Mutation analysis of the CHK2 gene in breast carcinoma and other cancers. Breast Cancer Res 4:R4, 2002
13. Hangaishi A, Ogawa S, Qiao Y, Wang L, Hosoya N, Yuji K, Imai Y, Takeuchi K, Miyawaki S, Hirai H: Mutations of Chk2 in primary hematopoietic neoplasms. Blood 99:3075-7, 2002
14. Aktas D, Arno M, Rassool F, Mufti G: Analysis of CHK2 in patients with myelodysplastic syndromes. Leuk Res 26:985, 2002
15. Tavor S, Takeuchi S, Tsukasaki K, Miller CW, Hofmann WK, Ikezoe T, Said JW, Koeffler HP: Analysis of the CHK2 gene in lymphoid malignancies. Leuk Lymphoma 42:517-20, 2001
16. Hofmann WK, Miller CW, Tsukasaki K, Tavor S, Ikezoe T, Hoelzer D, Takeuchi S, Koeffler HP: Mutation analysis of the DNA-damage checkpoint gene CHK2 in myelodysplastic syndromes and acute myeloid leukemias. Leuk Res 25:333-8, 2001
17. Haruki N, Saito H, Tatematsu Y, Konishi H, Harano T, Masuda A, Osada H, Fujii Y, Takahashi T: Histological type-selective, tumor-predominant expression of a novel CHK1 isoform and infrequent in vivo somatic CHK2 mutation in small cell lung cancer. Cancer Res 60:4689-92, 2000
18. Meijers-Heijboer H, van den Ouweland A, Klijn J, Wasielewski M, de Snoo A, Oldenburg R, Hollestelle A, Houben M, Crepin E, van Veghel-Plandsoen M, Elstrodt F, van Duijn C, Bartels C, Meijers C, Schutte M, McGuffog L, Thompson D, Easton D, Sodha N, Seal S, Barfoot R, Mangion J, Chang-Claude J, Eccles D, Eeles R, Evans DG, Houlston R, Murday V, Narod S, Peretz T, Peto J, Phelan C, Zhang HX, Szabo C, Devilee P, Goldgar D, Futreal PA, Nathanson KL, Weber B, Rahman N, Stratton MR: Low-penetrance susceptibility to breast cancer due to CHEK2(*)1100delC in noncarriers of BRCA1 or BRCA2 mutations. Nat Genet 31:55-9, 2002
19. Vahteristo P, Bartkova J, Eerola H, Syrjakoski K, Ojala S, Kilpivaara O, Tamminen A, Kononen J, Aittomaki K, Heikkila P, Holli K, Blomqvist C, Bartek J, Kallioniemi OP, Nevanlinna H: A CHEK2 genetic variant contributing to a substantial fraction of familial breast cancer. Am J Hum Genet 71:432-8, 2002
20. Wu TT, Watanabe T, Heitmiller R, Zahurak M, Forastiere AA, Hamilton SR: Genetic alterations in Barrett esophagus and adenocarcinomas of the esophagus and esophagogastric junction region. Am J Pathol 153:287-94, 1998

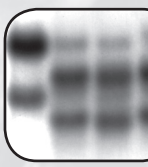
21. Casson AG, Mukhopadhyay T, Cleary KR, Ro JY, Levin B, Roth JA: p53 gene mutations in Barrett's epithelium and esophageal cancer. Cancer Res 51:4495-9, 1991
22. Neshat K, Sanchez CA, Galipeau PC, Blount PL, Levine DS, Joslyn G, Reid BJ: p53 mutations in Barrett's adenocarcinoma and high-grade dysplasia. Gastroenterology 106:1589-95, 1994
23. Jankowski JA, Wright NA, Meltzer SJ, Triadafilopoulos G, Geboes K, Casson AG, Kerr D, Young LS: Molecular evolution of the metaplasia-dysplasia-adenocarcinoma sequence in the esophagus. Am J Pathol 154:965-73, 1999
24. Jenkins GJ, Doak SH, Parry JM, D'Souza FR, Griffiths AP, Baxter JN: Genetic pathways involved in the progression of Barrett's metaplasia to adenocarcinoma. Br J Surg 89:824-37, 2002
25. Wijnhoven BP, Tilanus HW, Dinjens WN: Molecular biology of Barrett's adenocarcinoma. Ann Surg 233:322-37, 2001
26. Mandard AM, Hainaut P, Hollstein M: Genetic steps in the development of squamous cell carcinoma of the esophagus. Mutat Res 462:335-42, 2000
27. Bartkova J, Falck J, Rajpert-De Meyts E, Skakkebaek NE, Lukas J, Bartek J: Chk2 tumor suppressor protein in human spermatogenesis and testicular germ-cell tumors. Oncogene 20:5897-902, 2001
28. Lukas C, Bartkova J, Latella L, Falck J, Mailand N, Schroeder T, Sehested M, Lukas J, Bartek J: DNA damage-activated kinase Chk2 is independent of proliferation or differentiation yet correlates with tissue biology. Cancer Res 61:4990-3, 2001
29. Sodha N, Houlston RS, Williams R, Yuille MA, Mangion J, Eeles RA: A robust method for detecting CHK2/RAD53 mutations in genomic DNA. Hum Mutat 19:173-7, 2002

Chapter 12

Single Nucleotide Polymorphisms and susceptibility for cancer of the esophagus and the gastro-esophageal Junction

To be submitted

Linetta B Koppert
Fibo J ten Kate
Erwin CW Beerens
Albertina W van der Velden
Joyce Vermeulen
Harry FGM van den Ingh
Cees van Krimpen
Ann Driessen
Peter D Siersema
Herman van Dekken
Jan JB van Lanschot
Hugo W Tilanus
André G Uitterlinden
Winand NM Dinjens



ABSTRACT

Research has begun to identify common genetic variants, so called Single Nucleotide Polymorphisms (SNPs), that confer susceptibility to cancer. The aim of the present study was to analyse the relationship between SNPs and the susceptibility for cancer of the esophagus and gastro-esophageal junction (GEJ). SNPs were selected from the literature upon association with cancer predisposition in various other types of cancer. SNPs were genotyped in DNA from 254 GEJ adenocarcinomas and 189 esophageal squamous cell carcinomas obtained from resection specimens and in a control group of 235 healthy blood donors. The SNPs in p53 (exon 4 G72C and intron 6), Her-2/neu (exon 17 A655G), Cyclin D1 (exon 4 A870G) and STK15 (exon 5 T31A) were genotyped by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis. SNPs in p53 intron 3 (16 bp duplication), TGF β receptor 1*6A and E-cadherin (-347 G/GA) were visualized on agarose gels or denaturing gels. SNPs in E-cadherin (-160 C/A), TGF β (-509 G/A), p16 (exon 3 C540G), p73 (exon 2 G4C14-A4T14) and PPAR γ (exon 7 C34G) were analysed with the Taqman allelic discrimination assay. Significant differences between cases and controls were validated in separate validation cohorts of 326 GEJ adenocarcinomas and 209 squamous cell carcinomas. Subjects with the p16 exon 3 C/C genotype were at 1.5-fold reduced risk for squamous cell carcinoma as compared to those with C/G and G/G genotypes. Genotype frequencies were 51% (CC), 46% (CG) and 3% (GG) as compared to 75% , 23% and 2% GG in controls ($p<0.001$). Subjects with the Cyclin D1 exon 4 A/A genotype were at 1.3-fold reduced risk for squamous cell carcinoma as compared to the other genotypes. P73 exon 2 GC/GC genotype conferred a 1.2-fold increased risk for adenocarcinoma whereas the AT/AT genotype conferred a 1.99-fold increased risk for squamous cell carcinoma. These data suggest that the p16 exon 3 C/C wildtype genotype and the Cyclin D1 exon 4 A/A variant genotype might be protective for the development of squamous cell carcinoma. The p73 exon 2 SNP may be a genetic susceptibility factor for the onset of esophageal squamous cell carcinoma (AT/AT) and GEJ adenocarcinoma (GC/GC).

INTRODUCTION

Under current therapy modalities esophageal cancer remains a disease with poor prognosis. Alcohol and tobacco use are established risk factors for the development of esophageal squamous cell carcinomas [1]. Gastro-esophageal reflux disease (GERD) with formation of Barrett's esophagus is a well established risk factor for adenocarcinomas of the distal esophagus and the gastric cardia, the gastro-esophageal junction (GEJ) [2,3]. Despite these etiological conditions only a small group of patients suffering from predisposing disease or environmental exposure will ever develop cancer. It seems likely that progress with this cancer will be made only with early detection, prevention and a clearer understanding of its aetiology and tumor biology.

The number of known genetic mutations that are associated with cancer susceptibility is growing at an exponential rate [4]. With completion of the Human Genome project physicians are provided with an arsenal of genetic information that will hopefully lead to better understanding and treatment of approximately 4000 genetic diseases. An important example is the discovery of single nucleotide polymorphisms (SNPs) that occur about once every 1000 bases along the 3-billion base human genome. Individual variations in cancer risk have been associated with specific variant alleles of different genes, SNPs, that are present in a significant proportion of the normal population. The aetiology of specific cancers might therefore be associated with a set of SNPs, many of which could adversely interact with environmental factors. SNPs are thought to serve as genetic markers for identifying disease genes by linkage studies in families. Familial cases of esophageal cancer are however rare, and susceptibility genes for esophageal cancer are thus unlikely to be found by linkage analysis. Consequently, screening of candidate susceptibility genes may be a more feasible approach. Genes directly involved in tumor mutagenesis form the most interesting candidate susceptibility genes, i.e. tumor suppressor and proto-oncogenes. In this study SNPs with known susceptibility in several types of cancer were analysed in esophageal squamous cell carcinomas and GEJ adenocarcinomas. Selected SNPs concerned the tumor suppressor or proto-oncogenes p53 [5-21], p16 [22-24], Cyclin D1 [25-39], p73 [16,40-50], Transforming Growth Factor β (TGF β) receptor 1 [51-54], TGF β [55,56], E-cadherin [33,57-62], Peroxisome Proliferator-Activator Receptor-γ (PPAR-γ) [63-67], Serine/Threonine protein Kinase 15 (STK15) [68-71] and Her-2/neu [72-74] (Table 12.1).

MATERIALS AND METHODS

Study subjects

Cases included 254 GEJ adenocarcinoma patients and 189 esophageal squamous cell carcinoma patients who underwent esophagectomy at the Erasmus Medical Center (Rotterdam). Tissue was obtained from resection specimens. Validation cohorts of 326 adenocarcinomas and 209 squamous cell carcinomas were obtained from the archives of the Academic Medical

Table 12.1. Selection of SNPs from the literature.

	Gene function	Associated tumortypes	Literature references
P53 exon 4 G72C	Tumor suppressor	Esophageal squamous cell carcinomas	Lee et al. [9]
		Esophageal cancer	Lee et al. [10]
		Gastric cancer	Perez-Perez et al. [14]
		Lung cancer	Kawajiri et al. [8] Jin et al. [7] Wu et al. [20] Biros et al. [6] Birgander et al. [5] Liu et al. [11] Schabath et al. [16]
		Breast cancer	Själander et al. [18] Weston et al. [19]
		Hepatocellular carcinoma	Zhu et al. [83]
		Oral squamous cell cancer	Hsieh et al. [84]
		Colorecal cancer	Själander et al. [17]
		Liver cancer	Yu et al. [21]
P53 intron 3	Tumor suppressor	Lung cancer	Wu et al. [20] Biros et al. [6] Birgander et al. [5] Schabath et al. [16]
		Ovarian cancer	Runnebaum et al. [15]
		Colorecal cancer	Själander et al. [17] Gemignani et al. [85]
		Breast cancer	Weston et al. [19]
P53 intron 6	Tumor suppressor	Lung cancer	Wu et al. [20] Biros et al. [6] Birgander et al. [5] Schabath et al. [16]
		Ovarian	Mavridou et al. [12]
		Breast, colon cancer	Peller et al. [13]
		Colorecal cancer	Själander et al. [17]
		Breast cancer	Weston et al. [19]
P16 exon 3 C540G	Tumor suppressor	Melanomas	Kumar et al. [22] Sauroja et al. [24] Sakano et al. [23]
Cyclin D1 exon 4 A870G	Proto-oncogene	Colorectal cancer	Le Marchand et al. [27] McKay et al [30] Porter et al. [33]
		Colorectal adenomas	Lewis et al. [28]
		Lung cancer	Qiuling et al. [34]
		Bladder cancer	Ito et al. [26] Wang et al. [36]
		Esophageal squamous cell carcinoma and cardia carcinoma	Zhang et al. [38]
		Esophageal adenocarcinoma	Casson et al. [25]

		Head and neck squamous cell carcinoma	Matthias et al. [29] Zheng et al. [39] Nishimoto et al. [32] Monteiro et al. [31]
		Renal cell carcinoma	Yu et al. [37]
		Prostate cancer	Wang et al. [35]
P73 exon 2 G4C14-A4T14	Tumor suppressor/ Proto-oncogene	Esophageal carcinoma	Ryan et al. [50]
		Esophageal squamous cell carcinoma	Cai et al. [40]
		Uterine cervix cancer	Craveiro et al. [41] Niwa et al. [47]
		Endometrial cancer	Niwa et al. [48]
		Lung cancer	Mai et al. [46] Li et al. [45] Schabath et al. [16] Hu et al. [42]
		Head and neck squamous cell carcinoma	Li et al. [44]
		Colorectal cancer	Pfeifer et al. [49]
		Breast cancer	Huang et al. [43]
TGF β receptor 1	Tumor suppressor/ Proto-oncogene	Breast, ovarian, colorectal cancer Hematologic malignancies	Kaklamani et al. [53]
		Breast and ovarian cancer	Baxter et al. [51]
		Breast, colon, bladder cancer	Pasche et al. [54]
		Uterine cervix cancer	Chen et al. [52]
TGF β promotor –509 G/A	Tumor suppressor/ Proto-oncogene	Breast cancer Prostate cancer	Shu et al. [56] Ewart-Toland et al. [55]
E-cadherin promotor -347 G/GA	Tumor suppressor (putative)	Colorectal cancer	Shin et al. [59]
E-cadherin promotor -160 C/A	Tumor suppressor (putative)	Gastric carcinoma	Wu et al. [61] Humar et al. [57]
		Colorectal cancer	Porter et al. [33]
		Bladder cancer	Zhang et al. [62]
		Prostate cancer	Jonsson et al. [58] Verhage et al. [60]
PPAR γ exon 7 C34G	Proto-oncogene	Colorectal cancer	Landi et al. [65] Koh et al. [64]
		Colorectal adenomas	Gong et al. [63]
		Bladder cancer	Leibovici et al. [66]
		Renal cell carcinoma	Smith et al. [67]
STK15 exon 5 T31A	Proto-oncogene	Esophageal squamous cell carcinoma	Miao et al. [71]
		Esophageal cancer	Kimura et al. [70]
		Breast cancer	Cox et al. [68] Dai et al. [69]
Her-2/neu exon 17 A655G	Proto-oncogene	Breast cancer	Xie et al. [74] Cox et al. [72]
		Gastric cancer	Kuraoka et al. [73]

Centers (Amsterdam, Maastricht) and non-academic hospitals Medisch Centrum Rijnmond Zuid (Rotterdam), Reinier de Graaf Gasthuis (Delft) and analysed when significant differences were found between cases (Rotterdam) and controls. All control cohorts consisted of Dutch healthy blood donors. To compare frequencies of the 3 investigated p53 polymorphisms control cohort 1 was analysed (N=166). For the TGF β receptor 1 polymorphism a control cohort was used described in the literature (control cohort 2, N=183) [75]. For the other investigated polymorphisms control cohort 3 was used (N=235). A fourth control cohort was used when the genotype distribution was not in agreement with Hardy-Weinberg equilibrium (N=360).

SNP genotyping

Genomic DNA was extracted from frozen or paraffin-embedded formalin fixed normal (not tumor) tissues (patients) or from whole blood samples (controls). PCR-primer sequences are shown in Table 12.2. Used technique per SNP, flanking sequences, genotype and dbSNP ID rs number are mentioned in Table 12.3. For SNPs in E-cadherin promotor -347, p53 (exon 4 G72C, intron 3 and intron 6), Her-2/Neu (exon 17 A655G), Cyclin D1 (exon 4 A870G) and STK15 (exon 5 T31A) PCR conditions were standardised at 35 cycles of 95°C for 30 sec, 55°C for 45 sec, 72°C for 45 sec and 72°C for 10 min. Amplified PCR products using p53 intron 3 primers were visualized on 1.5% agarose gels. E-cadherin (promotor -347 G/GA) SNP was visualized on denaturing polyacrylamide gels (acrylamide/bisacrylamide 19:1). PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis was performed for determining genotypes of p53 (exon 4 and intron 6), Her-2/neu (exon 17 A655G), Cyclin D1 (exon 4 A870G), and STK15 (exon 5 T31A). Ten units of restriction enzym (BstU, MspI, APOI or BsmAI) were added to PCR products with 16 hours of incubation at 60°C (BstU), 37°C (MspI), 50°C (APOI) or 55°C (BsmAI) with a final inactivation at 80°C for 20 min. Digested products were visualized on 3% agarose gels.

Table 12.2. PCR-primer sequences.

SNP	Forward	Reverse
p53 exon 4	5'...ACCCAGGTCCAGATGAAGC…3'	5'...GATGACAGGGGCCAGGAG…3'
p53 intron 3	5'...AACGTTCTGGTAAGGACAAG…3'	5'...GAAAAGAGCAGTCAGAGGAC…3'
p53 intron 6	5'...GGGGTTAAGGGTGGTTGTC…3'	5'...CCCATTTACTTTGCACATCTC…3'
p16 exon 3	5'...CCCCGATTGAAAGAACCAGAGA…3'	5'...AGGACCTTCGGTGACTGATGAT…3'
Cyclin D1 exon 4	5'...CGCAGTGCAAGGCCTGAAC…3'	5'...CAAGGCTGCCTGGGACATC…3'
p73 exon 2	5'...TCAGGTGTCATTCCTTCCTTCCT…3'	5'...GGTGGACTGGGCCATCTTC…3'
TGF- β R1 exon 1	5'...CGTCGCCCCCGGGAGCAGCGCCGC…3'	5'...CCACAGGCGGTGGCGGCGGGACCATG…3'
TGF-β -509	5'...GGAGAAGAGGGTCTGTCAACATG…3'	5'...GGAGAGCAATTCTTACAGGTGTCT…3'
E-cadherin -347	5'...GGCCAGAGGACCGCTTGAG…3'	5'...GTTTGTTCGTTTTGGAGA…3'
E-cadherin -160	5'...CCACCTAGACCCTAGCAACTC…3'	5'...AGGGCGGAGCTGACG…3'
PPARγ exon 1	5'...GTTATGGGTGAAACTCTGGGAGATT…3'	5'...GCAGACAGTGTATCAGTGAAGGAAT…3'
STK15 exon 5	5'...TCCATTCTAGGCTACAGCTC…3'	5'...AAGAATTTGAAGGACACAAGAC…3'
Her2/neu exon 17	5'...AGCCCTCTGACGTCCATC…3'	5'...CTGCAGCAGTCTCCGCATC…3'

Table 12.3. Wildtype and SNP sequences, dbSNP ID number, used technique.

Polymorphism	Genotype	SNP ID rs number[a]	Wild type/polymorph sequence	Used technique
p53 exon 4	G/C	1042522	5'...TCCCC(G/C)CGTGCC…3'	RFLP (BstUI)
p53 intron 3	-/+ 16bp	-	5'…(1n/2n)CCAGGTCCCAGCCCT…3'	PCR
p53 intron 6	G/A	1800372	5'...CCCTCCG(G/A)GTGAG…3'	RFLP (MspI)
p16 exon 3	C/G	11515	5'…GTTTCC(C/G)GAGGTT…3'	Taqman
Cyclin D1 exon 4	A/G	11557584	5'…CAAGGG(A/G)AGATTG…3'	RFLP (MspI)
p73 exon 2	GC/AT	5031052	5'…CAGAGC(G/A)AGCTGC…3'	Taqman
TGF- β R1 exon 1	9A/6A	11466445	5'…GCGGCGGCG(GCGGCGGCG)GCGGCGGCG…3'	PCR
TGF- β –509	C/T	1800469	5'…CCATCC(C/T)TCAGGT…3'	Taqman
E-cadherin –347	G/GA	5030625	5'…AGTGAG(G/GA)CCCCAT…3'	Denaturing gel
E-cadherin –160	C/A	16260	5'…ACGCG(G/T)TGACC…3'	Taqman
PPARγ exon 1	C/G	1801282	5'…ATTGAC(C/G)CAGAAA…3'	Taqman
STK15 exon 5	A/T	2273535	5'…AAGGAA(A/G)TTGCTG…3'	RFLP (APOI)
Her-2/neu exon 17	A/G	11655866	5'…GGATGC(A/G)TCTGTG…3'	RFLP (BsmAI))

[a] dbSNP http://www.ncbi.nlm.nih.gov/SNP

A touchdown PCR was performed for the TGF β receptor 1*6A (deletion of three alanines (6A) from a nine-alanine stretch (9A)) SNP for 27 cycles of 95°C for 30 sec, 68°C (-0,5°C per cycle) for 30 sec, 72°C for 30 sec, 10 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec and 72°C for 10 min. Amplified products were analysed on 3% agarose gels.

E-cadherin (promotor -160 C/A), TGF β (promotor -509 G/A), p16 (exon 3 C540G), p73 (exon 2 G4C14-A4T14) and PPAR γ (exon 7 C34G) were genotyped in 5-ng genomic DNA with the Taqman allelic discrimination assay (Applied Biosystems, Foster City, Calif). PCR conditions were standardised at 95°C for 10 min, 40 cycli of 92°C for 15 sec and 60°C for 1 min. Reactions were performed with the Taqman Prism 7900HT 384 wells format. Primer sequences are reported in Table 12.2, probe sequences corresponded to wildtype or SNP sequence mentioned in Table 12.3. Examples of Taqman allelic discrimination analysis and PCR-RFLP analysis are shown in Figures 12.1 and 12.2.

Statistics

The χ^2 test was used to compare genotype frequencies between cases and controls and to compute deviations from Hardy-Weinberg equilibrium. The association between the variant genotypes and risk of esophageal and GEJ cancer was estimated by computing odds ratios (ORs) and their 95% confidence interval (CIs). Statistical significance was set at $p<0.05$. All analyses were 2-sided and conducted using SPSS software (version 11.0; SPSS, Inc., Chicago, IL).

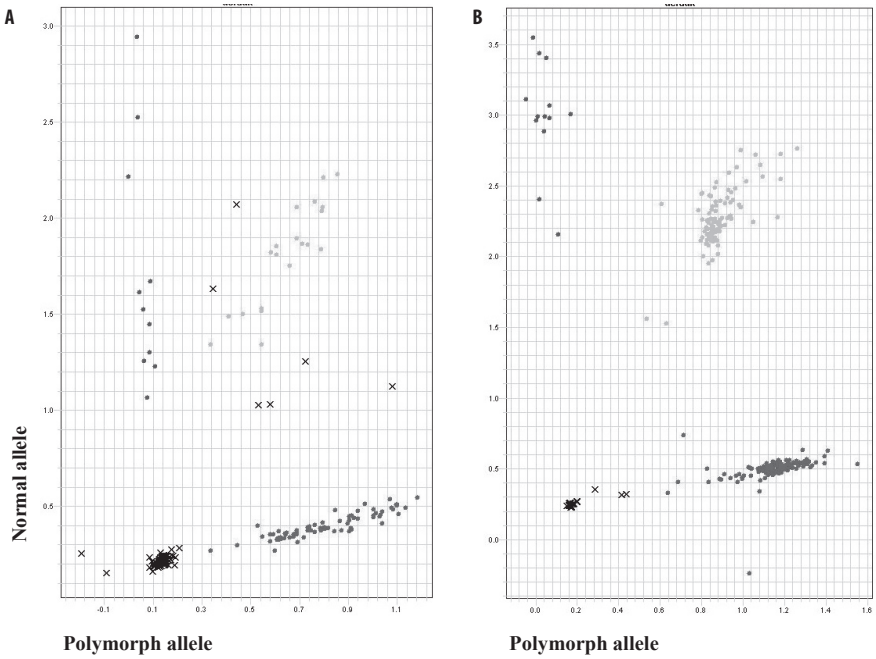


Figure 12.1. Taqman allelic discrimination assay concerning p73 exon 2 G4C14-A4T14 of 191 GEJ adenocarcinomas (A.) and 232 controls (B.). Distribution of homozygous normal (blue dots), heterozygous (green dots) and homozygous polymorphism (red dots). *Also see color figures page 291.*

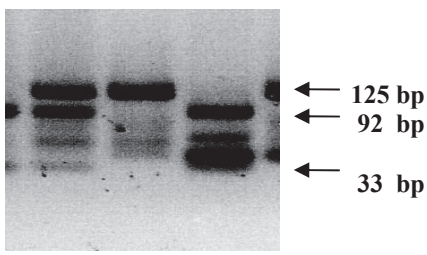


Figure 12.2. Example of PCR-RFLP analysis concerning Cyclin D1 exon 4 A870G. The polymorphic allele contains a restriction site resulting in two bands, 92 basepairs and 33 basepairs respectively (arrows). The normal allele, without a restriction site has a length of 125 basepairs. Examples shown are heterozygous (lane 1), homozygous normal (lane 2) and homozygous polymorphic (lane 3).

RESULTS

Mean age ± standard deviation (cases) and gender (cases and controls) are shown in Table 12.4. The distributions of genotype frequencies of the 13 investigated SNPs between the cases and controls are summarized in Table 12.5. Only informative samples were mentioned. For the p16 exon 3 C540G SNP in squamous cell carcinomas the frequencies of CC, CG and GG genotypes were 51%, 46% and 3% respectively which were significantly different from the frequencies in controls (75%, 23% and 2%, $p<0.001$, Table 12.5). After significant results in the cases in the first cohort, results were validated using a validation cohort with substantiation

Table 12.4. Characteristics of patients and controls.

Cohort	Age, years (mean ± SD[a])	Gender (M:F)
Adenocarcinomas		
N= 254	63.1 ± 10.2	223:31
Squamous cell carcinomas		
N=189	60.9 ± 9.5	121:68
Adenocarcinomas (validation cohort)		
N=326	64.3 ± 9.7	275:51
Squamous cell carcinomas (validation cohort)		
N=209	61.3 ± 9.6	115:94
Control cohort 1		
N=166	-[b]	83:83
Control cohort 2[75]		
N=183	-[b]	-[b]
Control cohort 3		
N=235	-[b]	145:82
Control cohort 4		
N=360	-[b]	-[b]

[a]Standard deviation; [b]Unknown

of significancy. Pooled results are depicted in Table 12.5. The odds ratio (OR) for development of squamous cell carcinoma in C/C homozygotes compared to the other genotypes was 0.68 (95% CI 0.60-0.77), corresponding to a 1.5-fold reduced risk in these individuals and therefore a protective effect of the C/C homozygote genotype. The OR in C/G heterozygotes as compared to the other genotypes was 1.99 (95% CI 1.53-2.59), corresponding to a 1.99-fold increased risk.

For the Cyclin D1 exon 4 A870G SNP in squamous cell carcinomas the frequencies of A/A, A/G and G/G genotypes were 18%, 52% and 30% respectively which were different from the frequencies in controls (23%, 52% and 25%, p=0.048, Table 12.5). The OR for development of squamous cell carcinoma in G/G homozygotes compared to the other genotypes was 1.21 (95% CI 0.99-1.49, p=0.07), corresponding to a 1.21-fold increased risk in these individuals or a 1.3-fold reduced risk for A/A homozygotes (OR 0.75 (95% CI 0.58-0.98, p=0.03). Examples of Cyclin D1 exon 4 A870G PCR-RFLP analysis are shown in Figure 12.2.

SNPs in the same chromosomal region are not inherited randomly, but as combinations of alleles which form haploblocks. The p73 exon 2 G4C14-A4T14 SNP is such an example. For this SNP in adenocarcinomas and squamous cell carcinomas frequencies of GC/GC, GC/AT, AT/AT genotypes were 70% and 65% respectively, 25% and 24% respectively and 5% and 10% respectively as compared to 57%, 37% and 5% in healthy blood donors which significantly differed (p=0.003 and p=0.003, Table 12.5). The OR for development of adenocarcinoma in GC/GC homozygotes compared to the other genotypes was 1.21 (95% CI 1.07-1.37). The OR for development of squamous cell carcinoma in AT/AT homozygotes compared to the other genotypes was 1.99 (95% CI 1.03-3.84).

Table 12.5. Distribution of SNP genotypes in GEJ adenocarcinomas and esophageal squamous cell carcinomas as compared to controls.

	N=	Homozygous Normal	Heterozygous	Homozygous Polymorphism	P-value[a]
P53 exon 4 G72C					
AC	165	89 (54)	60 (36)	16 (10)	0.7
SCC	180	103 (57)	62 (34)	15 (8)	0.5
Controls	166	85 (51)	67 (40)	14 (8)	
P53 intron 3					
AC	158	121 (77)	32 (20)	5 (3)	0.6
SCC	184	140 (76)	39 (21)	5 (3)	0.8
Controls	166	124 (75)	39 (23)	3 (2)	
P53 intron 6					
AC	159	120 (75)	37 (23)	2 (1)	0.7
SCC	177	143 (81)	32 (18)	2 (1)	0.4
Controls	166	125 (75)	37 (22)	4 (2)	
p16 exon 3 C540G					
AC	473	338 (71)	126 (27)	9 (2)	0.6
SCC	337	171 (51)	155 (46)	11 (3)	<0.001
Controls	229	171 (75)	53 (23)	5 (2)	
Cyclin D1 exon 4 A870G					
AC	552	141 (26)	253 (46)	158 (29)	0.1
SCC	392	69 (18)	205 (52)	118 (30)	0.048
Controls	587	137 (23)	304 (52)	146 (25)	
P73 exon 2 G4C14-A4T14					
AC	471	330 (70)	117 (25)	24 (5)	0.003
SCC	262	171 (65)	64 (24)	27 (10)	0.003
Controls	232	134 (57)	86 (37)	12 (5)	
TGF β receptor 1					
AC	73	61 (84)	10 (14)	2 (3)	0.7
SCC	185	150 (81)	34 (18)	1 (0.5)	0.6
Controls	183	148 (81)	32 (17)	3 (2)	
TGF β promotor –509 G/A					
AC	178	98 (55)	73 (41)	7 (4)	0.3
SCC	-	-	-	-	-
Controls	229	118 (52)	94 (41)	17 (7)	
E-cadherin promotor -347 G/GA					
AC	246	192 (78)	51 (21)	3 (1)	0.3
SCC	146	106 (73)	39 (27)	1 (1)	0.98
Controls	228	165 (72)	61 (27)	2 (1)	
E-cadherin promotor -160 C/A					
AC	519	277 (53)	200 (39)	42 (8)	0.8
SCC	322	142 (44)	155 (48)	25 (8)	0.2
Controls	231	118 (51)	93 (40)	20 (9)	
PPAR γ exon 7 C34G					
AC	109	79 (72)	27 (25)	3 (3)	0.7
SCC	-	-	-	-	-
Controls	222	153 (69)	64 (29)	5 (2)	

STK15 exon 5 T31A					
AC	251	147 (59)	93 (37)	11 (4)	0.6
SCC	183	103 (56)	70 (38)	10 (5)	0.6
Controls	227	123 (54)	95 (42)	9 (4)	
Her-2/neu exon 17 A655G					
AC	251	141 (56)	96 (38)	14 (6)	0.8
SCC	346	208 (60)	123 (36)	15 (4)	0.6
Controls	581	330 (57)	225 (39)	26 (4)	

[a]P-value as compared to controls by χ^2 test. AC: Adenocarcinoma. SCC: Squamous cell carcinoma.

The distribution of none of the other genotypes was associated with risk of esophageal squamous cell carcinoma or adenocarcinoma. Examples of the p73 exon 2 G4C14-A4T14 Taqman allelic discrimination assay in GEJ adenocarcinomas and controls is shown in Figure 12.1.
All genotype frequencies of controls were in Hardy-Weinberg equilibrium ($p>0.05$), except for Cyclin D1 exon 4 A870G and Her-2/neu exon 17 A655G (control cohort 1, $p=0.01$ and $p=0.005$ respectively). There could have been an influence from control selection. To overcome this problem a second control cohort was used, which was in agreement with the Hardy-Weinberg equilibrium ($p=0.6$ and $p=0.8$ respectively). When the two control cohorts were pooled it was in Hardy-Weinberg equilibrium (Table 12.5, $p=0.4$ and $p=0.1$ respectively). All genotype frequencies of the cases were in Hardy-Weinberg equilibrium except for the p16 exon 3 polymorphism (squamous cell carcinomas, $p<0.001$) and the p73 (squamous cell carcinomas and adenocarcinomas, $p=0.002$ and $p<0.001$ respectively), which gave significant results when distribution of genotypes were compared between cases and controls (Table 12.5).

DISCUSSION

The current study analysed the possible influence of common genetic variants, Single Nucleotide Polymorphisms (SNPs), for risk of esophageal squamous cell carcinoma and GEJ adenocarcinoma. Most SNPs are 'silent', i.e. do not alter the function or expression of a gene. The term 'mutation' is reserved for rare variants with a particularly high penetrance, usually associated with a detrimental phenotype, such as Hereditary Breast and Ovarian Cancer (BRCA1, BRCA2), Hereditary Non-Polyposis Colorectal cancer (MSH2, MSH6, MLH2, PMS1, PMS2) and Familial Adenomatous Polyposis (APC) amongst others. As classically thought, changes in either tumor suppressor or oncogenes sort 100% effect, in an on/off way. SNPs are associated with low penetrance and variable effects on wildtype activity. Interestingly, all SNPs with a significant effect on esophageal cancer susceptibility in this study (p16 exon 3 C540G, Cyclin D1 exon 4 A870G, p73 exon 2 G4C14-A4T14) did not lead to an amino acid sequence change. SNPs are, however, thought to be able to sort their effect by influence on splicing, by linkage with other SNPs (on the same or on a different gene) with functional effects or by mere coincidence. Even intronic SNPs, within non-coding regions of the corresponding genes, are

described to confer functional relevance without a proven explanation. It becomes increasingly apparent that cancer susceptibility might be related to polymorphic variations in the DNA sequence [76].

In the current study, healthy blood donors were chosen as controls. Although it is not clear if blood donors exactly mirror the genotypes of a population, e.g. due to ethnic asymmetries, blood donors are generally accepted as controls for SNP studies for practicability reasons [77]. However, as blood donors tend to be younger than cancer patients, no perfect match between patients and controls was gained in our study.

A 1.5-fold reduced risk was shown for squamous cell carcinoma subjects with the p16 exon 3 C540G C/C genotype as compared to those with C/G and G/G genotypes. This suggests a protective role for the C/C genotype in the development of squamous cell carcinoma. P16 regulates function in G1 cell cycle arrest and is therefore important in carcinogenesis. P16 exon 3 C540G SNP does not lead to an amino acid sequence change but has, however, been associated with tumor susceptibility in a number of studies [22-24]. The SNP has been associated with low expression of p53 [24]. In our series not the variant G/G genotype but the heterozygous C/G genotype was increased prevalent in squamous cell carcinoma cases, with an OR 1.99 (95% CI 1.53-2.59), corresponding to a 1.99-fold increased risk for squamous cell carcinomas as compared to the other genotypes. We were not able to test for linkage disequilibrium, described for melanomas in the literature, since we did not analyse the described p16 intronic SNP [24]. Analogous to Geddert and colleagues, who did not find an association of p16 exon 3 C540G SNP with esophageal adenocarcinomas, cardia adenocarcinomas and subcardia adenocarcinomas, we could also not find an association between this SNP and GEJ adenocarcinomas in our series [78].

Subjects with the Cyclin D1 exon 4 G/G genotype were at 1.21-fold increased risk for squamous cell carcinoma as compared to the other genotypes. The control cohort used (control cohort 3) was not in agreement with the Hardy-Weinberg equilibrium (HWE) and therefore was supplemented with a second control cohort (n=581 in total, HWE p=0.4). Cyclin D1 is a key protein for the regulation of cell cycle G1-S-transition. The Cyclin D1 exon 4 A870G SNP does not lead to an amino acid sequence change but it creates an alternative splice site in its mRNA, encoding a protein with an altered C-terminal domain. It has been suggested that DNA damage in cells with the A allele bypasses the G(1)/S checkpoint of the cell cycle more easily than damage in cells without the A allele. Several studies have suggested a significant association between the Cyclin D1 genotype and onset or progression of various cancers [25-39]. Interestingly, this SNP is in the literature contradictory reported as A>G and G>A. Within the dbSNP (http://www.ncbi.nlm.nih.gov/SNP) this SNP is reported as A870G, which was followed in our study. Concerning squamous cell carcinomas of head and neck and of esophagus, having comparable risk factors, significant associations between the A/A genotype and cancer risk were described, which is not in agreement with our data which show a protective role of the A/A genotype instead of susceptibility for esophageal squamous cell

carcinoma [38,39]. In these studies there was an adjustment for smoking habits within multivariable analyses. Unfortunately, since our study was not designed as a prospective case-control study, data on life style riskfactors were not available for cases or for controls. Interestingly, in a recently performed study the A/A genotype was shown to confer increased risk for GERD, Barrett's esophagus and esophageal adenocarcinoma [25]. In a northern Chinese population the A/A genotype conferred an increased risk for gastric cardia adenocarcinoma [38]. In our study, distribution of Cyclin D1 genotypes within 253 GEJ adenocarcinomas as compared to 587 controls did significantly differ (A/A 29% vs. 23%, p=0.01), but when a validation cohort existing of 299 adenocarcinomas was analysed, this significancy disappeared (22% vs. 23%, p=0.7; total group 26% vs. 23% p=0.1). Cyclin D1 exon 4 A870G therefore did not function as a genetic susceptibility factor in GEJ adenocarcinomas in our study.

P73 is a gene that is structurally similar to the p53 tumor suppressor gene and can inhibit cell growth and induce apoptosis. Two common SNPs in position 4 (G/A) and 14 (C/T) in the uncoding region of exon 2 of the p73 gene are in complete linkage disequilibrium with one another and form a haplotype block. The AT allele is described to possibly affect p73 function by altering the efficiency of translation initiation [79]. P73 exon 2 G4C14-A4T14 has been associated with several cancer types [16,40-50]. In our current study, p73 exon 2 GC/GC genotype conferred a 1.2-fold increased risk for adenocarcinoma whereas the AT/AT variant genotype conferred a 1.99-fold increased risk for squamous cell carcinoma. Analysing esophageal squamous cell and adenocarinomas (n=25 and n=59), Ryan et al. reported less prevalent AT/AT in the total group of patients as compared to controls (1.2% vs. 4%, p<0.02) [50]. When squamous cell carcinomas and adenocarcinomas were separately analysed genotype frequencies were 48% and 50.8% (GC/GC), 48% and 49.1% (GC/AT) and 4% and 0% (AT/AT) respectively [50]. In our study, using large patient numbers, genotype frequencies were 65% and 70% (GC/GC), 24% and 25% (GC/AT) and 10% and 5% (AT/AT) in squamous cell and adenocarcinomas respectively. AT/AT was increased prevalent in squamous cell carcinomas as compared to controls, in contrast to the Ryan-study. GC/GC was increased prevalent in GEJ adenocarcinomas. Since esophageal squamous cell and adenocarcinomas concern different tumortypes, harbouring different genetic alterations, we analysed them separately, with different outcomes concerning p73 (amongst others). Different results in the Ryan-study and our study might perhaps be explained by different ethnic background, although the control group used by Ryan et al. is a Caucasian one (homogeneous Irish) as are the control groups used in our study. Ethnicity is known to be an important confounding factor in epidemiologic studies involving genetic polymorphisms [80]. Interestingly, in the study performed by Ryan et al., LOH of p73 was shown in 37.8% of 37 heterozygote tumor tissues being exclusive loss of the AT allele, suggesting a specific functional role for the AT allele [50]. They speculate that the p73 AT allele might modulate the inflammatory response to GERD and its subsequent evolution of Barrett's esophagus, dysplasia and cancer because it may lead to difference in p73 function, perhaps due to splice variant expression [50]. We did not perform LOH analysis in our cases, which might be very

interesting. It could well be that this allele is lost in tumors of part of the patients harbouring the allele in their normal DNA. Since it could also be that the p73 SNP is in linkage disequilibrium with other functional polymorphisms, thereby altering the function of p73 or involving adjacent susceptibility loci, it would also be interesting to study multigenetic effects of variant alleles, for example from p53 exon 4, introns 3 and 6, and p73.

Over the past decades, there has been an increasing incidence of GEJ adenocarcinomas in developed countries. GEJ adenocarcinoma now exceeds the incidence of esophageal squamous cell carcinoma. GERD and Barrett's esophagus, the precursorlesion of GEJ adenocarcinoma, are highly prevalent in the population. Only a fraction of individuals show progression to adenocarcinoma. Endoscopic surveillance of Barrett's esophagus patients is a widely accepted and utilized strategy to detect early carcinoma, which is potentially curable. Most Barrett's patients however never develop carcinoma. It is likely that interactions between molecular and lifestyle risk factors confer individual susceptibility for neoplastic progression. SNPs may modify the effect of environmental exposures and therefore susceptibility for cancer [81]. As published previously for breast cancer, microarray technique allowing for the simultaneous detection of a high number of mutations and SNPs might provide prognostic information in addition to established clinical parameters [82]. Analysis of SNPs can be performed conveniently from a simple blood test. This might be of help to define subsets of Barrett's esophagus patients or patients suffering from GERD, who are at increased risk for adenocarcinoma and therefore to further stratify surveillance. There is no known precursor lesion of squamous cell carcinoma, although chronic irritation and inflammation of the esophagus in response to environmental factors such as alcohol or nicotine is thought to play a role in the development of cancer. Also in these patients at risk SNPs analysis might be helpful in the future. Our data confirm that SNPs confer risk for esophageal squamous cell carcinoma (p16 exon 2 C540G, Cyclin D1 exon 4 A870G and p73 exon 2 G4C14-A4T14) and for GEJ adenocarcinoma (p73 exon 2 G4C14-A4T14). Currently a large cohort of Barrett's esophagus patients, who did not develop cancer yet, is being analysed for p73 exon 2 G4C14-A4T14 genotype frequencies as well as for the other SNPs described in this study to reveal whether SNPs could be helpful to stratify surveillance.

ACKNOWLEDGMENTS

We greatly acknowledge Pascal Arp and Joyce van Meurs from the Department of Internal Medicine, Erasmus MC Rotterdam, for excellent technical assistance.

REFERENCES

1. Muir CS, McKinney PA: Cancer of the oesophagus: a global overview. Eur J Cancer Prev 1:259-64, 1992
2. Falk GW: Barrett's esophagus. Gastroenterology 122:1569-91, 2002
3. Tajima Y, Nakanishi Y, Yoshino T, Kokawa A, Kusano M, Shimoda T: Clinicopathological study of early adenocarcinoma of the gastric cardia: comparison with early adenocarcinoma of the distal stomach and esophagus. Oncology 61:1-9, 2001
4. American Society of Clinical Oncology policy statement update: genetic testing for cancer susceptibility. J Clin Oncol 21:2397-406, 2003
5. Birgander R, Sjalander A, Rannug A, Alexandrie AK, Sundberg MI, Seidegard J, Tornling G, Beckman G, Beckman L: P53 polymorphisms and haplotypes in lung cancer. Carcinogenesis 16:2233-6, 1995
6. Biros E, Kalina I, Kohut A, Stubna J, Salagovic J: Germ line polymorphisms of the tumor suppressor gene p53 and lung cancer. Lung Cancer 31:157-62, 2001
7. Jin X, Wu X, Roth JA, Amos CI, King TM, Branch C, Honn SE, Spitz MR: Higher lung cancer risk for younger African-Americans with the Pro/Pro p53 genotype. Carcinogenesis 16:2205-8, 1995
8. Kawajiri K, Nakachi K, Imai K, Yoshii A, Shinoda N, Watanabe J: Identification of genetically high risk individuals to lung cancer by DNA polymorphisms of the cytochrome P450IA1 gene. FEBS Lett 263:131-3, 1990
9. Lee JM, Lee YC, Yang SY, Shi WL, Lee CJ, Luh SP, Chen CJ, Hsieh CY, Wu MT: Genetic polymorphisms of p53 and GSTP1,but not NAT2,are associated with susceptibility to squamous-cell carcinoma of the esophagus. Int J Cancer 89:458-64, 2000
10. Lee JM, Shun CT, Wu MT, Chen YY, Yang SY, Hung HI, Chen JS, Hsu HH, Huang PM, Kuo SW, Lee YC: The associations of p53 overexpression with p53 codon 72 genetic polymorphism in esophageal cancer. Mutat Res 594:181-8, 2006
11. Liu G, Miller DP, Zhou W, Thurston SW, Fan R, Xu LL, Lynch TJ, Wain JC, Su L, Christiani DC: Differential association of the codon 72 p53 and GSTM1 polymorphisms on histological subtype of non-small cell lung carcinoma. Cancer Res 61:8718-22, 2001
12. Mavridou D, Gornall R, Campbell IG, Eccles DM: TP53 intron 6 polymorphism and the risk of ovarian and breast cancer. Br J Cancer 77:676-7, 1998
13. Peller S, Kopilova Y, Slutzki S, Halevy A, Kvitko K, Rotter V: A novel polymorphism in intron 6 of the human p53 gene: a possible association with cancer predisposition and susceptibility. DNA Cell Biol 14:983-90, 1995
14. Perez-Perez GI, Bosques-Padilla FJ, Crosatti ML, Tijerina-Menchaca R, Garza-Gonzalez E: Role of p53 codon 72 polymorphism in the risk of development of distal gastric cancer. Scand J Gastroenterol 40:56-60, 2005
15. Runnebaum IB, Tong XW, Konig R, Zhao H, Korner K, Atkinson EN, Kreienberg R, Kieback DG, Hong Z: p53-based blood test for p53PIN3 and risk for sporadic ovarian cancer. Lancet 345:994, 1995
16. Schabath MB, Wu X, Wei Q, Li G, Gu J, Spitz MR: Combined effects of the p53 and p73 polymorphisms on lung cancer risk. Cancer Epidemiol Biomarkers Prev 15:158-61, 2006
17. Sjalander A, Birgander R, Athlin L, Stenling R, Rutegard J, Beckman L, Beckman G: P53 germ line haplotypes associated with increased risk for colorectal cancer. Carcinogenesis 16:1461-4, 1995
18. Sjalander A, Birgander R, Hallmans G, Cajander S, Lenner P, Athlin L, Beckman G, Beckman L: p53 polymorphisms and haplotypes in breast cancer. Carcinogenesis 17:1313-6, 1996
19. Weston A, Pan CF, Ksieski HB, Wallenstein S, Berkowitz GS, Tartter PI, Bleiweiss IJ, Brower ST, Senie RT, Wolff MS: p53 haplotype determination in breast cancer. Cancer Epidemiol Biomarkers Prev 6:105-12, 1997
20. Wu X, Zhao H, Amos CI, Shete S, Makan N, Hong WK, Kadlubar FF, Spitz MR: p53 Genotypes and Haplotypes Associated With Lung Cancer Susceptibility and Ethnicity. J Natl Cancer Inst 94:681-90, 2002
21. Yu MW, Yang SY, Chiu YH, Chiang YC, Liaw YF, Chen CJ: A p53 genetic polymorphism as a modulator of hepatocellular carcinoma risk in relation to chronic liver disease, familial tendency, and cigarette smoking in hepatitis B carriers. Hepatology 29:697-702, 1999

22. Kumar R, Smeds J, Berggren P, Straume O, Rozell BL, Akslen LA, Hemminki K: A single nucleotide polymorphism in the 3'untranslated region of the CDKN2A gene is common in sporadic primary melanomas but mutations in the CDKN2B, CDKN2C, CDK4 and p53 genes are rare. Int J Cancer 95:388-93, 2001
23. Sakano S, Berggren P, Kumar R, Steineck G, Adolfsson J, Onelov E, Hemminki K, Larsson P: Clinical course of bladder neoplasms and single nucleotide polymorphisms in the CDKN2A gene. Int J Cancer 104:98-103, 2003
24. Sauroja I, Smeds J, Vlaykova T, Kumar R, Talve L, Hahka-Kemppinen M, Punnonen K, Jansen CT, Hemminki K, Pyrhonen S: Analysis of G(1)/S checkpoint regulators in metastatic melanoma. Genes Chromosomes Cancer 28:404-14, 2000
25. Casson AG, Zheng Z, Evans SC, Geldenhuys L, van Zanten SV, Veugelers PJ, Porter GA, Guernsey DL: Cyclin D1 polymorphism (G870A) and risk for esophageal adenocarcinoma. Cancer 104:730-9, 2005
26. Ito M, Habuchi T, Watanabe J, Higashi S, Nishiyama H, Wang L, Tsuchiya N, Kamoto T, Ogawa O: Polymorphism within the cyclin D1 gene is associated with an increased risk of carcinoma in situ in patients with superficial bladder cancer. Urology 64:74-8, 2004
27. Le Marchand L, Seifried A, Lum-Jones A, Donlon T, Wilkens LR: Association of the cyclin D1 A870G polymorphism with advanced colorectal cancer. Jama 290:2843-8, 2003
28. Lewis RC, Bostick RM, Xie D, Deng Z, Wargovich MJ, Fina MF, Roufail WM, Geisinger KR: Polymorphism of the cyclin D1 gene, CCND1, and risk for incident sporadic colorectal adenomas. Cancer Res 63:8549-53, 2003
29. Matthias C, Branigan K, Jahnke V, Leder K, Haas J, Heighway J, Jones PW, Strange RC, Fryer AA, Hoban PR: Polymorphism within the cyclin D1 gene is associated with prognosis in patients with squamous cell carcinoma of the head and neck. Clin Cancer Res 4:2411-8, 1998
30. McKay JA, Douglas JJ, Ross VG, Curran S, Murray GI, Cassidy J, McLeod HL: Cyclin D1 protein expression and gene polymorphism in colorectal cancer. Aberdeen Colorectal Initiative. Int J Cancer 88:77-81, 2000
31. Monteiro E, Varzim G, Pires AM, Teixeira M, Lopes C: Cyclin D1 A870G polymorphism and amplification in laryngeal squamous cell carcinoma: implications of tumor localization and tobacco exposure. Cancer Detect Prev 28:237-43, 2004
32. Nishimoto IN, Pinheiro NA, Rogatto SR, Carvalho AL, Simpson AJ, Caballero OL, Kowalski LP: Cyclin D1 gene polymorphism as a risk factor for squamous cell carcinoma of the upper aerodigestive system in non-alcoholics. Oral Oncol 40:604-10, 2004
33. Porter TR, Richards FM, Houlston RS, Evans DG, Jankowski JA, Macdonald F, Norbury G, Payne SJ, Fisher SA, Tomlinson I, Maher ER: Contribution of cyclin d1 (CCND1) and E-cadherin (CDH1) polymorphisms to familial and sporadic colorectal cancer. Oncogene 21:1928-33, 2002
34. Qiuling S, Yuxin Z, Suhua Z, Cheng X, Shuguang L, Fengsheng H: Cyclin D1 gene polymorphism and susceptibility to lung cancer in a Chinese population. Carcinogenesis 24:1499-503, 2003
35. Wang L, Habuchi T, Mitsumori K, Li Z, Kamoto T, Kinoshita H, Tsuchiya N, Sato K, Ohyama C, Nakamura A, Ogawa O, Kato T: Increased risk of prostate cancer associated with AA genotype of cyclin D1 gene A870G polymorphism. Int J Cancer 103:116-20, 2003
36. Wang L, Habuchi T, Takahashi T, Mitsumori K, Kamoto T, Kakehi Y, Kakinuma H, Sato K, Nakamura A, Ogawa O, Kato T: Cyclin D1 gene polymorphism is associated with an increased risk of urinary bladder cancer. Carcinogenesis 23:257-64, 2002
37. Yu J, Habuchi T, Tsuchiya N, Nakamura E, Kakinuma H, Horikawa Y, Inoue T, Ogawa O, Kato T: Association of the cyclin D1 gene G870A polymorphism with susceptibility to sporadic renal cell carcinoma. J Urol 172:2410-3, 2004
38. Zhang J, Li Y, Wang R, Wen D, Sarbia M, Kuang G, Wu M, Wei L, He M, Zhang L, Wang S: Association of cyclin D1 (G870A) polymorphism with susceptibility to esophageal and gastric cardiac carcinoma in a northern Chinese population. Int J Cancer 105:281-4, 2003
39. Zheng Y, Shen H, Sturgis EM, Wang LE, Eicher SA, Strom SS, Frazier ML, Spitz MR, Wei Q: Cyclin D1 polymorphism and risk for squamous cell carcinoma of the head and neck: a case-control study. Carcinogenesis 22:1195-9, 2001
40. Cai YC, Yang GY, Nie Y, Wang LD, Zhao X, Song YL, Seril DN, Liao J, Xing EP, Yang CS: Molecular alterations of p73 in human esophageal squamous cell carcinomas: loss of heterozygosity occurs

frequently; loss of imprinting and elevation of p73 expression may be related to defective p53. Carcinogenesis 21:683-9, 2000
41. Craveiro R, Costa S, Pinto D, Salgado L, Carvalho L, Castro C, Bravo I, Lopes C, Silva I, Medeiros R: TP73 alterations in cervical carcinoma. Cancer Genet Cytogenet 150:116-21, 2004
42. Hu Z, Miao X, Ma H, Tan W, Wang X, Lu D, Wei Q, Lin D, Shen H: Dinucleotide polymorphism of p73 gene is associated with a reduced risk of lung cancer in a Chinese population. Int J Cancer 114:455-60, 2005
43. Huang XE, Hamajima N, Katsuda N, Matsuo K, Hirose K, Mizutani M, Iwata H, Miura S, Xiang J, Tokudome S, Tajima K: Association of p53 codon Arg72Pro and p73 G4C14-to-A4T14 at exon 2 genetic polymorphisms with the risk of Japanese breast cancer. Breast Cancer 10:307-11, 2003
44. Li G, Sturgis EM, Wang LE, Chamberlain RM, Amos CI, Spitz MR, El-Naggar AK, Hong WK, Wei Q: Association of a p73 exon 2 G4C14-to-A4T14 polymorphism with risk of squamous cell carcinoma of the head and neck. Carcinogenesis 25:1911-6, 2004
45. Li G, Wang LE, Chamberlain RM, Amos CI, Spitz MR, Wei Q: p73 G4C14-to-A4T14 polymorphism and risk of lung cancer. Cancer Res 64:6863-6, 2004
46. Mai M, Yokomizo A, Qian C, Yang P, Tindall DJ, Smith DI, Liu W: Activation of p73 silent allele in lung cancer. Cancer Res 58:2347-9, 1998
47. Niwa Y, Hamajima N, Atsuta Y, Yamamoto K, Tamakoshi A, Saito T, Hirose K, Nakanishi T, Nawa A, Kuzuya K, Tajima K: Genetic polymorphisms of p73 G4C14-to-A4T14 at exon 2 and p53 Arg72Pro and the risk of cervical cancer in Japanese. Cancer Lett 205:55-60, 2004
48. Niwa Y, Hirose K, Matsuo K, Tajima K, Ikoma Y, Nakanishi T, Nawa A, Kuzuya K, Tamakoshi A, Hamajima N: Association of p73 G4C14-to-A4T14 polymorphism at exon 2 and p53 Arg72Pro polymorphism with the risk of endometrial cancer in Japanese subjects. Cancer Lett 219:183-90, 2005
49. Pfeifer D, Arbman G, Sun XF: Polymorphism of the p73 gene in relation to colorectal cancer risk and survival. Carcinogenesis 26:103-7, 2005
50. Ryan BM, McManus R, Daly JS, Carton E, Keeling PW, Reynolds JV, Kelleher D: A common p73 polymorphism is associated with a reduced incidence of oesophageal carcinoma. Br J Cancer 85:1499-503, 2001
51. Baxter SW, Choong DY, Eccles DM, Campbell IG: Transforming growth factor beta receptor 1 polyalanine polymorphism and exon 5 mutation analysis in breast and ovarian cancer. Cancer Epidemiol Biomarkers Prev 11:211-4, 2002
52. Chen T, de Vries EG, Hollema H, Yegen HA, Vellucci VF, Strickler HD, Hildesheim A, Reiss M: Structural alterations of transforming growth factor-beta receptor genes in human cervical carcinoma. Int J Cancer 82:43-51, 1999
53. Kaklamani VG, Hou N, Bian Y, Reich J, Offit K, Michel LS, Rubinstein WS, Rademaker A, Pasche B: TGFBR1*6A and cancer risk: a meta-analysis of seven case-control studies. J Clin Oncol 21:3236-43, 2003
54. Pasche B, Kolachana P, Nafa K, Satagopan J, Chen YG, Lo RS, Brener D, Yang D, Kirstein L, Oddoux C, Ostrer H, Vineis P, Varesco L, Jhanwar S, Luzzatto L, Massague J, Offit K: TbetaR-I(6A) is a candidate tumor susceptibility allele. Cancer Res 59:5678-82, 1999
55. Ewart-Toland A, Chan JM, Yuan J, Balmain A, Ma J: A gain of function TGFB1 polymorphism may be associated with late stage prostate cancer. Cancer Epidemiol Biomarkers Prev 13:759-64, 2004
56. Shu XO, Gao YT, Cai Q, Pierce L, Cai H, Ruan ZX, Yang G, Jin F, Zheng W: Genetic polymorphisms in the TGF-beta 1 gene and breast cancer survival: a report from the Shanghai Breast Cancer Study. Cancer Res 64:836-9, 2004
57. Humar B, Graziano F, Cascinu S, Catalano V, Ruzzo AM, Magnani M, Toro T, Burchill T, Futschik ME, Merriman T, Guilford P: Association of CDH1 haplotypes with susceptibility to sporadic diffuse gastric cancer. Oncogene 21:8192-5, 2002
58. Jonsson BA, Adami HO, Hagglund M, Bergh A, Goransson I, Stattin P, Wiklund F, Gronberg H: -160C/A polymorphism in the E-cadherin gene promoter and risk of hereditary, familial and sporadic prostate cancer. Int J Cancer 109:348-52, 2004
59. Shin Y, Kim IJ, Kang HC, Park JH, Park HW, Jang SG, Lee MR, Jeong SY, Chang HJ, Ku JL, Park JG: A functional polymorphism (-347 G-->GA) in the E-cadherin gene is associated with colorectal cancer. Carcinogenesis 25:2173-6, 2004

60. Verhage BA, van Houwelingen K, Ruijter TE, Kiemeney LA, Schalken JA: Single-nucleotide polymorphism in the E-cadherin gene promoter modifies the risk of prostate cancer. Int J Cancer 100:683-5, 2002
61. Wu MS, Huang SP, Chang YT, Lin MT, Shun CT, Chang MC, Wang HP, Chen CJ, Lin JT: Association of the -160 C --> a promoter polymorphism of E-cadherin gene with gastric carcinoma risk. Cancer 94:1443-8, 2002
62. Zhang X, Ma X, Zhu QG, Li LC, Chen Z, Ye ZQ: Association between a C/A single nucleotide polymorphism of the E-cadherin gene promoter and transitional cell carcinoma of the bladder. J Urol 170:1379-82, 2003
63. Gong Z, Xie D, Deng Z, Bostick RM, Muga SJ, Hurley TG, Hebert JR: The PPAR{gamma} Pro12Ala polymorphism and risk for incident sporadic colorectal adenomas. Carcinogenesis 26:579-85, 2005
64. Koh WP, Yuan JM, Van Den Berg D, Ingles SA, Yu MC: Peroxisome proliferator-activated receptor (PPAR) {gamma} gene polymorphisms and colorectal cancer risk among Chinese in Singapore. Carcinogenesis, 2006
65. Landi S, Moreno V, Gioia-Patricola L, Guino E, Navarro M, de Oca J, Capella G, Canzian F: Association of common polymorphisms in inflammatory genes interleukin (IL)6, IL8, tumor necrosis factor alpha, NFKB1, and peroxisome proliferator-activated receptor gamma with colorectal cancer. Cancer Res 63:3560-6, 2003
66. Leibovici D, Grossman HB, Dinney CP, Millikan RE, Lerner S, Wang Y, Gu J, Dong Q, Wu X: Polymorphisms in inflammation genes and bladder cancer: from initiation to recurrence, progression, and survival. J Clin Oncol 23:5746-56, 2005
67. Smith WM, Zhou XP, Kurose K, Gao X, Latif F, Kroll T, Sugano K, Cannistra SA, Clinton SK, Maher ER, Prior TW, Eng C: Opposite association of two PPARG variants with cancer: overrepresentation of H449H in endometrial carcinoma cases and underrepresentation of P12A in renal cell carcinoma cases. Hum Genet 109:146-51, 2001
68. Cox DG, Hankinson SE, Hunter DJ: Polymorphisms of the AURKA (STK15/Aurora Kinase) Gene and Breast Cancer Risk (United States). Cancer Causes Control 17:81-3, 2006
69. Dai Q, Cai QY, Shu XO, Ewart-Toland A, Wen WQ, Balmain A, Gao YT, Zheng W: Synergistic effects of STK15 gene polymorphisms and endogenous estrogen exposure in the risk of breast cancer. Cancer Epidemiol Biomarkers Prev 13:2065-70, 2004
70. Kimura MT, Mori T, Conroy J, Nowak NJ, Satomi S, Tamai K, Nagase H: Two functional coding single nucleotide polymorphisms in STK15 (Aurora-A) coordinately increase esophageal cancer risk. Cancer Res 65:3548-54, 2005
71. Miao X, Sun T, Wang Y, Zhang X, Tan W, Lin D: Functional STK15 Phe31Ile polymorphism is associated with the occurrence and advanced disease status of esophageal squamous cell carcinoma. Cancer Res 64:2680-3, 2004
72. Cox DG, Hankinson SE, Hunter DJ: The erbB2/HER2/neu receptor polymorphism Ile655Val and breast cancer risk. Pharmacogenet Genomics 15:447-50, 2005
73. Kuraoka K, Matsumura S, Hamai Y, Nakachi K, Imai K, Matsusaki K, Oue N, Ito R, Nakayama H, Yasui W: A single nucleotide polymorphism in the transmembrane domain coding region of HER-2 is associated with development and malignant phenotype of gastric cancer. Int J Cancer 107:593-6, 2003
74. Xie D, Shu XO, Deng Z, Wen WQ, Creek KE, Dai Q, Gao YT, Jin F, Zheng W: Population-based, case-control study of HER2 genetic polymorphism and breast cancer risk. J Natl Cancer Inst 92:412-7, 2000
75. van Tilborg AA, de Vries A, Zwarthoff EC: The chromosome 9q genes TGFBR1, TSC1, and ZNF189 are rarely mutated in bladder cancer. J Pathol 194:76-80, 2001
76. Ponder BA: Cancer genetics. Nature 411:336-41, 2001
77. Hemminki K, Forsti A: Proper controls for SNP studies? Carcinogenesis 23:1405, 2002
78. Geddert H, Kiel S, Zotz RB, Zhang J, Willers R, Gabbert HE, Sarbia M: Polymorphism of p16 (INK4A) and cyclin D1 in adenocarcinomas of the upper gastrointestinal tract. J Cancer Res Clin Oncol 131:803-8, 2005

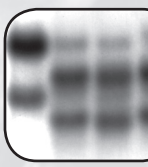
79. Kaghad M, Bonnet H, Yang A, Creancier L, Biscan JC, Valent A, Minty A, Chalon P, Lelias JM, Dumont X, Ferrara P, McKeon F, Caput D: Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell 90:809-19, 1997
80. Ambrosone CB, Kadlubar FF: Toward an integrated approach to molecular epidemiology. Am J Epidemiol 146:912-8, 1997
81. Perera FP, Weinstein IB: Molecular epidemiology: recent advances and future directions. Carcinogenesis 21:517-24, 2000
82. van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, Witteveen A, Glas A, Delahaye L, van der Velde T, Bartelink H, Rodenhuis S, Rutgers ET, Friend SH, Bernards R: A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 347:1999-2009, 2002
83. Zhu ZZ, Cong WM, Liu SF, Dong H, Zhu GS, Wu MC: Homozygosity for Pro of p53 Arg72Pro as a potential risk factor for hepatocellular carcinoma in Chinese population. World J Gastroenterol 11:289-92, 2005
84. Hsieh LL, Huang TH, Chen IH, Liao CT, Wang HM, Lai CH, Liou SH, Chang JT, Cheng AJ: p53 polymorphisms associated with mutations in and loss of heterozygosity of the p53 gene in male oral squamous cell carcinomas in Taiwan. Br J Cancer 92:30-5, 2005
85. Gemignani F, Moreno V, Landi S, Moullan N, Chabrier A, Gutierrez-Enriquez S, Hall J, Guino E, Peinado MA, Capella G, Canzian F: A TP53 polymorphism is associated with increased risk of colorectal cancer and with reduced levels of TP53 mRNA. Oncogene 23:1954-6, 2004

Chapter 13

Searching for early tumor markers in gastro-esophageal junction adenocarcinomas

Submitted

Linetta B Koppert
Henk WPC van de Meerendonk
Monique MCP Hoogmans
Bas PL Wijnhoven
Peter D Siersema
Herman van Dekken
Hugo W Tilanus
Winand NM Dinjens



ABSTRACT

Barrett's metaplasia (MET) or intestinal differentiation in columnar lined esophagus can progress to low grade dysplasia (LGD), high grade dysplasia (HGD) and ultimately to invasive carcinoma (CA). Since the genetic abnormalities involved in this multistep process are still poorly understood, we aimed to identify molecular markers that predict progression to malignancy in Barrett's patients. We investigated first whether gastro-esophageal (GEJ) adenocarcinomas are monoclonal or polyclonal proliferations. To this end, multiple biopsies per carcinoma were selected from 14 GEJ adenocarcinoma resection specimens. These were investigated for loss of heterozygosity (LOH) of chromosomal loci 17p (p53), 9p (p16), 5q (APC, MCC), 13q (Rb) and 18q (DCC, DPC4/SMAD4), for p53 mutations (exon 5 to 8) and for mutations in the homopolymeric C-stretch (D310) within the mitochondrial DNA (mtDNA) displacement loop. We then investigated the usefulness of these aberrations as molecular progression markers in the MET-LGD-HGD-CA sequence in Barrett's esophagus from 31 GEJ resection specimens. Results of the genetic analyses were compared to the histology of the specimens. Identical 17p LOH patterns in multiple biopsies from one tumor were seen in 10 out of 11 informative tumors and, especially, identical intratumor p53 mutations in all nine tumors with a p53 mutation indicated monoclonality of GEJ adenocarcinomas. LOH of 9p was detected in 76% of MET, p53 aberrations appeared in around 60% of LGD, 13q LOH and 18q (DCC and DPC4) LOH appeared in around 45% of LGD adjacent to carcinoma. Consistent LOH and mutation patterns, i.e. loss of the same allele or identical mutations, were found in the majority of cases within the MET-LGD-HGD-CA sequence. A combination of the investigated markers, especially p53 mutations, 17p LOH, 9p LOH, 13q LOH and 18q LOH may be used in conjunction with the histopathological diagnosis for detection of neoplastic progression in Barrett's esophagus.

INTRODUCTION

Chronic gastro-esophageal reflux disease leads to Barrett's esophagus (BE) in around 10% of the patients suffering from this common disease. BE is characterized by columnar metaplasia with intestinal differentiation that has replaced the normally present stratified squamous epithelium in the lower third of the esophagus. BE harbors the risk of progression to dysplasia and adenocarcinoma, with 0.2-2% of patients with Barrett's metaplasia eventually developing a carcinoma [1]. Progression is considered to follow the 'metaplasia (MET)-low grade dysplasia (LGD)-high grade dysplasia (HGD)-carcinoma (CA) sequence', characterized by accumulation of cell cycle abnormalities, genetic abnormalities and aneuploidy [2-4]. Patients with BE are mostly subjected to intensive endoscopic surveillance with biopsy sampling to identify those with neoplastic progression. The histological identification of dysplasia in tissue biopsies is of major importance in determining the intensity of both surveillance and treatment [5]. The histological diagnosis of the grade of dysplasia, however, is characterized by problems concerning biopsy sampling error and intra- and interobserver variation [6].

Preferably, BE patients at high risk for neoplastic progression should be identified before histologic presence of dysplasia. From the literature it is known that aneuploidy as well as genetic abnormalities could be predictors of neoplastic progression, demonstrating a clinical application of early tumor markers in BE surveillance programs [7,8]. From this perspective, it is important to know whether GEJ cancer develops via a process of monoclonal or polyclonal expansion. Monoclonal development would enhance the reliability of molecular markers, since the genetic aberrant clone that represents a possible developing tumor could then be harvested from the premalignant Barrett's epithelium. If we assume that progression from premalignant Barrett's epithelium to carcinoma is a monoclonal process, we expect intratumor homogeneity of early genetic alterations.

In the current study we investigated whether GEJ adenocarcinomas are monoclonal or polyclonal proliferations. Furthermore we investigated the value of several candidate molecular markers as predictors of neoplastic progression from Barrett's metaplasia to adenocarcinoma. We hypothesized that genetic alterations may occur before phenotypic changes in Barrett's epithelium take place. The most promising candidate markers are genetic alterations that are known to occur early in GEJ carcinogenesis as shown by Loss of Heterozygosity (LOH) studies and Comparative Genomic Hybridization (CGH) studies reported in the literature [2,9-28]. In addition, mitochondrial DNA (mtDNA) mutation analyses were performed of a homopolymeric C stretch (D310), a mutational hotspot in the mitochondrial genome. MtDNA mutations have been suggested to be indicators of clonal expansion and as such can be considered as cancer biomarkers [29,30]. The molecular aberrations detected were evaluated for their use as early tumor markers in 31 GEJ resection specimens containing several combinations of MET-LGD-HGD and adenocarcinoma.

MATERIALS AND METHODS

Patients and specimens

Resection specimens of patients with GEJ adenocarcinomas were obtained from 35 patients (33 male, 2 female, mean age at the time of cancer diagnosis 65.4 ± 8.4 years, age range 50-80 years) treated between July 1999 and April 2002 at the Erasmus MC, Rotterdam. All patients underwent a transhiatal resection of the tumor with restoration of the continuity of the gastrointestinal tract by a gastric tube with cervical anastomosis. In thirty-one patients Barrett's epithelium was present. In one patient high grade dysplastic Barrett's epithelium without invasive carcinoma was present. In 4 patients no Barrett's epithelium was found. Thirty-one tumors were located at the distal esophagus, 2 were located at the GEJ and 2 carcinomas were located at the gastric cardia.

Tissue samples and DNA isolation

Tissue biopsies were derived from premalignant and malignant tissue from the 35 GEJ adenocarcinoma resection specimens who were all embedded in paraffin. From 14 GEJ adenocarcinomas multiple, macroscopically separated, tumor biopsies were collected (Figure 13.1). From 31 GEJ resection specimens, 10 of which were also used for the analysis of multiple tumor samples, both premaligant and malignant tissue was present and collected (11 cases MET-LGD-HGD-CA, 6 cases LGD-HGD-CA, 4 cases HGD-CA, 3 cases MET-LGD-CA, 2 cases MET-HGD-CA, 3 cases MET-CA, 1 case LGD-CA, 1 case MET-LGD-HGD). Five-μm thick sections of the paraffine embedded tissue biopsies were mounted on uncoated glass-slides. After deparaffinisation, the sections were stained with eosine and Mayer's haematoxylin solution, analysed by an experienced GI-pathologist (HvD) and finally areas of interest were isolated by Laser Capture Microdissection (LCM, Figure 13.2). In all 35 cases tumor-negative lymph nodes were used as normal non-cancerous tissue. Microdissected tissues and multiple sections of normal tissue were incubated in 50μl of lysis buffer (10 mmol/L Tris-HCl, 1mmol/L EDTA and 1% Tween 20 pH 8.0) plus 1μl proteinase K (20μg/ml) for at least 48 hours. Every 24 hours, 1 μl of proteinase K was added to the samples. Thereafter samples were heated for 10 minutes at 95 °C to inactivate proteinase K.

PCR amplification

DNA was amplified by a radioactive 15μl Polymerase Chain Reaction (PCR). The PCR mix per sample consisted of 10μl distilled H_2O, 1.5μl goldbuffer, 1.5μl 25 mM $MgCl_2$-solution, 0.3μl 10mM dGTP, dTTP, dCTP, 0.3μl 1mM dATP, 0.9 Units (5U/μl x 0.18μl) Amplitaq polymerase (Perkin-Elmer, Wellesley, MA, USA), 30 ng (0.1μg/μl x 0.3μl) of forward and reverse primer, 0.075μl αP^{32}-dATP and 1μl DNA sample. Before amplification, the PCR-mix was heated at 95°C for 5 minutes to activate the Amplitaq polymerase. The temperatures for amplification were 95°C for 30 seconds, 55°C for 45 seconds and 72°C for 45 seconds. These steps were repeated

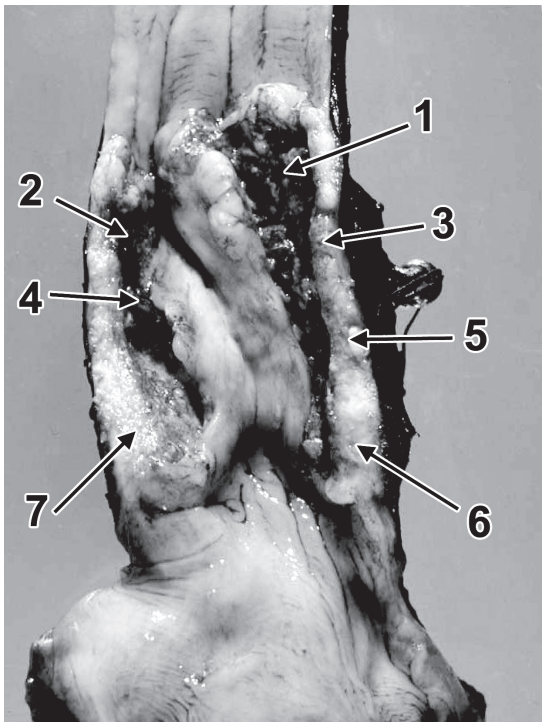


Figure 13.1 Collection of macroscopically separated tumor biopsies from a distal esophageal adenocarcinoma.

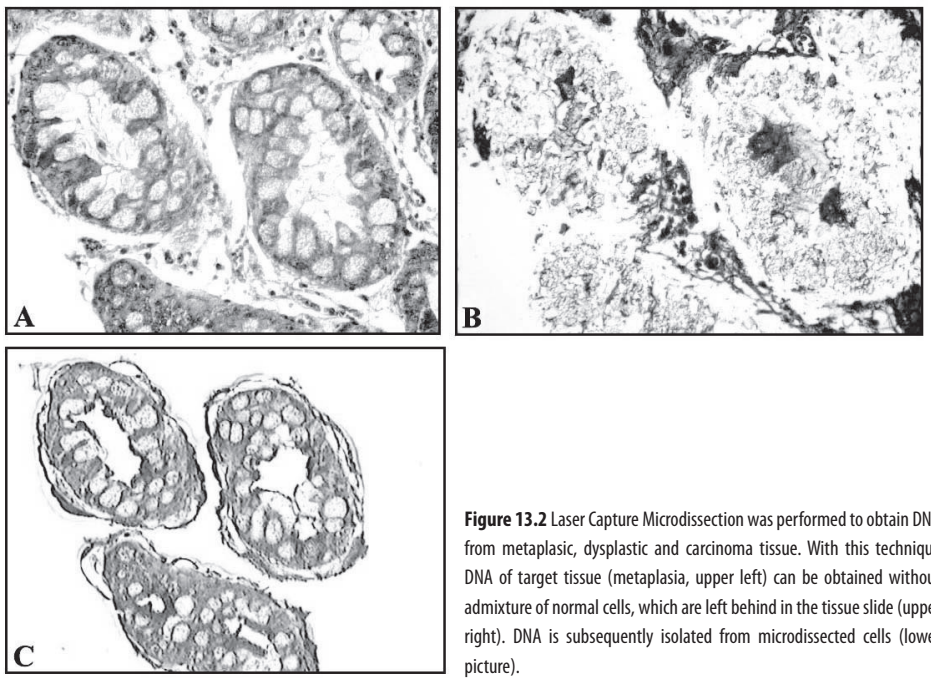


Figure 13.2 Laser Capture Microdissection was performed to obtain DNA from metaplasic, dysplastic and carcinoma tissue. With this technique DNA of target tissue (metaplasia, upper left) can be obtained without admixture of normal cells, which are left behind in the tissue slide (upper right). DNA is subsequently isolated from microdissected cells (lower picture).

for 35 cycles followed by a final extension at 72°C for 10 minutes.

LOH and Single Strand Conformation Polymorphism (SSCP) analysis

For LOH analysis, highly polymorphic dinucleotide repeat markers were amplified. The markers used were located at chromosomal regions known to be frequently deleted in GEJ adenocarcinomas [2,9-28]. The chromosome loci selected harbor the tumor suppressor genes p53 (17p), p16 (9p), Adenomatous Polyposis Coli (APC, 5q), Mutated in Colorectal Cancer (MCC, 5q), Retinoblastoma (Rb, 13q), Deleted in Colorectal Cancer (DCC, 18q), and Deleted in Pancreatic Cancer (DPC4/SMAD4, 18q). Exons 5 to 8 of the p53 gene were investigated by SSCP analysis. Each exon was amplified in 2 overlapping fragments. In addition, deletion/insertion mutations in the mitochondrial D310 fragment were investigated according to the revised human mitochondrial DNA Cambridge sequence [31]. For the mtDNA experiments DNA samples from the mtDNA-less 143B206 ρ° cells and parental 143B206 cells were used as negative and positive controls (cell lines were kind gifts of Dr. G.P. Comi, Milan, Italy, and Dr. D.M. Turnbull, Newcastle upon Tyne, United Kingdom). All primers used are listed in Table 1. PCR products (15μl) were diluted with 14μl loading buffer (95% formamide, 10 mM EDTA pH 8, 0.05% bromophenol blue and 0.05% xylene cyanol) and denaturated at 95°C for 5 minutes. Solutions were chilled on ice and, per sample, 4μl was loaded on a 15% polyacrylamide gel (acrylamide to bisacrylamide 19:1) for LOH analysis, or on an 8% polyacrylamide gel (acrylamide to bisacrylamide 49:1) containing 10% of glycerol for SSCP analysis. Electrophoresis was performed at 65W at room temperature for 1.5-2 hours for LOH analysis, and at 8W overnight at room temperature for SSCP analysis. The gels were vacuum dried at 80°C and exposed to X-ray films. For LOH as well as for SSCP analyses, tumor DNA was always compared with normal DNA from the same patient.

Table 13.1. LOH microsatellite markers, p53 exon 5-8 and D310 mtDNA PCR Primer information.

Chromosome	Marker		Primer sequences	Productsize (bp)	Heterozygosity
17p (p53)	TP53CA	F	AGG GAT ACT ATT CAG CCC GAG GTG	< 130	?
		R	ACT GCC ACT CCT TGC CCC ATT C		
17p (p53)	D17S786	F	TAC AGG GAT AGG TAG CCG AG	135-157	0.77
		R	GGA TTT GGG CTC TTT TGT AA		
p53 exon 5/1		F	CCT GAC TTT CAA CTC TTG CTC	158	Not applicable
		R	ACT GCT TGT AGA TGG CCA TG		
p53 exon 5/2		F	CAG CTG TGG GTT GAT TCC AC	176	Not applicable
		R	CTG GGG ACC CTG GGC AAC		
p53 exon 6/1		F	AGG CCT CTG ATT CCT CAC TG	127	Not applicable
		R	GCA CCA CCA CAC TAT GTC GA		
p53 exon 6/2		F	CTC CTC AGC ATC TTA TCC GA	159	Not applicable
		R	CCA CTG ACA ACC ACC CTT		
p53 exon 7/1		F	AGG CGC ACT GGC CTC ATC TT	141	Not applicable

		R	TCC AGT GTG ATG ATG GTG AGG		
p53 exon 7/2		F	CAT GTG TAA CAG TTC CTG CAT G	135	Not applicable
		R	GCG GCA AGC AGA GGC TGG		
p53 exon 8/1		F	CCT TAC TGC CTC TTG CTT CTC	130	Not applicable
		R	CTT GCG GAG ATT CTC TTC CTC		
p53 exon 8/2		F	TTG TGC CTG TCC TGG GAG AG	127	Not applicable
		R	CTC CAC CGC TTC TTG TCC T		
9p (p16)	D9S1748	F	GTG TGC TTG AAA TAC ACC TTT C	110	0.87
		R	AGT GAG TCC CGA ATA TCC TG		
9p (p16)	D9S942	F	TCC TGC GGA AAC CAT TAT AAC	100	0.78
		R	CAA GAT TCC AAA CAG TAA ACA G		
9p (p16)	D9SREP1	F	ATG AGA AGA TAC TTA TTC CCA G	110	0.78
		R	ACA AGA GTG AAA CTC CAT CTC		
9p (p16)	D9S1870	F	GGG GTT TTC ATT CTA GAA CAG	130	0.67
		R	ATA TGT GGG AAA GTG GGT ATG		
9p (p16)	D9S269	F	GAA ACT ATA ATC TCA AAG GAT G	130	0.64
		R	TGT GAC TAA ATC TGG CCA ATG		
9p (p16)	D9S274	F	CTT CAC GGG TCA ATC CAT TTC	115	0.78
		R	GCA ATT TCT TCC TTC AGC ATT G		
9p (p16)	D9S304	F	GAT GAT AGA TGA TTG ATA GAT TG	110	0.83
		R	GTA TAT GTG CCC ACA CAC ATC		
5q (APC)	D5S346	F	ACT CAC TCT AGT GAT AAA TCG GG	110	0.82
		R	AGC AGA TAA GAC AGT ATT ACT AGT T		
5q (APC)	D5S82	F	TCA CCT ACC TGC CCC AAT TG	110	0.76
		R	AAT TAT ACA TGC ACA CAC ATA TC		
5q (MCC)	D5S659	F	TGT ATT TTA ATG GTC TGG TTG C	134	0.81
		R	TCA ACT TTC ATG GTA CCC TTC		
5q (MCC)	D5S2001	F	ATA TTT TTC CAG AGT CTT CAT TC	130	88
		R	GTC TCT GGG GAG GAG TAA G		
13q (Rb)	D13S118	F	GTC TAT CTC TGT CTC TTT ATT TC	95	0.72
		R	TAT ATA ACT TGT GTG AGC ACA G		
13q (Rb)	D13S1307	F	CCT CAC TGA TAA ATG GGC TG	95	0.71
		R	CTC ATC TAC TCC TTC AAA CAG		
18q (DCC)	D18S484	F	TTT AGA AAC CCT AAA ATG TGA AG	120	0.72
		R	GAT TTC TGT GAC ATA TTC CTT G		
18q (DCC)	D18S35	F	GCT AGA TTT TTA CTT CTC TGA C	110	0.70
		R	CCT GGT TGT ACA TGC CTG AC		
18q (DPC4)	D18S474	F	CAC CCA CTA GAT GTC AGT AG	120	0.80
		R	GCC TTG GAC TGG CTA ATG AG		
18q (DPC4)	D18S1110	F	GAA ACC AAT GTG ACA GTT CTT TG	75	0.75
		R	AAA CTC AGA GTG AAA ACA G		
Mitochondrial	D310	F	TTG AAT GTC TGC ACA GCC AC	112	Not applicable
		R	GGG GTT TGG CAG AGA TGT G		

F=Forward primer, R=Reverse primer

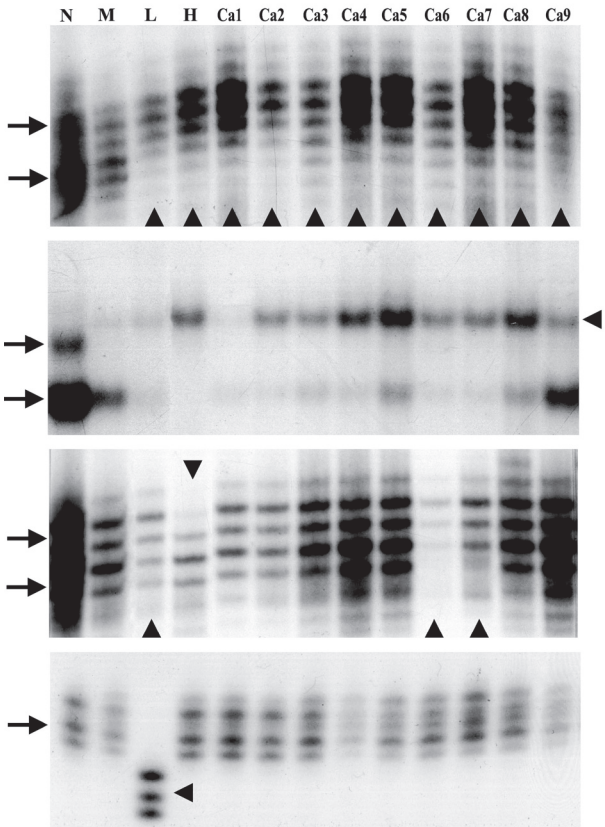


Figure 13.3 PCR-LOH 17p (p53, marker TP53CA), PCR-SSCP p53 (exon 7), PCR-LOH 9p (p16, marker D9S1748) and PCR-SSCP MtDNA (marker D310) analyses of GEJ adenocarcinoma with MET-LGD-HGD and 9 carcinoma samples (see also Table 13.2, Table 13.3 patient 12). Black arrows at the left side point to allelic patterns. The upper picture demonstrates loss of the lower allele (arrow heads) in LGD, HGD and in all carcinoma (Ca) samples as compared to corresponding Normal DNA (N). The second picture demonstrates an identical p53 mutation in MET (M), LGD (L), HGD (H) and all Ca samples (arrow head points to mutation pattern). The third picture demonstrates loss of the lower allele in L, Ca6 and Ca7 (arrow heads) and loss of the upper allele in H (arrow head) as compared to N. The fourth picture demonstrates a MtDNA mutation in L (arrow head).

Sequencing

P53 mutations indicated by SSCP analysis were identified by direct sequencing (Figure 13.4). PCR products were used for cycle sequencing using a Dye Terminator Cycle Sequencing Ready Reaction and analysed on an ABI Prism 3100 genetic analyser according to the instructions of the manufacturer (PE Biosystems, Foster City, CA).

Statistics

The mean number of molecular aberrations was compared between the total groups of MET and LGD, between total groups of LGD and HGD, between total groups of HGD and carcinoma and between MET/LGD and HGD/carcinoma using the independent samples T-test. The number of molecular aberrations was calculated as the sum of p53 mutation, LOH at 7 investigated loci (wit maximum count of 7) and D310 mutation.

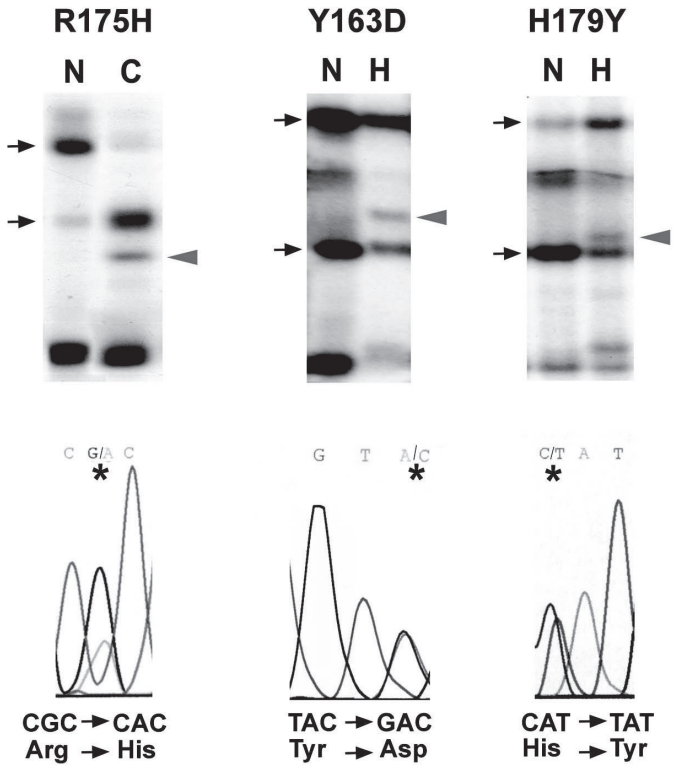


Figure 13.4 PCR-SSCP and sequencing analyses of three p53 mutations in High grade dysplasia (H), Carcinoma (C) and corresponding normal DNA (N). Black arrows point to allelic patterns. Red arrow heads point to mutated alleles. The sequencing chromatograms below each autoradiograph show the alterations (note the substituted nucleotide marked by an asterisk), which all represent p53 mutations. *Also see color figures page 291.*

RESULTS

Clonal analysis

A mean number of six biopsies (range 2-10) was taken from different areas within the tumor. LOH (17p, 9p, 5q, 13q and 18q), p53 exon 5 to 8 and D310 mutation analyses were thus investigated in a total number of 78 samples from 14 tumors. The LOH and mutation data of 1 of the 14 tumors are shown in Table 13.2 and Figure 13.3.

In 9 out of 14 tumors, p53 mutation analysis showed complete intratumor homogeneity, i.e. all biopsies from one tumor harbored an identical p53 mutation. In 5 out of 14 tumors no p53 mutation could be detected in all tumor biopsies (Table 13.3). With regard to the intratumor clonality of 17p LOH (p53), 11 patients had an informative LOH pattern for at least one of the microsatellite markers and 10 of the tumors demonstrated LOH with intratumor homogeneity (Table 13.3). In the remaining informative tumor, no p53 locus LOH was detected in all tumor biopsies. Thirteen tumors showed LOH of the p16 locus with 9 tumors demonstrating intratumor homogeneity and 4 tumors heterogeneity of 9p LOH (Table 13.3). Of the 14 informative tumors for the 5q (APC) locus, 10 showed LOH, with 6 tumors intratumor homogene-

ity (14/10/6, Table 13.3). For the 5q (MCC) locus these Figures were 14/10/5, for 13q 13/8/3, for 18q (DCC) 11/9/4, and for the 18q (DPC4/SMAD4) locus 13/11/4 (Table 13.3). Intratumor consistent LOH patterns were detected in all 14 tumors for at least one microsatellite marker. In 6 tumors mtDNA D310 mutations were found. In 3 of these tumors there was intratumor homogeneity with regard to the D310 mutations and in 3 tumors with D310 mutation heterogeneity was observed (Table 13.3).

Table 13.2 LOH and mutation data in GEJ adenocarcinoma with MET-LGD-HGD and 9 carcinoma samples (see also Table 13.2 patient 12 and Figure 13.3).

	Normal	MET	LGD	HGD	Ca1	Ca2	Ca3	Ca4	Ca5	Ca6	Ca7	Ca8	Ca9
p53, D17S786	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni
P53, TP53CA			L	L	L	L	L	L	L	L	L	L	L
P53, exon 5													
p53, exon 6													
p53, exon 7													
p53, exon 8													
p16, D9S1748			L	U						L	L		
p16, D9S942	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni
p16, REP 1				L									
p16, D9S1870	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni
p16, D9S269	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni
p16, D9S274													
p16, D9S304	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni
APC, D5S346													
APC, D5S82													
MCC, D5S659													
MCC, D5S2001													
Rb, D13S118			U	U					U	U			
Rb, D13S1307													
DCC, D18S484	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni
DCC, D18S35				U		U	U			U	U		
SMAD 4, D18S474	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni
SMAD 4, D18S1110	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni	ni
D310													

Normal = normal tissue; MET = Metaplasia; LGD = Low grade dysplasia; HGD = High grade dysplasia; Ca = carcinoma; ni= not informative.
■=LOH; ▒=mutation; □= no LOH/ no mutation; U=Upper allele; L=Lower allele.

Molecular analysis in MET-LGD-HGD-CA

Table 13.4 summarizes the LOH analysis and mutation data for the 31 patients with GEJ adenocarcinoma and adjacent premalignant lesions. In 25 of the 31 investigated cases one or more p53 mutation was found, in 21 cases in the CA sample. Examples of PCR-SSCP and sequencing analyses are shown in Figure 13.4. In 11 resection specimens from which MET, LGD, HGD and CA were microdissected, we found 9 p53 mutations. In one case the same p53

Table 13.3 Results LOH and mutation analysis in 14 patients with multiple tumor samples.

Patient	p53 LOH	p53 mutation	p16 LOH	APC LOH	MCC LOH	Rb LOH	DCC LOH	DPC4 LOH	MtDNA mutation
1.	m	-	m	no LOH	p	no LOH	p	m	p
2.	m	m	m	m	p	ni	no LOH	p	-
3.	m	-	m	m	m	m	ni	p	-
4.	no LOH	m	m	m	m	no LOH	ni	p	m
5.	ni	m	p	m	no LOH	m	p	p	p
6.	m	-	m	m	m	p	m	m	m
7.	m	-	m	no LOH	p	no LOH	no LOH	no LOH	-
8.	m	m	m	p	p	p	p	p	p
9.	ni	m	m	no LOH	p	m	m	no LOH	-
10.	m	m	no LOH	p	no LOH	no LOH	ni	m	-
11.	m	m	p	p	no LOH	no LOH	m	p	m
12.	m	m	p	no LOH	no LOH	p	p	ni	-
13.	m	-	m	m	m	p	m	m	-
14.	ni	m	p	p	m	p	p	p	-
monoclonal (%)	10/11 (91)	9/9 (100)	9/13 (69)	6/10 (60)	5/10 (50)	3/8 (38)	4/9 (44)	4/11 (36)	3/6 (50)
polyclonal (%)	0/11 (0)	0/9 (0)	4/13 (31)	4/10 (40)	5/10 (50)	5/8 (63)	5/9 (56)	7/11 (64)	3/6 (50)

m=monoclonal; p=polyclonal; -=no mutations in carcinoma samples; ni=not informative.

mutation was present in MET, LGD, HGD and CA, and in 6 cases an identical p53 mutation was found in LGD, HGD and CA with no mutation in the MET samples. In 2 cases the same mutation was found only in the HGD and CA samples, with no mutation in MET and LGD. In addition, in 6 cases from which we had only LGD, HGD and CA 2 cases had an identical mutation in the LGD, HGD and CA samples, 2 cases had no p53 mutation, one case had a p53 mutation only in the LGD and one case only in the HGD sample.

In 19 of 21 MET samples, at least one molecular aberration was found (p53 mutation, LOH of one of 7 investigated loci, D310 mutation). In 13 of these cases at least one aberration in the MET was also found in all of the more advanced stages.

As shown in Table 13.4, p53 mutations were found in 10% (MET), 59% (LGD), 68% (HGD) and 71% (CA). P53 allelic loss increased from 0% (MET), to 67% (LGD), to 76% (HGD) and to 93% (CA). P16 allelic loss increased from 76% (MET), to 91% (LGD), 91% (HGD) and 91% (CA). For APC and MCC allelic loss percentages were: 16% and 29% (MET), 23% and 32% (LGD), 48% and 59% (HGD) and 50% and 51% (CA) respectively. Rb allelic loss was detected in 26% (MET), 45% (LGD), 50% (HGD) and 52% (CA). Finally, DCC and DPC4/SMAD4 allelic loss increased with 5% and 26% (MET), 48% and 43% (LGD), 45% and 62% (HGD) to 63% and 65% (CA) respectively.

Table 13.4 Results of LOH and mutation analysis in 31 patients within Metaplasia-Dysplasia-Carcinoma sequence.

	Metaplasia	Low grade dysplasia	High grade dysplasia	Carcinoma
p53 LOH[a] (%)	0/20 (0)	12/18 (67)	13/17 (76)	28/30 (93)
p53 mutation (%)	2/21 (10)	13/22 (59)	15/22 (68)	25/35 (71)
p16 LOH[a] (%)	16/21 (76)	20/22 (91)	20/22 (91)	33/35 (91)
APC LOH[a] (%)	3/19 (16)	5/22 (23)	10/21 (48)	16/32 (50)
MCC LOH[a] (%)	6/21 (29)	7/22 (32)	13/22 (59)	18/35 (51)
Rb LOH[a] (%)	5/19 (26)	9/20 (45)	10/20 (50)	17/33 (52)
DCC LOH[a] (%)	1/20 (5)	10/21 (48)	9/20 (45)	19/30 (63)
DPC4 LOH[a] (%)	5/19 (26)	9/21 (43)	13/21 (62)	22/34 (65)
MtDNA mutation (%)	4/18 (22)	6/22 (27)	6/19 (32)	11/30 (37)

[a] Number of patients with LOH out of total number of informative patients.

Of 30 cases from which we obtained D310 mutation data, 12 cases had one or more D310 insertion/deletion mutation. Four of 18 MET, 6 of 22 LGD, 6 of 19 HGD and 11 of 30 CA samples had D310 mutations. In 3 of the 4 MET samples with the D310 mutation, the same mutation was also found in the more advanced histological stages. In 5 cases with a D310 mutation in LGD, and without a MET sample or with a MET sample without D310 mutation, 3 mutations were also found in more advanced lesions from the same cases.

Within MET, a mean number of 1.95 ± 1.28 molecular aberrations (p53 mutation, LOH of one of 7 investigated loci, D310 mutation) was found. Within LGD a mean number of 4.0 ± 1.54, within HGD 4.91 ± 1.69 and within CA a mean number of 5.03 ± 1.74 molecular aberrations

was found. This number differed significantly between MET and LGD ($p<0.001$). No significant differences in mean number of molecular aberrations were found when LGD and HGD were compared or when HGD and CA were compared ($p=0.07$ and $p=0.8$ respectively). The mean number of molecular aberrations was significantly lower in the combined group MET and LGD (3.0 ± 1.74) as compared to the combined group HGD and CA (5.0 ± 1.71, $p<0.001$).

DISCUSSION

Currently the histological diagnosis of dysplasia is still the gold standard for the risk stratification for malignant progression in patients with BE. In recent years, many studies have reported that (combinations of) molecular markers could be of additional value in predicting neoplastic progression. In this respect, a monoclonal development of adenocarcinoma in BE would be a prerequisite to find molecular markers that predict progression. Monoclonality in human cancers has been reported for several cancers, amongst which endometrial cancer, head and neck cancer, and bladder cancer [32-34]. To investigate whether GEJ adenocarcinomas are monoclonal or polyclonal proliferations, we performed p53 exon 5 to 8 mutation analyses, LOH analyses of 7 loci with 19 microsatellite markers and mtDNA D310 mutation analyses on a total of 78 samples from 14 GEJ adenocarcinomas. Nine of 14 GEJ adenocarcinomas showed identical p53 mutations in multiple carcinoma biopsies within each tumor, whereas the other 5 carcinomas had no p53 mutation in their carcinoma biopsies. All 5 carcinomas without p53 mutations demonstrated however homogeneity for p53 locus LOH and/or p16 locus LOH. From 13 tumors with LOH at the p16 locus, 9 showed intratumor homogeneity and 4 intratumor heterogeneity. Three of these latter 4 had homogeneous p53 mutations in all tumor biopsies per case. The fourth tumor without p53 mutation demonstrated homogeneous p53 locus LOH. These results strongly indicate that GEJ adenocarcinomas are monoclonal in origin and that p53 and p16 genomic aberrations are early events in GEJ adenocarcinogenesis, as has been proposed by other investigators [2,3,9,11,14,21,25,26,35-37]. The patterns of allelic loss of MCC, Rb, DCC and DPC4/SMAD4 showed higher percentages of heterogeneity in different carcinoma samples (Table 13.2A and 13.3), and might therefore be later events in GEJ adenocarcinogenesis. Miyazono et al. investigated mtDNA D310 mutations in 20 Barrett's adenocarcinomas and adjacent Barrett's epithelium [30]. They found two D310 c-track mutations (10%), both one nucleotide deletions, in only two carcinoma samples [30]. We found D310 c-track mutations in six of 14 (43%) GEJ adenocarcinomas. Three tumors with D310 mutations showed intratumor homogeneity and three heterogeneity. This indicates that D310 c-track mutations have limited value as clonality marker in GEJ adenocarcinomas.

Our finding that GEJ adenocarcinomas are monoclonal in origin is in agreement with several other studies. Nowell et al. proposed that tumors may be monoclonal in origin and share, common, early genetic abnormalities predisposing to the evolution of clones with accumu-

lated, different, late genetic abnormalities [38,39]. Several studies on GEJ adenocarcinomas with or without metaplastic areas and studies on high grade dysplasia without carcinoma show evidence for a monoclonal origin of these lesions by use of comparative genomic hybridisation (CGH), LOH analysis, mutation analysis, X-chromosome inactivation analysis as well as methylation analysis [27,40-46].

Supported by this evidence for monoclonality of GEJ adenocarcinomas, molecular tumor markers can potentially be found which identify BE patients at increased or decreased risk for neoplastic progression. Due to good accessibility of the distal esophagus, tissue biopsies and thus DNA from Barrett's epithelium can readily become available for molecular investigation. DNA in our study was derived from routine formalin fixed and paraffin embedded tissue specimens after LCM. We were able to perform genetic analyses on isolated epithelium from MET, LGD, HGD and CA with minimal contamination of stromal cells (Figure 13.2).

Next to the molecular clonality analyses in 14 GEJ adenocarcinomas molecular aberrations were determined in 31 cases of different combinations of the MET-LGD-HGD-CA sequence. LOH as well as p53 mutations were found in premalignant lesions already. 9p LOH was found in 76% of MET. Within LGD 9p LOH and p53 alterations comprised 91% and about 60%, respectively. LOH of 13q, 18q (DCC) and 18q (DPC4/SMAD4) was also found in LGD in about 45% of cases. We found consistent LOH and p53 mutation patterns, i.e. loss of the same allele or identical mutations, in the majority of cases within the MET-LGD-HGD-CA sequence. This in accordance with Zhuang et al. who found identical allelic loss patterns of the APC gene in metaplasia, dysplasia and adjacent carcinoma as well as the same X-chromosome inactivation patterns in female cases [41]. In that study, metaplasia distant from dysplasia, however, did not show identical LOH patterns as compared to dysplasia and carcinoma [41]. Wu et al. report the same allelic losses of 17p, 18q and 5q in Barrett's metaplasia and carcinoma in 59%, 62% and 27%, respectively [11]. After flow cytometric cell sorting, the same p53 mutations were found in both carcinoma and surrounding HGD in their study as well as in diploid and aneuploid cell populations in another study [11,27]. Walch et al. used CGH analysis and found a high concordance between specific aberration patterns in the MET-LGD-HGD-CA sequence within several samples [9]. Gleeson et al. showed dysplastic Barrett's epithelium and adjacent carcinoma to have the same pattern of novel microsatellite alleles at a number of loci in three cases [14]. In a CGH study by Riegman et al. about one-third of the aberrations present in the dysplasias was also found in the adjacent carcinomas [2]. Identical 18q (DCC), 5q (APC) and 17p (p53) LOH was detected in premalignant and malignant tissues in 4 of 17 patients by Dolan et al. [13]. In contrast to our findings and that of others, Raja et al. found that the genotype of dysplastic Barrett crypts did not necessarily mirror that of the adjacent adenocarcinoma [36]. Finally, Galipeau et al. reported different 9p and 17p LOH patterns in patients with HGD using purified cell fractions by flow cytometry [35]. Flow cytometric cell sorting selects cells with aberrant DNA content, whereas in our study cells were selected by histology.

Our results and literature data demonstrate that part of the DNA aberrations present in the GEJ adenocarcinomas are also present in the tumor adjacent precursor lesions. These molecular results provide evidence that in the investigated GEJ adenocarcinoma resection specimens the precursor lesions progressed to malignancy. This implies that molecular aberrations present in premalignant lesions potentially can predict disease progression. In the current study all resection specimens contained next to premalignant lesions, HGD or adenocarcinoma, indicating that progression had occurred. In the literature few studies have reported on LOH or p53 mutations in premalignant Barrett's epithelium without simultaneous, or in follow up, HGD or cancer. Also in these studies on premalignant Barrett's epithelia without evidence of progression the number of molecular aberrations increases with increasing histological grade [21,25,26,37,47].

Interestingly, in a few cases the premalignant lesions and the adjacent CA samples did show both LOH of a specific locus, though with different allelic loss (i.e. upper allele could be lost in MET whereas lower allele was lost in LGD, HGD and/or CA (Figure 13.3). However, consistent patterns of LOH or absence of LOH in all premalignant lesions and carcinoma were demonstrated in 84% of p53 LOH informative patients (16/19), in 75% of p53 mutation patients (12/16), in 63% of p16 LOH informative patients (17/27), in 61% and 62% of APC and MCC LOH informative patients (14/23 and 16/26), in 68% of Rb LOH informative patients (17/25) and in 57% and 62% of DCC and DPC4 LOH informative patients (12/21 and 13/21). In the cases with slightly different locus LOH patterns clonal expansion may have occurred from a different cell population than the sampled precursor lesion, as suggested by Gleeson et al. [14]. Hypothetically, Barrett's epithelium may comprise multiple genetically different clones while one of these clones progresses, representing the genetic alterations of the tumor. Such a development is known in polyclonal colorectal adenoma and the eventually monoclonal adenocarcinoma [48]. In our study we provided evidence for a monoclonal origin of Barrett's adenocarcinoma, thus minimizing 'genetic sampling error'.

In summary, the MET-LGD-HGD-CA sequence is complex from a molecular point of view. We presented molecular evidence that GEJ adenocarcinomas are monoclonal in orgin. Most likely, a monoclonal tumor harbors clonal diversity concerning later genetic events. Supported by evidence for monoclonality by this current study, we think that tumor markers potentially can be applied in conjunction with histopathology to identify Barrett patients at risk for neoplastic progression. Since the mean number of molecular aberrations (p53 mutation, LOH of one of 7 investigated loci, D310 mutation) significantly differed between MET/LGD as compared to HGD/CA these aberrations potentially can be used as diagnostic markers in tissue biopsies showing indefinite for dysplasia. Importantly, we have to test this hypothesis in a cohort of non-progressive versus progressive BE patients. To date, 17p LOH and DNA content as measured by flow cytometry represent markers which might be prospectively predictive of progression, although routine clinical use is not recommended yet [7,8,49]. In general, a 'multimarker' approach seems more promising than a 'single marker' approach.

Fearon and Vogelstein proposed 'accumulation of changes, rather than order, to be most important in (colorectal) tumor progression' [50]. The development of a 'combined histological and molecular staging system' seems to be a realistic aim to classify patients into various risk groups very early on in the development of malignancy, allowing a tailor-made program for follow-up, screening and therapeutic interventions [51]. The burden of proof lies in prospective future studies. An eventual management strategy could be proposed restricting endoscopic surveillance for neoplastic progression of BE to BE patients with more than a certain number of genetic aberrations in a panel of markers [52]. We suggest that a combination of the investigated markers, especially p53 mutations, 17p LOH, 9p LOH, 13q LOH and 18q LOH might be part of the panel of markers, in conjunction with histopathological diagnosis.

ACKNOWLEDGMENTS

We would like to thank Frank van der Panne for photographic work.

REFERENCES

1. Hage M, Siersema PD, van Dekken H, Steyerberg EW, Dees J, Kuipers EJ: Oesophageal cancer incidence and mortality in patients with long-segment Barrett's oesophagus after a mean follow-up of 12.7 years. Scand J Gastroenterol 39:1175-9, 2004
2. Riegman PH, Vissers KJ, Alers JC, Geelen E, Hop WC, Tilanus HW, van Dekken H: Genomic alterations in malignant transformation of Barrett's esophagus. Cancer Res 61:3164-70, 2001
3. Reid BJ: p53 and neoplastic progression in Barrett's esophagus. Am J Gastroenterol 96:1321-3, 2001
4. Jankowski JA, Wright NA, Meltzer SJ, Triadafilopoulos G, Geboes K, Casson AG, Kerr D, Young LS: Molecular evolution of the metaplasia-dysplasia-adenocarcinoma sequence in the esophagus. Am J Pathol 154:965-73, 1999
5. Sampliner RE: Appearance and prognosis of dysplasia in Barrett's esophagus. Chest Surg Clin N Am 12:69-76, ix, 2002
6. Montgomery E: Is there a way for pathologists to decrease interobserver variability in the diagnosis of dysplasia? Arch Pathol Lab Med 129:174-6, 2005
7. Reid BJ, Levine DS, Longton G, Blount PL, Rabinovitch PS: Predictors of progression to cancer in Barrett's esophagus: baseline histology and flow cytometry identify low- and high-risk patient subsets. Am J Gastroenterol 95:1669-76, 2000
8. Reid BJ, Prevo LJ, Galipeau PC, Sanchez CA, Longton G, Levine DS, Blount PL, Rabinovitch PS: Predictors of progression in Barrett's esophagus II: baseline 17p (p53) loss of heterozygosity identifies a patient subset at increased risk for neoplastic progression. Am J Gastroenterol 96:2839-48, 2001
9. Walch AK, Zitzelsberger HF, Bruch J, Keller G, Angermeier D, Aubele MM, Mueller J, Stein H, Braselmann H, Siewert JR, Hofler H, Werner M: Chromosomal imbalances in Barrett's adenocarcinoma and the metaplasia-dysplasia-carcinoma sequence. Am J Pathol 156:555-66, 2000
10. van Dekken H, Geelen E, Dinjens WN, Wijnhoven BP, Tilanus HW, Tanke HJ, Rosenberg C: Comparative genomic hybridization of cancer of the gastroesophageal junction: deletion of 14Q31-32.1 discriminates between esophageal (Barrett's) and gastric cardia adenocarcinomas. Cancer Res 59:748-52, 1999
11. Wu TT, Watanabe T, Heitmiller R, Zahurak M, Forastiere AA, Hamilton SR: Genetic alterations in Barrett esophagus and adenocarcinomas of the esophagus and esophagogastric junction region. Am J Pathol 153:287-94, 1998
12. Dolan K, Garde J, Gosney J, Sissons M, Wright T, Kingsnorth AN, Walker SJ, Sutton R, Meltzer SJ, Field JK: Allelotype analysis of oesophageal adenocarcinoma: loss of heterozygosity occurs at multiple sites. Br J Cancer 78:950-7, 1998
13. Dolan K, Garde J, Walker SJ, Sutton R, Gosney J, Field JK: LOH at the sites of the DCC, APC, and TP53 tumor suppressor genes occurs in Barrett's metaplasia and dysplasia adjacent to adenocarcinoma of the esophagus. Hum Pathol 30:1508-14, 1999
14. Gleeson CM, Sloan JM, McGuigan JA, Ritchie AJ, Weber JL, Russell SE: Barrett's oesophagus: microsatellite analysis provides evidence to support the proposed metaplasia-dysplasia-carcinoma sequence. Genes Chromosomes Cancer 21:49-60, 1998
15. El-Rifai W, Frierson HF, Jr., Moskaluk CA, Harper JC, Petroni GR, Bissonette EA, Jones DR, Knuutila S, Powell SM: Genetic differences between adenocarcinomas arising in Barrett's esophagus and gastric mucosa. Gastroenterology 121:592-8, 2001
16. Barrett MT, Sanchez CA, Prevo LJ, Wong DJ, Galipeau PC, Paulson TG, Rabinovitch PS, Reid BJ: Evolution of neoplastic cell lineages in Barrett oesophagus. Nat Genet 22:106-9, 1999
17. Nobukawa B, Abraham SC, Gill J, Heitmiller RF, Wu TT: Clinicopathologic and molecular analysis of high-grade dysplasia and early adenocarcinoma in short- versus long-segment Barrett esophagus. Hum Pathol 32:447-54, 2001
18. El-Rifai W, Harper JC, Cummings OW, Hyytinen ER, Frierson HF, Jr., Knuutila S, Powell SM: Consistent genetic alterations in xenografts of proximal stomach and gastro-esophageal junction adenocarcinomas. Cancer Res 58:34-7, 1998

19. Moskaluk CA, Hu J, Perlman EJ: Comparative genomic hybridization of esophageal and gastroesophageal adenocarcinomas shows consensus areas of DNA gain and loss. Genes Chromosomes Cancer 22:305-11, 1998
20. van Dekken H, Alers JC, Riegman PH, Rosenberg C, Tilanus HW, Vissers K: Molecular cytogenetic evaluation of gastric cardia adenocarcinoma and precursor lesions. Am J Pathol 158:1961-7, 2001
21. Barrett MT, Sanchez CA, Galipeau PC, Neshat K, Emond M, Reid BJ: Allelic loss of 9p21 and mutation of the CDKN2/p16 gene develop as early lesions during neoplastic progression in Barrett's esophagus. Oncogene 13:1867-73, 1996
22. Wong DJ, Paulson TG, Prevo LJ, Galipeau PC, Longton G, Blount PL, Reid BJ: p16(INK4a) lesions are common, early abnormalities that undergo clonal expansion in Barrett's metaplastic epithelium. Cancer Res 61:8284-9, 2001
23. Huang Y, Boynton RF, Blount PL, Silverstein RJ, Yin J, Tong Y, McDaniel TK, Newkirk C, Resau JH, Sridhara R, et al.: Loss of heterozygosity involves multiple tumor suppressor genes in human esophageal cancers. Cancer Res 52:6525-30, 1992
24. Sarbia M, Tekin U, Zeriouh M, Donner A, Gabbert HE: Expression of the RB protein, allelic imbalance of the RB gene and amplification of the CDK4 gene in metaplasias, dysplasias and carcinomas in Barrett's oesophagus. Anticancer Res 21:387-92, 2001
25. Sanz-Ortega J, Hernandez S, Saez MC, Sierra E, Sanz-Ortega G, Torres A, Balibrea JL, Sanz-Esponera J, Merino MJ: 3p21, 5q21, 9p21 and 17p13.1 allelic deletions are potential markers of individuals with a high risk of developing adenocarcinoma in Barrett's epithelium without dysplasia. Hepatogastroenterology 50:404-7, 2003
26. Jenkins GJ, Doak SH, Griffiths AP, Tofazzal N, Shah V, Baxter JN, Parry JM: Early p53 mutations in nondysplastic Barrett's tissue detected by the restriction site mutation (RSM) methodology. Br J Cancer 88:1271-6, 2003
27. Neshat K, Sanchez CA, Galipeau PC, Blount PL, Levine DS, Joslyn G, Reid BJ: p53 mutations in Barrett's adenocarcinoma and high-grade dysplasia. Gastroenterology 106:1589-95, 1994
28. Bektas N, Donner A, Wirtz C, Heep H, Gabbert HE, Sarbia M: Allelic loss involving the tumor suppressor genes APC and MCC and expression of the APC protein in the development of dysplasia and carcinoma in Barrett esophagus. Am J Clin Pathol 114:890-5, 2000
29. Jakupciak JP, Wang W, Markowitz ME, Ally D, Coble M, Srivastava S, Maitra A, Barker PE, Sidransky D, O'Connell CD: Mitochondrial DNA as a cancer biomarker. J Mol Diagn 7:258-67, 2005
30. Miyazono F, Schneider PM, Metzger R, Warnecke-Eberz U, Baldus SE, Dienes HP, Aikou T, Hoelscher AH: Mutations in the mitochondrial DNA D-Loop region occur frequently in adenocarcinoma in Barrett's esophagus. Oncogene 21:3780-3, 2002
31. Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, Howell N: Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nat Genet 23:147, 1999
32. Mutter GL, Chaponot ML, Fletcher JA: A polymerase chain reaction assay for non-random X chromosome inactivation identifies monoclonal endometrial cancers and precancers. Am J Pathol 146:501-8, 1995
33. Moniz S, Catarino AL, Marques AR, Cavaco B, Sobrinho L, Leite V: Clonal origin of non-medullary thyroid tumors assessed by non-random X-chromosome inactivation. Eur J Endocrinol 146:27-33, 2002
34. Sidransky D, Frost P, Von Eschenbach A, Oyasu R, Preisinger AC, Vogelstein B: Clonal origin bladder cancer. N Engl J Med 326:737-40, 1992
35. Galipeau PC, Prevo LJ, Sanchez CA, Longton GM, Reid BJ: Clonal expansion and loss of heterozygosity at chromosomes 9p and 17p in premalignant esophageal (Barrett's) tissue. J Natl Cancer Inst 91:2087-95, 1999
36. Raja S, Finkelstein SD, Baksh FK, Gooding WE, Swalsky PA, Godfrey TE, Buenaventura PO, Luketich JD: Correlation between dysplasia and mutations of six tumor suppressor genes in Barrett's esophagus. Ann Thorac Surg 72:1130-5, 2001
37. Suspiro A, Pereira AD, Afonso A, Albuquerque C, Chaves P, Soares J, Leitao CN: Losses of heterozygosity on chromosomes 9p and 17p are frequent events in Barrett's metaplasia not associated with dysplasia or adenocarcinoma. Am J Gastroenterol 98:728-34, 2003

38. Nowell PC: The clonal evolution of tumor cell populations. Science 194:23-8, 1976
39. Anderson GR, Stoler DL, Brenner BM: Cancer: the evolved consequence of a destabilized genome. Bioessays 23:1037-46, 2001
40. Owonikoko T, Rees M, Gabbert HE, Sarbia M: Intratumoral genetic heterogeneity in Barrett adenocarcinoma. Am J Clin Pathol 117:558-66, 2002
41. Zhuang Z, Vortmeyer AO, Mark EJ, Odze R, Emmert-Buck MR, Merino MJ, Moon H, Liotta LA, Duray PH: Barrett's esophagus: metaplastic cells with loss of heterozygosity at the APC gene locus are clonal precursors to invasive adenocarcinoma. Cancer Res 56:1961-4, 1996
42. Blount PL, Meltzer SJ, Yin J, Huang Y, Krasna MJ, Reid BJ: Clonal ordering of 17p and 5q allelic losses in Barrett dysplasia and adenocarcinoma. Proc Natl Acad Sci U S A 90:3221-5, 1993
43. Romagnoli S, Roncalli M, Graziani D, Cassani B, Roz E, Bonavina L, Peracchia A, Bosari S, Coggi G: Molecular alterations of Barrett's esophagus on microdissected endoscopic biopsies. Lab Invest 81:241-7, 2001
44. Prevo LJ, Sanchez CA, Galipeau PC, Reid BJ: p53-mutant clones and field effects in Barrett's esophagus. Cancer Res 59:4784-7, 1999
45. Eads CA, Lord RV, Kurumboor SK, Wickramasinghe K, Skinner ML, Long TI, Peters JH, DeMeester TR, Danenberg KD, Danenberg PV, Laird PW, Skinner KA: Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma. Cancer Res 60:5021-6, 2000
46. van Dekken H, Vissers CJ, Tilanus HW, Tanke HJ, Rosenberg C: Clonal analysis of a case of multifocal oesophageal (Barrett's) adenocarcinoma by comparative genomic hybridization. J Pathol 188:263-6, 1999
47. Gonzalez MV, Artimez ML, Rodrigo L, Lopez-Larrea C, Menendez MJ, Alvarez V, Perez R, Fresno MF, Perez MJ, Sampedro A, Coto E: Mutation analysis of the p53, APC, and p16 genes in the Barrett's oesophagus, dysplasia, and adenocarcinoma. J Clin Pathol 50:212-7, 1997
48. Shibata D, Schaeffer J, Li ZH, Capella G, Perucho M: Genetic heterogeneity of the c-K-ras locus in colorectal adenomas but not in adenocarcinomas. J Natl Cancer Inst 85:1058-63, 1993
49. Reid BJ, Blount PL, Rabinovitch PS: Biomarkers in Barrett's esophagus. Gastrointest Endosc Clin N Am 13:369-97, 2003
50. Fearon ER, Vogelstein B: A genetic model for colorectal tumorigenesis. Cell 61:759-67, 1990
51. Bronchud MH: Is cancer really a 'local' cellular clonal disease? Med Hypotheses 59:560-5, 2002
52. Weissfeld JL: Cyclin D1 and esophageal adenocarcinoma risk: how good does a marker have to be? J Natl Cancer Inst 92:1282-3, 2000

Summary, Conclusions and Epilogue

Part VI

Chapter 14

Summary and Conclusions
Samenvatting en Conclusies

SUMMARY

Adenocarcinoma of the distal esophagus or gastric cardia, the gastro-esophageal junction, is an aggressive disease with an extremely poor prognosis as reflected by a 5-year survival rate of less than 25% after surgery. Most of these cancers are detected at an advanced stage when they cannot be cured surgically. Barrett's esophagus is the only known precursor to adenocarcinoma. Barrett's esophagus is an acquired metaplastic change that occurs in the distal esophagus secondary to chronic gastro-esophageal reflux. The progression towards adenocarcinoma is believed to follow the 'metaplasia-low grade dysplasia-high grade dysplasia-carcinoma sequence'. This neoplastic progression involves an accumulation of genetic and cell cycle abnormalities. Actual knowledge on these genetic pathways remains limited, especially in relation to clinicopathologic characteristics. The interest of the studies described in this thesis was to analyze genetic alterations within GEJ adenocarcinomas and Barrett's esophagus in order to develop molecular markers that in the end could predict which Barrett's patients are at high risk for adenocarcinoma formation and which patients are not. Genetic alterations were related to tumor and patient characteristics as well as to patient outcome. Moreover epidemiological and treatment aspects were highlighted.

The current literature on the molecular biology of esophageal adenocarcinoma was reviewed in **Chapter 2**. Findings on proto-oncogenes, tumor suppressor genes, mismatch repair genes and mitotic checkpoint genes, as the targets of genomic instability, were summarized. From Chapter 2 it was concluded that within the multistep process of esophageal adenocarcinogenesis, no single molecular marker came forward yet able to predict who will and who will not develop cancer in the setting of Barrett's esophagus. Instead, combinations of markers will perhaps lead to a further differentiation in the prediction of neoplastic risk.

Part II concerns molecular biological studies in GEJ adenocarcinomas. The contribution of the Wnt signaling pathway in GEJ adenocarcinomas was studied in **Chapter 3 and 4.** As in colorectal carcinomas the Wnt pathway is suggested to play a key role in GEJ adenocarcinomas. Nuclear β-catenin expression is generally believed to indicate activated Wnt signaling. In chapter 4 it was demonstrated that nuclear β-catenin expression in GEJ adenocarcinoma cell lines indeed correlated with T-cell factor 4 (TCF4)-mediated transcription activation and therefore with activated Wnt signaling. In several tumor types, TCF/β-catenin activation is caused by mutations in either Adenomatous Polyposis Coli (APC), β-catenin exon 3, AXIN1, AXIN2 or β-Transducin repeat-Containing Protein (β-TrCP). In GEJ adenocarcinomas very few APC and β-catenin mutations have been described in the literature. As described in chapter 4 we did not find AXIN1 gene mutations in 17 GEJ tumors with nuclear β-catenin expression (without β-catenin exon 3 mutations). Therefore the mechanism of Wnt pathway activation in GEJ adenocarcinomas still remains unclear. To elucidate a possible prognostic role of the diverse components of the Wnt pathway, an immunohistochemical study was undertaken

in 164 GEJ adenocarcinomas and in 5 human GEJ adenocarcinoma cell lines (chapter 3). Expression of β-catenin, phospho-β-catenin Ser33/37/Thr41, phospho-β-catenin Thr41/Ser45, dephosphorylated 'active' β-catenin, Lymphoid Enhancer-binding Factor 1 (LEF1), TCF4, and the Wnt target genes CD44V6 and Ephrin receptor B2 (EphB2) was analysed in formalin-fixed, paraffin embedded tumor tissues. Thirty-five % of tumors expressed nuclear β-catenin in more than 10% of tumor cells, which indicated that activated Wnt signaling might probably be of less importance in GEJ adenocarcinomas than previously thought. However, loss of expression of the Wnt target gene EphB2 in the membrane or the cytoplasm correlated with worse tumor differentiation grade (p=0.05 and p=0.04) and with worse overall survival (p=0.04 and p=0.04 respectively).

Within the search for genetic alterations that play a role in GEJ adenocarcinogenesis, recent work demonstrated chromosome 14q31-32.1 to be frequently deleted in GEJ adenocarcinomas, suggesting the presence of a tumor suppressor gene in the deleted region. In **Chapter 5** a detailed Loss of Heterozygosity (LOH) analysis was performed in 34 GEJ adenocarcinomas and one tumor-corresponding dysplastic Barrett's epithelium sample with 37 polymorphic microsatellite markers. Fourteen of 34 tumor samples (41%) showed loss of 14q markers. A minimal region of allelic loss of 7,105,440 base pairs was identified between markers D14S1000 and D14S256 at cytogenetic location 14q31.1-32.11 containing 7 known genes. The identification of this minimal deletion and the data base information on the genes present in this region facilitate the search for the candidate GEJ adenocarcinoma suppressor gene(s).

In **Chapter 6** the prevalence and prognostic significance of neuroendocrine (NE) cells was estimated in a series of 208 GEJ adenocarcinomas and 56 Barrett's mucosas adjacent to carcinoma, using immunohistochemistry with chromogranin A (CGA) antibodies. CGA was positive in 49% (102/208) of GEJ adenocarcinomas and in 68% (38/56) of Barrett's mucosas. In multivariate analysis, CGA in Barrett adjacent to carcinoma was an independent prognostic factor for better survival after surgery. To our knowledge this is the first example that in the concurrent presence of a premalignant lesion and a carcinoma a characteristic of the premalignant lesion has prognostic significance. An explanation for this finding remains obscure. Although, its finding might be helpful in the prognostic assessment of patient outcome.

In **Part III** of the thesis epidemiological aspects of GEJ adenocarcinomas as well as gastric carcinomas are discussed. Similarities in epidemiological and histomorphological features haven been described between patients with adenocarcinomas of the distal esophagus and patients with adenocarcinomas of the gastric cardia as well as parallel, rising incidence rates. This formed the assumption for pooling adenocarcinomas of the GEJ within the studies described in this thesis. In **Chapter 7** it was questionized whether distal esophageal adenocarcinoma patients and gastric cardia carcinoma patients were comparable concerning comorbidity. Data on comorbidity at diagnosis (previous cancers, Chronic Obstructive Pulmonary

Diseases (COPD), cardiovascular and cerebrovascular diseases, hypertension, ulcerative digestive tract diseases, liver diseases and diabetes) were derived from a population-based database in the Netherlands (Eindhoven Cancer Registry/IKZ) to compare comorbidity profiles for 479 esophageal squamous cell carcinomas, 339 distal esophageal adenocarcinomas, 570 cardia adenocarcinomas and 1965 subcardia cancers. In terms of comorbidity at diagnosis, cardia adenocarcinoma patients resembled distal esophageal adenocarcinoma patients with likewise equal age and gender distribution.
Additionally, the influence of comorbidity on treatment choice (either curative (surgery) or palliative) and its prognostic value was analyzed within 430 esophageal squamous cell carcinomas, 392 distal esophageal adenocarcinomas, 494 cardia adenocarcinomas and 1708 subcardia cancers in a retrospective study described in **Chapter 8**. It was concluded from multivariable analyses with adjustment for TNM stage and applied therapy that esophageal and gastric carcinoma patients with ≥2 comorbidities had a lower chance of receiving surgery as well as worse overall survival. The dismal prognosis for patients with ≥ 2 comorbidities at cancer diagnosis was likely to be a direct effect of the poor condition of the patient stressing importance of further refinement in perioperative care.

Part IV of the thesis describes several aspects of surgery. Since surgical resection of cancers of the GEJ is associated with substantial morbidity and mortality, one might wonder if this is also necessary in patients with early malignancies, i.e. high grade dysplasias, and intramucosal or submucosal (T1) tumors. Local endoscopic techniques seem to offer a safe alternative to surgery in these patients. A retrospective study described in **Chapter 9** evaluated the outcome of transhiatal esophagectomy in 120 patients with early GEJ adenocarcinoma (13 high grade dysplasias and 107 T1 tumors). Tumors were subdivided into six different depths of invasion ('T1-mucosal' m1- m3, 'T1-submucosal' sm1-sm3). T1m1-m3/sm1 GEJ adenocarcinomas showed a very low risk of lymphatic dissemination and are therefore eligible for local endoscopic therapy. In contrast, almost half of the patients with T1sm2-sm3 lesions developed recurrent disease within 5 years, and therefore need aggressive therapy to improve survival.
With less than 25% of patients surviving at 5 years after surgical resection, the risk in the short-term and the potential loss in quality of life have to be weighed against the long-term benefit. It is well known that the short-term surgical risk varies by clinical characteristics, such as age and comorbidity. In **Chapter 10** 3592 esophagectomy patients from 4 cohorts were analyzed to develop and validate a risk score able to predict mortality occurring within 30 days after esophagectomy. The risk score combines age, comorbidity (cardiac, pulmonary, renal, hepatic, and diabetes), preoperative radiotherapy or combined chemoradiotherapy along with hospital volume.

In **Part V** of the thesis it was aimed to identify Barrett's patients at high risk for developing GEJ adenocarcinoma using molecular biology. The identification of genetic alterations that

confer susceptibility for adenocarcinoma formation in Barrett's esophagus would imply improved manageability of patients with Barrett's esophagus. CHEK2 was investigated as a candidate susceptibility gene within a cohort of adenocarcinomas and (dysplastic) Barrett's epithelia as well as in esophageal squamous cell carcinomas in **Chapter 11**. CHEK2*1100delC is a truncating germline variant of CHEK2 that abrogates kinase activity and confers low-penetrance susceptibility to breast cancer. CHEK2*1100delC was found in 1.5% of 196 esophageal adenocarcinomas, 3.0% of 99 Barrett's metaplasias, 1.5% of 66 dysplastic Barrett's epithelia and in 0.5% of 190 esophageal squamous cell carcinomas. Since these CHEK2*1100delC mutation frequencies did not significantly differ as compared to healthy individuals it was concluded that the mutation has no major contribution in esophageal carcinogenesis. Therefore CHEK2*1100delC is not suitable for risk prediction of neoplastic progression in Barrett's patients.

Individual variations in cancer risk have been associated with specific variant alleles of different genes, Single Nucleotide Polymorphisms (SNPs). SNPs are present in a significant proportion of the normal population. **Chapter 12** investigated the distribution of various SNPs in GEJ adenocarcinoma and esophageal squamous cell carcinoma patients. Frequencies were compared between patients (cases) and healthy blood donors (controls), in order to investigate a possible significance for cancer susceptibility. Selected SNPs concerned the tumor suppressor or proto-oncogenes p53, p16, Cyclin D1, p73, Transforming Growth Factor β (TGF β) receptor 1, TGF β, E-cadherin, Peroxisome Proliferator-Activator Receptor-γ (PPAR-γ), Serine/Threonine protein Kinase 15 (STK15) and Her-2/neu. The value of several candidate molecular markers as predictors of neoplastic progression in Barrett's metaplasia was investigated in **Chapter 13**. Application of such markers in the future would be favoured by a monoclonal growth pattern from the Barrett's esophagus towards adenocarcinoma. A monoclonal growth pattern would enhance the reliability of molecular markers, since the genetic aberrant clone that represents a possible developing tumor could then be harvested from the premalignant Barrett's epithelium. Multiple biopsies per carcinoma were selected from 14 GEJ adenocarcinoma resection specimens and obtained by Laser Capture Microdissection (LCM). LCM allows isolation of target cells without admixture of normal cells. DNA was investigated for LOH of several chromosomal loci (17p (p53), 9p (p16), 5q (APC, Mutated in Colorectal Cancer (MCC)), 13q (Retinoblastoma (Rb)) and 18q (Deleted in Colorectal Cancer (DCC), Deleted in Pancreatic Cancer (DPC4/SMAD4)), for p53 mutations (exon 5 to 8) and for mutations in the homopolymeric C-stretch (D310) within the mitochondrial DNA (mtDNA) displacement loop. In 9 out of 14 tumors, p53 mutation analysis showed complete intratumor homogeneity, i.e. all biopsies from one tumor harbored an identical p53 mutation and therefore evidence for a monoclonal origin of GEJ adenocarcinomas was provided. Next to the clonality analyses, the mentioned molecular aberrations were determined in 31 cases of different combinations of the 'metasplasia-low grade dysplasia-high grade dysplasia-carcinoma sequence', again isolated by LCM. Part of the DNA aberrations present in the GEJ adenocarci-

nomas was also present in the tumor adjacent precursor lesions and therefore could be able to predict adenocarcinoma formation. A combination of the investigated markers, especially p53 mutations, 17p LOH, 9p LOH, 13q LOH and 18q LOH might be part of a future panel of molecular markers, in conjunction with histopathological diagnosis.

SAMENVATTING

Klierbuisvormende tumoren (adenocarcinomen) van het onderste gedeelte van de slokdarm of van het bovenste gedeelte van de maag, de slokdarm-maag overgang (SMO), zijn agressieve tumoren met een zeer slechte prognose. De 5-jaarsoverleving bedraagt <25% na chirurgie. De meeste van deze tumoren worden pas in een vergevorderd stadium gediagnostiseerd als er geen mogelijkheden meer zijn om te opereren. Het enige bekende voorloperstadium van deze tumoren vormt de zogenaamde Barrett slokdarm. Dit is een verworven weefselverandering (metaplasie) in het onderste gedeelte van de slokdarm die ontstaat na langdurige expositie aan zure reflux ('zuurbranden'). Eventuele progressie naar kanker vindt plaats volgens de 'Barrett metaplasie-laaggradige dysplasie-hooggradige dysplasie-carcinoom sequentie'. Deze maligne ontaarding gaat gepaard met een opeenstapeling van genetische afwijkingen en disregulatie van de celcyclus. Gedetailleerde kennis over deze cascade van moleculair genetische gebeurtenissen is beperkt en met name kennis over het verband tussen genetische afwijkingen en patiënt- en tumorkenmerken ontbreekt. De studies in dit proefschrift hadden tot doel moleculair genetische afwijkingen in SMO adenocarcinomen en Barrett slokdarm te analyseren om zodoende moleculaire markers te vinden. In de toekomst zouden moleculaire markers wellicht behulpzaam kunnen zijn in het voorspellen welke Barrett patiënten een hoog risico lopen op het krijgen van kanker en welke Barrett patiënten niet. De gevonden genetische afwijkingen werden gecorreleerd aan patiënt- en tumorkenmerken en overleving. Tevens werden epidemiologische aspecten en aspecten van de behandeling van SMO adenocarcinomen belicht.

In een review in **Hoofdstuk 2** werd de bestaande literatuur over de moleculaire biologie van slokdarm adenocarcinomen samengevat. Afwijkingen in proto-oncogenen, tumorsuppressor genen, mismatch repair genen en mitotische checkpoint genen resulteren in genomische instabiliteit. Gevonden afwijkingen in deze groepen genen in slokdarm adenocarcinomen werden beschreven. De conclusie luidde dat in het uitgebreide proces van slokdarm adenocarcinogenese nog geen unieke moleculaire marker is gevonden die kan voorspellen welke Barrett patiënt een hoog risico heeft op het krijgen van kanker en welke patiënt niet. In plaats daarvan zullen naar verwachting combinaties van moleculaire markers in de toekomst bijdragen aan risico predictie.

Deel II betreft moleculair biologische studies in SMO adenocarcinomen. Het aandeel van de zogenaamde 'Wnt pathway' in SMO adenocarcinomen werd onderzocht in **Hoofdstuk 3** en **4**. De Wnt pathway of, beter gezegd, afwijkingen in de Wnt pathway waardoor de pathway wordt geactiveerd, spelen een sleutelrol in dikke darm tumoren (colon en rectum). De aanwezigheid van ß-catenine in de kern van tumorcellen wordt in het algemeen beschouwd als uiting van een geactiveerde pathway en daarmee als tumorigeen. In **Hoofdstuk 4** werd aangetoond dat kernexpressie van ß-catenine correleert met T-cell factor 4 (TCF-4) geme-

dieerde transcriptie activatie en daardoor met een geactiveerde pathway. In verscheidene typen tumoren wordt TCF-4/ ß-catenine activatie veroorzaakt door mutaties in Adenomatous Polyposis Coli (APC), ß-catenine exon 3, AXIN1, AXIN2 of ß-Transducin repeat-Containing Protein (ß-TrCP). In SMO adenocarcinomen zijn in de literatuur slechts enkele gevallen beschreven van APC en ß-catenine mutante tumoren en daarom lijken mutaties in APC en ß-catenine geen verklaring voor activatie van de Wnt pathway in SMO adenocarcinomen met nucleair ß-catenine. Zoals in **Hoofdstuk 4** beschreven werden in een serie van 17 SMO adenocarcinomen met ß-catenine kernexpressie (en zonder ß-catenine exon 3 mutatie) geen AXIN1 mutaties gevonden. Het Wnt pathway mechanisme in SMO adenocarcinomen is dus nog altijd onduidelijk. In **Hoofdstuk 3** werd een immunohistochemische studie verricht met de vraagstelling of componenten van de Wnt pathway een prognostische betekenis hebben in SMO adenocarcinomen. Expressie van verschillende eiwitten werd onderzocht in formaline gefixeerd paraffine materiaal van 164 SMO adenocarcinomen en 5 SMO tumor cellijnen. De onderzochte eiwitten waren: ß-catenine, phospho-ß-catenine Ser33/37/Thr41, phospho-ß-catenine Thr41/Ser45, gedefosforyleerd 'active' ß-catenine, Lymphoid Enhancer-binding Factor 1 (LEF1), TCF4 en de Wnt target genen CD44V6 en Ephrin receptor B2 (EphB2). In 35% van de tumoren werd kernexpressie van ß-catenine aangetoond in de kern in >10% van de tumorcellen, erop duidend dat de Wnt pathway mogelijk een minder belangrijke rol speelt in SMO adenocarcinomen dan aanvankelijk gedacht. Desalniettemin correleerde afwezigheid van EphB2 in celmembraan of het cytoplasma met een slechtere tumor differentiatiegraad (p=0.05 en p=0.04) en slechtere survival (p=0.04 en p=0.04).

In de zoektocht naar genetische afwijkingen welke een rol spelen in SMO adenocarcinogenese werd recent gevonden dat een gebied op de lange arm van chromosoom 14 frequent verloren was in SMO adenocarcinomen. Dit zou kunnen duiden op de lokalisatie van een tumorsuppressor gen in dat gebied. In **Hoofdstuk 5** werd een gedetailleerde Loss of Heterozygosity (LOH) analyse verricht in 34 SMO adenocarcinomen en 1 dysplastisch Barrett epitheel gelegen naast een SMO adenocarcinoom, met behulp van 37 polymorfe microsatelliet markers. In 14 van de 34 samples (41%) werd 14q LOH aangetoond. Tussen de markers D14S1000 en D14S256 werd een minimale regio van verlies gevonden van 7105440 baseparen (14q31.1-32.11), een gebied waar zich 7 bekende genen bevinden. Identificatie van deze regio in combinatie met database informatie over de genen in de minimale verlies regio vergemakkelijken de zoektocht naar een kandidaat SMO adenocarcinoom tumorsuppressor gen/genen.

In **Hoofdstuk 6** werd de prevalentie en eventueel prognostische betekenis van neuroendocriene cellen onderzocht in 208 SMO adenocarcinomen en 56 Barrett epithelia gelegen naast tumor. Hiertoe werd Chromogranine A (CGA) immunohistochemie verricht. CGA werd aangetoond in 49% (102/108) van de tumoren en in 68% (38/56) van de Barrett epithelia. In multivariate analyse bleek CGA in Barrett epitheel gelegen naast tumor een onafhankelijke prognostische factor voor betere overleving na chirurgie. Dat een kenmerk van een prema-

ligne lesie, gelegen naast tumor, prognostische betekenis heeft, is niet eerder aangetoond. Een mogelijke verklaring voor deze bevinding hebben we vooralsnog niet gevonden. Toch zou deze uitkomst van prognostische betekenis kunnen zijn in patiënten met SMO adenocarcinomen.

In **Deel III** van dit proefschrift werden epidemiologische aspecten bediscussieerd. In de literatuur worden overeenkomsten in epidemiologische factoren en histomorfologische kenmerken beschreven tussen patiënten met adenocarcinomen van het onderste deel van de slokdarm en patiënten met adenocarcinomen van het bovenste gedeelte van de maag, de cardia. Ook wordt een vergelijkbare, parallel stijgende incidentie van deze tumoren beschreven. Dit vormde de aanname om in de studies beschreven in dit proefschrift de tumoren van onderste gedeelte van slokdarm en bovenste gedeelte van maag als één klinische entiteit te beschouwen en als zodanig te analyseren.
In **Hoofdstuk 7** werd onderzocht of de aanwezigheid van comorbiditeit verschilde tussen patiënten met adenocarcinomen van het onderste deel van de slokdarm en patiënten met adenocarcinomen van de cardia. Gegevens over aanwezige comorbiditeit ten tijde van diagnose (eerdere maligniteit, Chronic Obstructive Pulmonary Disease (COPD), cardiovasculaire en cerebrovasculaire ziekten, hypertensie, ulcuslijden van het maagdarmstelsel, leverziekten en diabetes) werden verkregen uit een Nederlandse populatie database (Kankerregistratie Eindhoven/IKZ). Comorbiditeitsprofielen van 479 slokdarm plaveiselcelcarcinomen, 339 slokdarm adenocaricnomen, 570 cardia adenocarcinomen en 1965 subcardia carcinomen werden met elkaar vergeleken. Patiënten met cardia adenocarcinomen waren qua comorbiditeitspatroon vergelijkbaar met patiënten met onderste slokdarm adenocarcinomen, met eveneens vergelijkbare leeftijds- en geslachtsverdeling.
Vervolgens werd de invloed van comorbiditeit op keuze van therapie (curatief (chirurgie) of palliatief) onderzocht in 430 slokdarm plaveiselcelcarcinomen, 392 slokdarm adenocarcinomen, 494 cardia adenocarcinomen en 1708 subcardia carcinomen in een retrospectieve studie in **Hoofdstuk 8**. Multivariate analyse met correctie voor TNM stadium en therapie wees uit dat patiënten met slokdarm- of maagtumoren bij aanwezigheid van ≥2 comorbiditeiten een kleinere kans hadden op het krijgen van chirurgie. Tevens was er een slechtere overleving. De slechtere prognose van patiënten met ≥2 comorbiditeiten leek een direct effect te zijn van hun slechtere conditie. Dit legt de nadruk op het belang van zorgvuldige perioperatieve zorg.

In **Deel IV** van het proefschrift werden aspecten van chirurgie belicht. Chirurgische resectie van SMO tumoren is geassocieerd met substantiële morbiditeit en mortaliteit. Mogelijk zou dit met een minder invasieve behandeling bij patiënten met vroege tumoren bespaard kunnen blijven. Vroege tumoren zijn hooggradige dysplasie en intramucosale en submucosale tumoren (T1 tumoren). Lokale endoscopische technieken lijken een veilig alternatief voor

chirurgie in deze patiënten. In **Hoofdstuk 9** werd een retrospectieve studie beschreven waarin de uitkomsten na chirurgie werden geanalyseerd in 120 patiënten met vroege SMO tumoren (13 hooggradige dysplasiëen en 107 intramucosale of submucosale tumoren). Deze groep werd onderverdeeld in 6 verschillende niveaus van invasie ('T1-mucosaal' m1-m3 en 'T1-submucosaal' sm1-sm3). T1m1-m3/sm1 SMO tumoren hadden een laag risico op lymfkliermetastasering en lijken daardoor veilig toegankelijk voor locale endoscopische therapie. Daarentegen bijna de helft van de patiënten met T1sm2-sm3 tumoren ontwikkelde tumorrecidief binnen 5 jaar en deze tumoren lijken agressief te moeten worden behandeld om overleving te verbeteren.
Met <25% 5-jaars overleving na chirurgie moeten de risico's van chirurgie op korte termijn en de daardoor potentieel verminderde kwaliteit van leven worden afgewogen tegen de baten op lange termijn. De risico's van chirurgie worden beïnvloed door patiënt kenmerken zoals leeftijd en comorbiditeit. In **Hoofdstuk 10** werden 3592 patiënten uit 4 verschillende cohorten na chirurgie geanalyseerd en werd een risico score model ontwikkeld en gevalideerd waarmee 30-dagen mortaliteit na chirurgie kon worden voorspeld. De score combineert gegevens van leeftijd, comorbiditeit (cardiaal, pulmonaal, renaal, leverziekten en diabetes), preoperatieve radiotherapie of gecombineerde chemoradiotherapie en ziekenhuisvolume.

In **Deel V** van het proefschrift werd geprobeerd moleculaire biologie te gebruiken voor het identificeren van Barrett patiënten met een hoog risico op het krijgen van kanker. Met het identificeren van genetische afwijkingen, geassocieerd met een verhoogde kans op het ontstaan van kanker in Barrett epitheel, zou surveillance kunnen worden verbeterd als ook behandeling en informatievoorziening van Barrett patiënten. In **Hoofdstuk 11** werd het CHEK2 gen onderzocht als mogelijk kandidaatgen voor deze toepassing in een cohort van adenocarcinomen, (dysplastische) Barrett epithelia en slokdarm plaveiselcelcarcinomen. CHEK2*1100delC is een truncerende kiembaanmutatie die kinase activiteit blokkeert. Deze mutatie is geassocieerd met een hogere kans op borstkanker (lage penetrantie). CHEK2*1100delC was aanwezig in 1.5% van 196 adenocarcinomen, 3.0% van 99 Barrett's metaplasieën, 1.5% van 66 dysplastische Barrett epithelia en 0.5% van 190 plaveiselcelcarcinomen. Aangezien deze CHEK2*1100delC mutatiefrequenties niet significant verschilden met de mutatiefrequentie in een cohort gezonde vrijwilligers luidde de conclusie van **Hoofdstuk 11** dat CHEK2*1100delC geen grote bijdrage levert aan het ontstaan van slokdarmkanker. CHEK2*1100delC is daarom geen geschikte kandidaat voor het identificeren van hoog risico patiënten. In de recente literatuur komt naar voren dat Single Nucleotide Polymorfismen (SNPs) geassocieerd zijn met het risico op het ontwikkelen van verschillende tumoren. Een genpolymorphisme is een variante sequentie van kiembaan DNA die frequent voorkomt in de populatie. In **hoofdstuk 12** werd de frequentie van verschillende SNPs onderzocht in patiënten met adenocarcinoom en plaveiselcelcarcinoom van de slokdarm. De frequentie van SNPs werd vergeleken met de frequentie in controles (gezonde bloeddonoren) om zodoende vast

te stellen of een of meerdere van de onderzochte SNPs geassocieerd waren met het krijgen van slokdarmcarcinoom. De onderzochte genen betroffen de tumorsuppressorgenen of proto-oncogenen p53, p16, Cycline D1, p73, Transforming Growth Factor β (TGF β) receptor 1, TGF β, E-cadherine, Peroxisome Proliferator-Activator Receptor-γ (PPAR-γ), Serine/Threonine proteïne Kinase 15 (STK15) en Her-2/neu. In **Hoofdstuk 13** werd de waarde van verscheidene kandidaat moleculaire markers onderzocht als voorspellers van neoplastische progressie in Barrett epitheel. Eventuele toepasbaarheid van moleculaire markers in de toekomst zou aangemoedigd worden als er een monoclonaal groeipatroon van Barrett metaplasie richting tumor zou bestaan. Dit betekent dat de voorlopercellen waaruit kanker ontstaat dezelfde genetische afwijkingen hebben als de uiteindelijke tumor. Een monoclonaal groeipatroon zou de betrouwbaarheid van moleculaire markers verhogen omdat de genetisch afwijkende cel welke een mogelijk ontwikkelende tumor representeert dan geoogst zou kunnen worden uit Barrett epitheel. Om dit te onderzoeken werden van 14 SMO adenocarcinomen per tumor meerdere biopten verzameld waarna DNA werd geïsoleerd na Laser Capture Microdissection (LCM). Met de LCM techniek werden selectief de cellen van interesse verkregen voor het isoleren van DNA zonder bijmenging van normale cellen. Vervolgens werd LOH analyse verricht van verscheidende chromosoom loci (17p (p53), 9p (p16), 5q (APC, Mutated in Colorectal Cancer (MCC)), 13q (Retinoblastoma (Rb)) en 18q (Deleted in Colorectal Cancer (DCC), Deleted in Pancreatic Cancer (DPC4/SMAD4)), werd mutatie analyse van p53 (exon 5 tot en met 8) verricht en werd mutatie analyse verricht van mitochondriaal DNA (mtDNA, D310). In 9 van de 14 tumoren liet p53 mutatie analyse complete intratumor homogeniteit zien, dat wil zeggen alle biopten van dezelfde tumor hadden dezelfde, identieke, p53 mutatie waarmee een bewijs werd geleverd voor het monoclonaal ontstaan van SMO adenocarcinomen. Vervolgens werden de LOH en mutatie analyses verricht in 31 verschillende combinaties van de 'metaplasie-laaggradige dyplasie-hooggradige dysplasie-carcinoom sequentie'. Een deel van de genetische afwijkingen aanwezig in de SMO adenocarcinomen was ook aanwezig in de, naast de tumor gelegen, voorloperstadia en zou daarmee geschikt zijn voor het voorspellen van adenocarcinoomvorming in Barrett patiënten. Een combinatie van de onderzochte moleculaire markers, met name p53 mutaties, 17p LOH, 9p LOH, 13q LOH, en 18q LOH zou in de toekomst deel uit kunnen maken van een set van moleculaire markers in Barrett patiënten in combinatie met histopathologie.

Chapter 15

Epilogue: general discussion, clinical implications and future research

Adenocarcinomas of the GEJ are often diagnosed at an advanced disease state and associated with a poor prognosis. These carcinomas are thought to arise within the 'Barrett's metaplasia-low grade dysplasia-high grade dysplasia-carcinoma sequence'. Therefore the detection of preneoplastic lesions is important to ensure early and appropriate treatment. Presently, the histological detection of dysplasia is the best available marker for cancer risk. Intensive endoscopic surveillance, including tissue biopsies, is aiming at early detection of high-grade dysplasia and early adenocarcinoma. Probably, molecular genetic analysis could be of help to identify high risk patients. This assumption formed the aim of this thesis. Genetic and clinical studies were performed and, as described in part V of the thesis, combined in order to build a bridge from Laboratory to Clinic.

MOLECULAR BIOLOGY

The formation of adenocarcinoma in Barrett's esophagus is complex from a molecular point of view and in this thesis it was aimed to further unravel the complex molecular alterations present in GEJ adenocarcinomas and its precursor lesions. Moreover it was aimed to correlate these molecular changes to clinicopathologic characteristics.

Many questions still exist and could not be answered in this thesis. Wnt signaling, having importance in a number of developmental processes, is able to contribute to tumorigenesis by altering the state and activity of β-catenin, and as such plays an important role in colorectal adenocarcinoma formation, amongst others. In the absence of Wnt signals β-catenin is located at the plasma membrane, linked to E-cadherin, and functions in cell-cell adhesion. Excess cytoplasmic β-catenin is sequestered, phosphorylated and eventually targeted to degradation in a protein complex comprised of adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK-3β) and AXIN1 or AXIN2. Activated Wnt signaling inhibits the phosphorylation and therefore degradation of β-catenin, which translocates to the nucleus where it activates genes containing T-cell factor (TCF)-binding sites, a family of transcription factors. In addition to work from others, who did not find mutations in β-catenin nor in APC to be responsible for Wnt signaling in GEJ adenocarcinomas [1-4], neither AXIN1 mutations nor AXIN1 LOH were shown to be responsible for Wnt signaling. Therefore, the mechanism of Wnt activation remains obscure in these tumors. We did show however that loss of expression of the recently identified Wnt target gene EphB2 was shown to be significantly correlated with worse survival and poor tumor differentiation grade.

We identified the commonly deleted region of allelic loss of chromosome 14q31-32.1, occurring in more than 40% of all GEJ adenocarcinomas, and determined an overlapping region of LOH with a size of about 7.1 Mb. This suggests that this region contains one or more tumor suppressor genes involved in GEJ tumorigenesis.

The presence of neuroendocrine cells, as shown by chromogranin immunohistochemistry, in Barrett's epithelium adjacent to tumor was an independent prognostic factor for better survival after surgery. Using mouse models and transfection of chromogranin cDNA a slower progression of tumor growth was demonstrated in the literature, probably being an explanation for the findings in this thesis [5]. Future experiments focusing on transfection of chromogranin cDNA in Barrett's metaplasia cell lines could possibly gain more insight in the role of CGA in GEJ adenocarcinoma development. It also needs to be established which kind of hormone(s) is/are being secreted by the neuroendocrine cells in Barrett and GEJ adenocarcinomas to unravel their function. A large panel of candidate hormones has being tested already at the Department of Pathology of the Erasmus MC but none of these has provided a firm conclusion yet.

EPIDEMIOLOGY

Concerning the epidemiology of esophageal cancer, remarkable changes have taken place over the past decades. About 50 years ago, the incidence of esophageal adenocarcinoma was lower than the incidence of squamous cell carcinoma [6]. To date, adenocarcinoma is the most common esophageal carcinoma and moreover, carcinomas from the gastric cardia show a parallel increasing incidence. Both esophageal and cardia adenocarcinomas occur mainly in Caucasian men. The risk of both esophageal and cardia adenocarcinomas is increased in people with Gastro-Esophageal Reflux Disease. Although studies concerning molecular biology produced conflicting results, many have demonstrated the similarity of these tumors [7-11]. In this thesis it was investigated whether distribution of comorbidity (previous cancers, Chronic Obstructive Pulmonary Diseases (COPD), cardiovascular and cerebrovascular diseases, hypertension, ulcerative digestive tract diseases, liver diseases and diabetes) differed between esophageal squamous cell carcinoma, distal esophageal adenocarcinoma, cardia adenocarcinoma and subcardia carcinoma patients within a large population based database (Eindhoven Cancer Registry). Cardia adenocarcinoma patients resembled distal esophageal adenocarcinoma patients considerably more than subcardia carcinoma patients concerning comorbidity, with likewise equal age and gender distribution and therefore again it seems justified to evaluate these tumors similarly.

Importantly, there is evidence that the likelihood and pattern of lymph node metastases as well as overall survival are similar in esophageal and cardia adenocarcinoma patients [12-14]. Therefore treatment should also be similar. In a retrospective study it was shown that the presence of comorbidity influenced the choice of treatment in patients with esophageal and gastric carcinoma.

TREATMENT

Surgery

Esophageal resection with extensive lymph node dissection is still considered the standard therapy for early GEJ lesions, i.e. high grade dysplasia or T1 adenocarcinoma, at many institutions. We showed lymph node metastases in only one out of 79 T1 tumors (1%) with location in the mucosa or superficial submucosa as compared to 18 out of 41 T1 tumors (44%) with location in the deep submucosa. Moreover, recurrence free period differed significantly between these two groups (p log rank < 0.0001, with 5-year recurrence free percentages of 97% and 57% respectively). Less invasive and more limited surgical procedures and endoscopic procedures might therefore be appropriate alternatives for high grade dysplasia and T1 tumors (intramucosal or superficial submucosa). Hence, an individualized treatment strategy should be employed based on depth of tumor penetration into the mucosa or submucosa, presence of lymph node metastases, endoscopic appearance concerning multicentricity of tumor growth and length of the underlying Barrett's mucosa and comorbidity of the affected patient. Esophageal resection is associated with substantial morbidity and compromised postoperative quality of life. Moreover, the average reported mortality nowadays varies from 4% in specialized centres to more than 10% in low volume hospitals [15,16]. We attempted to develop a simple and robust prediction model for surgical mortality in esophageal cancer patients. Mortality after esophagectomy was found to be related to patient characteristics (age and comorbidity (pulmonary, diabetes)), neoadjuvant therapy and hospital volume. Interestingly, neoadjuvant treatment with radiotherapy was associated with a substantially higher surgical mortality risk, as was neoadjuvant chemoradiotherapy.

Neoadjuvant therapy

In the literature, irradical resections are reported to occur frequently after esophageal resection, varying from 25-41% [17-19]. Adequate loco-regional control is an important issue and might be improved by neoadjuvant therapy. Concerning neoadjuvant chemotherapy, available randomised phase III studies and reviews show that the possible benefit, if any, is considered to be small and it is uncertain whether such a potential survival benefit outweighs the morbidity caused by such a treatment [17,19-26]. From several studies on neoadjuvant radiotherapy it was learned that concurrent chemoradiotherapy is recommended compared to radiotherapy alone since chemotherapy and radiotherapy can interact in several ways. [27-33]. To date several randomized controlled trials have been reported in which neoadjuvant chemoradiotherapy was applied [24,34-39]. Only one trial could show a survival benefit for the chemoradiotherapy-followed-by-surgery-arm and the reliability of the results in this study have been debated [6,39]. Complete pathologic response rate seems to be crucial for a beneficial effect of neoadjuvant chemoradiotherapy. A phase II study has recently been performed at the Erasmus MC, studying neoadjuvant paclitaxel and carboplatin in combination with radiotherapy prior to

surgery within 50 patients having resectable adenocarcinomas or squamous cell carcinomas of the esophagus. All patients received 100% of the planned dose of chemotherapy and radiotherapy with mild adverse effects. All but one patient had a radical surgical resection (R0-resection). Approximately 25% of patients had a complete pathological response. All but one had negative lymphnodes. The concept that this regimen of preoperative chemoradiotherapy improves the overall survival has, however, to be proven in a randomized phase III prospective trial, which is currently being performed in a multicenter setting.

Evidence is mounting that patients who do not respond to chemoradiotherapy may have worse survival due to delay of surgery. Molecular genetic analysis might be helpful in the identification of patients who are most likely to achieve a complete response before initiation of neoadjuvant chemoradiotherapy. Currently, GEJ adenocarcinoma cell lines as well as esophageal squamous cell carcinoma cell lines are being treated with chemoradiotherapy at the Department of Pathology to reveal genetic alterations that can predict therapy resistance/response.

SUSCEPTIBILITY

The identification of genes that confer susceptibility for adenocarcinoma formation in Barrett's esophagus would imply improved manageability of patients with Barrett's esophagus. However, familial cases of esophageal cancer are rare, and susceptibility genes are thus unlikely to be found by linkage analysis. Consequently, screening of candidate susceptibility genes may be a more feasible approach. A study of a large and unselected series of Barrett's metaplasias and dysplasias, esophageal adenocarcinomas and squamous cell carcinomas was performed in which the candidate susceptibility germline CHEK2*1100delC mutation was screened. This mutation had no major contribution in esophageal carcinogenesis. Genetic polymorphisms may play a significant role in person-to-person variability in cancer susceptibility. In this thesis the distribution of numerous Single Nucleotide Polymorphisms (SNPs) was screened in GEJ adenocarcinomas and squamous cell carcinomas and compared to a cohort of healthy blood donors. Interestingly, data confirm that SNPs influence risk for esophageal squamous cell carcinoma (p16 exon 2 C540G, Cyclin D1 exon 4 A870G and p73 exon 2 G4C14-A4T14) and for GEJ adenocarcinoma (p73 exon 2 G4C14-A4T14). SNPs might be used in the setting of surveillance of patients with Barrett's esophagus. Currently a large cohort of Barrett's esophagus patients, who did not develop cancer yet, is being analysed for p73 exon 2 G4C14-A4T14 genotype frequencies as well as for the other SNPs described in this study to reveal whether SNPs could be helpful to stratify surveillance.

FROM BARRETT'S ESOPHAGUS TOWARDS ADENOCARCINOMA: FROM LABORATORY TO CLINIC

At the Erasmus MC, patients with Barrett's metaplasia undergo surveillance endoscopy with tissue biopsies once in two years and patients with low grade dysplasia once a year. Importantly, high grade dysplasia harbors areas of invasive carcinoma in 30-60% already, which is a reason for surgical resection in many institutions. An alternative option for patients with high grade dysplasia forms intensive endoscopic follow-up (once in 3 months) until invasion is observed. Patients with high grade dysplasia or early adenocarcinoma have a survival rate of 80% after surgical resection as compared to 20% after surgery for invasive adenocarcinoma. This stresses the importance of early detection.

Interestingly, new endoscopic ablative techniques have emerged over the past two decades. These techniques have been developed primarily to treat dysplasia and early carcinoma. New techniques include endoscopic mucosal resection, photodynamic therapy, laser therapy, multipolar electrocoagulation, argon plasma coagulation, radiofrequency ablation and cryotherapy [40]. It has yet to be determined whether the risks associated with ablation therapy are less than the risk of Barrett's esophagus progressing to cancer. Whether ablation therapy eliminates or significantly reduces the risk of cancer is subject of currently performed comparative trials.

Endoscopic recognition of preneoplastic lesions might be difficult. The same holds for the histological classification of dyplasia. Significant intra- and interobserver variation exists in classifying dysplasia whereas the decision to treat or to follow-up largely depends on this diagnosis. New developments in both endoscopy technique and tissue sampling aim to improve diagnosis of high grade dysplasia and early cancer [41].

To date it is not possible to predict which patients with Barrett's esophagus will progress to invasive carcinoma. Stratification of patients with Barrett's esophagus who are at risk of esophageal adenocarcinoma and those who are not, is therefore urgently needed. This would allow more aggressive or preventative treatment of the people at risk, but also release the vast majority of people that are not at risk from years of unnecessary endoscopic surveillance.

Molecular genetic analysis might be helpful for two reasons:

1. The histological classification of dysplasia could be supplemented by the screening for genetic alterations to ensure accurate diagnosis. In this thesis areas of Barrett's metaplasia, low and high grade dysplasia and carcinoma were selected in H&E stained slides by a specialised GE pathologist. However, a major obstacle to apply molecular biologic techniques effectively to the genetic analysis of (pre)neoplastic tissue is the presence of abundant nonneoplastic elements in the analyzed specimen. These nonneoplastic elements, including reactive fibrous cells, vascular cells, and a variety of infiltrating white blood cells, may mask

genetic alterations that otherwise would be easily detectable if the (pre)neoplastic cells were procured selectively. In chapter 13 DNA from (pre)neoplastic areas was obtained after Laser Capture Microdissection technique. This technique allows to selectively procure and genetically analyze small populations of (pre)neoplastic cells from the glass slides. We compared the mean number of investigated molecular aberrations (i.e. LOH at p53 (17p), p16 (9p), Adenomatous Polyposis Coli (APC, 5q), Mutated in Colorectal Cancer (MCC, 5q), Retinoblastoma (Rb, 13q), Deleted in Colorectal Cancer (DCC, 18q), Deleted in Pancreatic Cancer (DPC4/SMAD4, 18q), mutation of the p53 gene, and mutation of mitochondrial DNA). The mean number was significantly lower in the combined group metaplasia/low grade dysplasia (3.0 ± 1.74) as compared to the combined group high grade dysplasia/carcinoma (5.0 ± 1.71, $p<0.001$). Screening for molecular aberrations might therefore indeed be valuable and add to histology within patients with difficulty to diagnose grade of dysplasia.

2. Endoscopic biopsy samples of Barrett's esophagus that histologically appear as low risk for neoplastic progression might in fact harbor genetic alterations that are indicative for a high risk profile (Figure 15.1). Therefore genetic alterations should perhaps also be investigated within evident metaplastic or low grade dysplastic lesions. A more intense surveillance should perhaps be offered to the patients harboring genetic alterations in their histologically unsuspected metaplasia or low grade dysplasia. It has been estimated that at least 1% of the Caucasian population may have Barrett's metaplasia and that from the patients with Barrett's esophagus 0.5-1% will develop adenocarcinoma [42]. It would be of great importance to be able to discriminate at an early stage which metaplasia- or low grade dysplasia patients will develop invasive disease and which will not.

Numerous genetic aberrations are known to frequently occur in esophageal adenocarcinoma. However, knowledge of the timing of these genetic events during esophageal adenocarcinogenesis is limited. In particular, few studies have analysed genetic aberrations in Barrett's epithelia that did not progress to esophageal adenocarcinoma. At the Erasmus MC, there is access to longitudinal esophageal biopsies from a cohort of at least 550 patients with Barrett's esophagus who underwent annual endoscopic surveillance since 1973. The cohort includes patients that have developed esophageal adenocarcinoma as well as patients that are still disease-free for more than 5 years. This collection of biopsies provides an unique opportunity to compare genetic aberrations in lesions from patients with Barrett's esophagus that are at risk of esophageal adenocarcinoma and those that are not at risk. Within a present study performed at the Departments of Pathology, Gastroenterology, Surgery and Epidemiology it is aimed to identify genetic markers that distinguish Barrett's epithelia that will progress to esophageal adenocarcinoma from those that will not progress to malignancy.

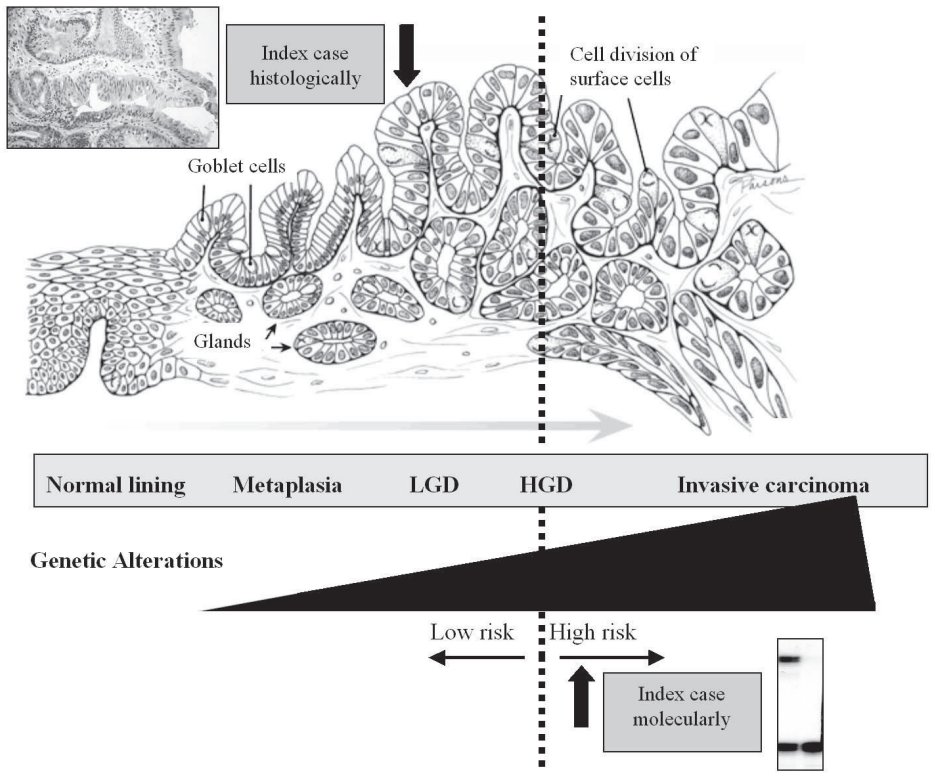


Figure 15.1 Metaplasia-Dysplasia-Carcinoma sequence. Index case is histologically at low risk for neoplastic progression, however genetic alterations are indicative for high risk profile. *Also see color figures page 291.*

FUTURE PERSPECTIVES

Targeted therapy

The sequence of the human genome has enabled systematic approaches to identify cancer genome alterations including point mutations, copy number increases and decreases, loss of allelic heterozygosity and chromosome translocations [43]. Molecular targets for therapy are becoming more widely known and include epidermal growth factors, tyrosine kinases, vascular endothelial growth factor and intracellular signaling pathways. With further development of targeted cancer therapies and improvement in genome analysis technology, genome-wide surveys of cancer will likely become tools for diagnosis as well as discovery [43-45]. Microarray analysis to study gene expression is able to rapidly identify genes which can function as a guide for tailored (neoadjuvant chemoradiation) therapy [46,47]. Examples of cancer-targeted therapy are the drugs Herceptin, used to treat c-erbB2 overexpressing breast cancer, Gleevec, used to treat a type of leukemia and certain rare cancers, and Gefitinib (Iressa), used to treat

lung cancers with EGFR mutations and probably other epithelial cancers with similar mutations in the near future. Intervention trials are underway in which neoadjuvant chemoradiotherapy is combined with EGFR-inhibitor Iressa prior to surgery for resectable esophageal cancer [48]. The concept of a cancer stem cell population as described in the literature gives hope that the isolation of these cells, coupled with the knowledge of the mutations causing cancer, will result in ways to eliminate cancer cells while sparing normal tissues [49]. In search for the Achilles' heel for cancer, future targeted cancer therapies will become available, although the pace at which molecular developments have been translated in clinical applications have been slow to date.

Molecular markers

Concerning Barrett's metaplasia and its malignant potential there is still need for improved understanding of the molecular biology in order to develop a more scientific approach to cancer prevention in these patients. Biopsy samples from patients with Barrett's esophagus might histologically appear as low risk for neoplastic progression whereas genetic alterations might be indicative for a high risk profile (Figure 15.1). Since the annual incidence rate for developing esophageal adenocarcinoma in Barrett's esophagus is as low as 0.5-1%, a cancer preventive strategy needs to be cost effective and acceptable to a large number of patients at relatively low risk [50]. It has been propagated that endoscopic cancer surveillance should therefore be limited to patients with increased cancer risk (for example Caucasian men, 55-70 years age, people with GERD) [51]. Perhaps it may someday be possible to identify a molecular marker that identifies GERD patients at high risk for Barrett's esophagus who might benefit from endoscopic surveillance [52]. Despite ongoing efforts to characterize the molecular changes leading to esophageal adenocarcinoma, no clinically applicable molecular markers for prediction of prognosis and/or response to therapy came forward to date. Several studies performed and described in this thesis intended to identify genetic alterations in GEJ adenocarcinogenesis and to develop molecular markers applicable in Barrett's patients. Unfortunately many patients with GEJ adenocarcinoma present with advanced stage of disease since they were not known to harbor Barrett's esophagus. Having realized this, one of the other main concerns in the finding of molecular markers forms the suspected existence of intralesional heterogeneity of genetic alterations in Barrett's epithelium. In the literature, FISH analysis, LOH and gene amplification analysis showed intratumoral heterogeneity in Barrett's adenocarcinomas [53,54]. This highlights one of the main problems associated with surveillance of Barrett's patients namely sampling error. From other studies it seemed likely that the acquisition of 9p LOH and 17p LOH predisposes to the evolution of aneuploid cell populations and other genetic abnormalities that culminate in the development of cancer [55-57]. Tumors may be monoclonal in origin and share, common, early genetic abnormalities predisposing to the evolution of clones with accumulated, different, late genetic abnormalities [58,59]. Several studies on GEJ adenocarcinomas with or without metaplastic areas and studies on high

grade dysplasia without carcinoma indeed show evidence for a monoclonal origin of these lesions by use of comparative genomic hybridisation (CGH), LOH analysis, mutation analysis, X-chromosome inactivation analysis as well as methylation analysis [54,56,60-65].

This issue of clonality of genetic alterations was investigated in this thesis. We presented molecular evidence that GEJ adenocarcinomas are monoclonal in orgin concerning early genetic alterations, i.e. especially p53 mutations, 17p LOH, 9p LOH, 13q LOH and 18q LOH. Mitochondrial DNA mutations have been suggested to be indicators of clonal expansion [66,67] and were also analyzed but they seemed to have limited value as clonality marker in GEJ adenocarcinomas. Most likely, a *monoclonal* tumor concerning *early genetic alterations* harbors *clonal diversity* concerning *later genetic alterations*. Such early genetic events in the neoplastic progression of Barrett's metaplasia concern loss of p16 and p53 but also loss of APC, Rb and DCC with aneuploidy as a probable driving force; additional genetic alterations superpose. Since this thesis provided evidence for monoclonality concerning early genetic alterations, molecular markers potentially can be applied in conjunction with histopathology to identify Barrett's patients at risk for neoplastic progression.

Most probably no single 'universal' genetic marker is sufficient to enable prediction of which patient will and which patient will not develop cancer in the setting of Barrett's esophagus. Thus a 'multimarker' approach seems more promising than a 'single marker' approach. A combination of the investigated markers, especially p53 mutations, 17p LOH, 9p LOH, 13q LOH and 18q LOH might be part of a future panel of markers, in conjunction with histopathological diagnosis. Early genetic alterations should be given highest priority in the ongoing search for molecular markers both for diagnostic and prognostic use. This will hopefully lead to a further differentiation in the prediction of neoplastic risk in Barrett's esophagus patients with early intervention and individualized treatment as ultimate goal.

REFERENCES

1. Bian YS, Osterheld MC, Bosman FT, Fontolliet C, Benhattar J: Nuclear accumulation of beta-catenin is a common and early event during neoplastic progression of Barrett esophagus. Am J Clin Pathol 114:583-90, 2000
2. Choi YW, Heath EI, Heitmiller R, Forastiere AA, Wu TT: Mutations in beta-catenin and APC genes are uncommon in esophageal and esophagogastric junction adenocarcinomas. Mod Pathol 13:1055-9, 2000
3. Gonzalez MV, Artimez ML, Rodrigo L, Lopez-Larrea C, Menendez MJ, Alvarez V, Perez R, Fresno MF, Perez MJ, Sampedro A, Coto E: Mutation analysis of the p53, APC, and p16 genes in the Barrett's oesophagus, dysplasia, and adenocarcinoma. J Clin Pathol 50:212-7, 1997
4. Wijnhoven BP, Nollet F, De Both NJ, Tilanus HW, Dinjens WN: Genetic alterations involving exon 3 of the beta-catenin gene do not play a role in adenocarcinomas of the esophagus. Int J Cancer 86:533-7, 2000
5. Colombo B, Curnis F, Foglieni C, Monno A, Arrigoni G, Corti A: Chromogranin A expression in neoplastic cells affects tumor growth and morphogenesis in mouse models. Cancer Res 62:941-6, 2002
6. DeMeester SR: Adenocarcinoma of the esophagus and cardia: a review of the disease and its treatment. Ann Surg Oncol 13:12-30, 2006
7. Ireland AP, Shibata DK, Chandrasoma P, Lord RV, Peters JH, DeMeester TR: Clinical significance of p53 mutations in adenocarcinoma of the esophagus and cardia. Ann Surg 231:179-87, 2000
8. Lord RV, Tsai PI, Danenberg KD, Peters JH, Demeester TR, Tsao-Wei DD, Groshen S, Salonga D, Park JM, Crookes PF, Kiyabu M, Chandrasoma P, Danenberg PV: Retinoic acid receptor-alpha messenger RNA expression is increased and retinoic acid receptor-gamma expression is decreased in Barrett's intestinal metaplasia, dysplasia, adenocarcinoma sequence. Surgery 129:267-76, 2001
9. Mendes de Almeida JC, Chaves P, Pereira AD, Altorki NK: Is Barrett's esophagus the precursor of most adenocarcinomas of the esophagus and cardia? A biochemical study. Ann Surg 226:725-33; discussion 733-5, 1997
10. Ruol A, Parenti A, Zaninotto G, Merigliano S, Costantini M, Cagol M, Alfieri R, Bonavina L, Peracchia A, Ancona E: Intestinal metaplasia is the probable common precursor of adenocarcinoma in barrett esophagus and adenocarcinoma of the gastric cardia. Cancer 88:2520-8, 2000
11. Sarbia M, Geddert H, Klump B, Kiel S, Iskender E, Gabbert HE: Hypermethylation of tumor suppressor genes (p16INK4A, p14ARF and APC) in adenocarcinomas of the upper gastrointestinal tract. Int J Cancer 111:224-8, 2004
12. Dolan K, Sutton R, Walker SJ, Morris AI, Campbell F, Williams EM: New classification of oesophageal and gastric carcinomas derived from changing patterns in epidemiology. Br J Cancer 80:834-42, 1999
13. Nigro JJ, DeMeester SR, Hagen JA, DeMeester TR, Peters JH, Kiyabu M, Campos GM, Oberg S, Gastal O, Crookes PF, Bremner CG: Node status in transmural esophageal adenocarcinoma and outcome after en bloc esophagectomy. J Thorac Cardiovasc Surg 117:960-8, 1999
14. Wijnhoven BP, Siersema PD, Hop WC, van Dekken H, Tilanus HW: Adenocarcinomas of the distal oesophagus and gastric cardia are one clinical entity. Rotterdam Oesophageal Tumor Study Group. Br J Surg 86:529-35, 1999
15. Katariya K, Harvey JC, Pina E, Beattie EJ: Complications of transhiatal esophagectomy. J Surg Oncol 57:157-63, 1994
16. Tilanus HW, Hop WC, Langenhorst BL, van Lanschot JJ: Esophagectomy with or without thoracotomy. Is there any difference? J Thorac Cardiovasc Surg 105:898-903, 1993
17. Surgical resection with or without preoperative chemotherapy in oesophageal cancer: a randomised controlled trial. Lancet 359:1727-33, 2002
18. Hulscher JB, van Sandick JW, de Boer AG, Wijnhoven BP, Tijssen JG, Fockens P, Stalmeier PF, ten Kate FJ, van Dekken H, Obertop H, Tilanus HW, van Lanschot JJ: Extended transthoracic resection compared with limited transhiatal resection for adenocarcinoma of the esophagus. N Engl J Med 347:1662-9, 2002

19. Kelsen DP, Ginsberg R, Pajak TF, Sheahan DG, Gunderson L, Mortimer J, Estes N, Haller DG, Ajani J, Kocha W, Minsky BD, Roth JA: Chemotherapy followed by surgery compared with surgery alone for localized esophageal cancer. N Engl J Med 339:1979-84, 1998
20. Ancona E, Ruol A, Santi S, Merigliano S, Sileni VC, Koussis H, Zaninotto G, Bonavina L, Peracchia A: Only pathologic complete response to neoadjuvant chemotherapy improves significantly the long term survival of patients with resectable esophageal squamous cell carcinoma: final report of a randomized, controlled trial of preoperative chemotherapy versus surgery alone. Cancer 91:2165-74, 2001
21. Kok TC, Van der Gaast A, Dees J, Eykenboom WM, Van Overhagen H, Stoter G, Tilanus HW, Splinter TA: Cisplatin and etoposide in oesophageal cancer: a phase II study. Rotterdam Oesophageal Tumor Study Group. Br J Cancer 74:980-4, 1996
22. Law S, Fok M, Chow S, Chu KM, Wong J: Preoperative chemotherapy versus surgical therapy alone for squamous cell carcinoma of the esophagus: a prospective randomized trial. J Thorac Cardiovasc Surg 114:210-7, 1997
23. Maipang T, Vasinanukorn P, Petpichetchian C, Chamroonkul S, Geater A, Chansawwaang S, Kuapanich R, Panjapiyakul C, Watanaarepornchai S, Punperk S: Induction chemotherapy in the treatment of patients with carcinoma of the esophagus. J Surg Oncol 56:191-7, 1994
24. Nygaard K, Hagen S, Hansen HS, Hatlevoll R, Hultborn R, Jakobsen A, Mantyla M, Modig H, Munck-Wikland E, Rosengren B, et al.: Pre-operative radiotherapy prolongs survival in operable esophageal carcinoma: a randomized, multicenter study of pre-operative radiotherapy and chemotherapy. The second Scandinavian trial in esophageal cancer. World J Surg 16:1104-9; discussion 1110, 1992
25. Roth JA, Pass HI, Flanagan MM, Graeber GM, Rosenberg JC, Steinberg S: Randomized clinical trial of preoperative and postoperative adjuvant chemotherapy with cisplatin, vindesine, and bleomycin for carcinoma of the esophagus. J Thorac Cardiovasc Surg 96:242-8, 1988
26. Schlag PM: Randomized trial of preoperative chemotherapy for squamous cell cancer of the esophagus. The Chirurgische Arbeitsgemeinschaft Fuer Onkologie der Deutschen Gesellschaft Fuer Chirurgie Study Group. Arch Surg 127:1446-50, 1992
27. Hatlevoll R, Hagen S, Hansen HS, Hultborn R, Jakobsen A, Mantyla M, Modig H, Munck-Wikland E, Nygaard K, Rosengren B, et al.: Bleomycin/cis-platin as neoadjuvant chemotherapy before radical radiotherapy in localized, inoperable carcinoma of the esophagus. A prospective randomized multicentre study: the second Scandinavian trial in esophageal cancer. Radiother Oncol 24:114-6, 1992
28. Roussel A, Bleiberg H, Dalesio O, Jacob JH, Haegele P, Jung GM, Paillot B, Heintz JF, Gignoux M, Nasca S, et al.: Palliative therapy of inoperable oesophageal carcinoma with radiotherapy and methotrexate: final results of a controlled clinical trial. Int J Radiat Oncol Biol Phys 16:67-72, 1989
29. Zhou JC: [Randomized trial of combined chemotherapy including high dose cisplatin and radiotherapy for esophageal cancer]. Zhonghua Zhong Liu Za Zhi 13:291-4, 1991
30. Herskovic A, Martz K, al-Sarraf M, Leichman L, Brindle J, Vaitkevicius V, Cooper J, Byhardt R, Davis L, Emami B: Combined chemotherapy and radiotherapy compared with radiotherapy alone in patients with cancer of the esophagus. N Engl J Med 326:1593-8, 1992
31. Schaake-Koning C, van den Bogaert W, Dalesio O, Festen J, Hoogenhout J, van Houtte P, Kirkpatrick A, Koolen M, Maat B, Nijs A, et al.: Effects of concomitant cisplatin and radiotherapy on inoperable non-small-cell lung cancer. N Engl J Med 326:524-30, 1992
32. Hennequin C, Favaudon V: Biological basis for chemo-radiotherapy interactions. Eur J Cancer 38:223-30, 2002
33. Tannock IF: Treatment of cancer with radiation and drugs. J Clin Oncol 14:3156-74, 1996
34. Apinop C, Puttisak P, Preecha N: A prospective study of combined therapy in esophageal cancer. Hepatogastroenterology 41:391-3, 1994
35. Bosset JF, Gignoux M, Triboulet JP, Tiret E, Mantion G, Elias D, Lozach P, Ollier JC, Pavy JJ, Mercier M, Sahmoud T: Chemoradiotherapy followed by surgery compared with surgery alone in squamous-cell cancer of the esophagus. N Engl J Med 337:161-7, 1997
36. Burmeister BH, Smithers BM, Gebski V, Fitzgerald L, Simes RJ, Devitt P, Ackland S, Gotley DC, Joseph D, Millar J, North J, Walpole ET, Denham JW: Surgery alone versus chemoradiotherapy

followed by surgery for resectable cancer of the oesophagus: a randomised controlled phase III trial. Lancet Oncol 6:659-68, 2005
37. Le Prise E, Etienne PL, Meunier B, Maddern G, Ben Hassel M, Gedouin D, Boutin D, Campion JP, Launois B: A randomized study of chemotherapy, radiation therapy, and surgery versus surgery for localized squamous cell carcinoma of the esophagus. Cancer 73:1779-84, 1994
38. Urba SG, Orringer MB, Turrisi A, Iannettoni M, Forastiere A, Strawderman M: Randomized trial of preoperative chemoradiation versus surgery alone in patients with locoregional esophageal carcinoma. J Clin Oncol 19:305-13, 2001
39. Walsh TN, Noonan N, Hollywood D, Kelly A, Keeling N, Hennessy TP: A comparison of multimodal therapy and surgery for esophageal adenocarcinoma. N Engl J Med 335:462-7, 1996
40. Johnston MH: Technology insight: ablative techniques for Barrett's esophagus--current and emerging trends. Nat Clin Pract Oncol 2:323-30, 2005
41. Kuipers EJ, Haringsma J: Diagnostic and therapeutic endoscopy. J Surg Oncol 92:203-9, 2005
42. Tytgat GN: Does endoscopic surveillance in esophageal columnar metaplasia (Barrett's esophagus) have any real value? Endoscopy 27:19-26, 1995
43. Weir B, Zhao X, Meyerson M: Somatic alterations in the human cancer genome. Cancer Cell 6:433-8, 2004
44. Brabender J, Marjoram P, Salonga D, Metzger R, Schneider PM, Park JM, Schneider S, Holscher AH, Yin J, Meltzer SJ, Danenberg KD, Danenberg PV, Lord RV: A multigene expression panel for the molecular diagnosis of Barrett's esophagus and Barrett's adenocarcinoma of the esophagus. Oncogene 23:4780-8, 2004
45. Miller CT, Moy JR, Lin L, Schipper M, Normolle D, Brenner DE, Iannettoni MD, Orringer MB, Beer DG: Gene amplification in esophageal adenocarcinomas and Barrett's with high-grade dysplasia. Clin Cancer Res 9:4819-25, 2003
46. Dahlberg PS, Ferrin LF, Grindle SM, Nelson CM, Hoang CD, Jacobson B: Gene expression profiles in esophageal adenocarcinoma. Ann Thorac Surg 77:1008-15, 2004
47. Hourihan RN, O'Sullivan GC, Morgan JG: Transcriptional gene expression profiles of oesophageal adenocarcinoma and normal oesophageal tissues. Anticancer Res 23:161-5, 2003
48. Gibson MK, Abraham SC, Wu TT, Burtness B, Heitmiller RF, Heath E, Forastiere A: Epidermal growth factor receptor, p53 mutation, and pathological response predict survival in patients with locally advanced esophageal cancer treated with preoperative chemoradiotherapy. Clin Cancer Res 9:6461-8, 2003
49. Clarke MF: Neurobiology: at the root of brain cancer. Nature 432:281-2, 2004
50. Fitzgerald RC: Ablative mucosectomy is the procedure of choice to prevent Barrett's cancer. Gut 52:16-7, 2003
51. Gudlaugsdottir S, van Blankenstein M, Dees J, Wilson JH: A majority of patients with Barrett's oesophagus are unlikely to benefit from endoscopic cancer surveillance. Eur J Gastroenterol Hepatol 13:639-45, 2001
52. Souza RF, Spechler SJ: Concepts in the prevention of adenocarcinoma of the distal esophagus and proximal stomach. CA Cancer J Clin 55:334-51, 2005
53. Walch AK, Zitzelsberger HF, Bruch J, Keller G, Angermeier D, Aubele MM, Mueller J, Stein H, Braselmann H, Siewert JR, Hofler H, Werner M: Chromosomal imbalances in Barrett's adenocarcinoma and the metaplasia-dysplasia-carcinoma sequence. Am J Pathol 156:555-66, 2000
54. Owonikoko T, Rees M, Gabbert HE, Sarbia M: Intratumoral genetic heterogeneity in Barrett adenocarcinoma. Am J Clin Pathol 117:558-66, 2002
55. Galipeau PC, Prevo LJ, Sanchez CA, Longton GM, Reid BJ: Clonal expansion and loss of heterozygosity at chromosomes 9p and 17p in premalignant esophageal (Barrett's) tissue. J Natl Cancer Inst 91:2087-95, 1999
56. Prevo LJ, Sanchez CA, Galipeau PC, Reid BJ: p53-mutant clones and field effects in Barrett's esophagus. Cancer Res 59:4784-7, 1999
57. Barrett MT, Sanchez CA, Prevo LJ, Wong DJ, Galipeau PC, Paulson TG, Rabinovitch PS, Reid BJ: Evolution of neoplastic cell lineages in Barrett oesophagus. Nat Genet 22:106-9, 1999
58. Anderson GR, Stoler DL, Brenner BM: Cancer: the evolved consequence of a destabilized genome. Bioessays 23:1037-46, 2001
59. Nowell PC: The clonal evolution of tumor cell populations. Science 194:23-8, 1976

60. Blount PL, Meltzer SJ, Yin J, Huang Y, Krasna MJ, Reid BJ: Clonal ordering of 17p and 5q allelic losses in Barrett dysplasia and adenocarcinoma. Proc Natl Acad Sci U S A 90:3221-5, 1993
61. Eads CA, Lord RV, Kurumboor SK, Wickramasinghe K, Skinner ML, Long TI, Peters JH, DeMeester TR, Danenberg KD, Danenberg PV, Laird PW, Skinner KA: Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma. Cancer Res 60:5021-6, 2000
62. Neshat K, Sanchez CA, Galipeau PC, Blount PL, Levine DS, Joslyn G, Reid BJ: p53 mutations in Barrett's adenocarcinoma and high-grade dysplasia. Gastroenterology 106:1589-95, 1994
63. Romagnoli S, Roncalli M, Graziani D, Cassani B, Roz E, Bonavina L, Peracchia A, Bosari S, Coggi G: Molecular alterations of Barrett's esophagus on microdissected endoscopic biopsies. Lab Invest 81:241-7, 2001
64. van Dekken H, Vissers CJ, Tilanus HW, Tanke HJ, Rosenberg C: Clonal analysis of a case of multifocal oesophageal (Barrett's) adenocarcinoma by comparative genomic hybridization. J Pathol 188:263-6, 1999
65. Zhuang Z, Vortmeyer AO, Mark EJ, Odze R, Emmert-Buck MR, Merino MJ, Moon H, Liotta LA, Duray PH: Barrett's esophagus: metaplastic cells with loss of heterozygosity at the APC gene locus are clonal precursors to invasive adenocarcinoma. Cancer Res 56:1961-4, 1996
66. Jakupciak JP, Wang W, Markowitz ME, Ally D, Coble M, Srivastava S, Maitra A, Barker PE, Sidransky D, O'Connell CD: Mitochondrial DNA as a cancer biomarker. J Mol Diagn 7:258-67, 2005
67. Miyazono F, Schneider PM, Metzger R, Warnecke-Eberz U, Baldus SE, Dienes HP, Aikou T, Hoelscher AH: Mutations in the mitochondrial DNA D-Loop region occur frequently in adenocarcinoma in Barrett's esophagus. Oncogene 21:3780-3, 2002

Appendices

Contributing authors
Acknowledgments / Dankwoord
List of publications
Color figures

CONTRIBUTING AUTHORS

Prof.dr. HW Tilanus
Department of Surgery
Erasmus Medical Center
Rotterdam, The Netherlands

Dr. WNM Dinjens
Department of Pathology
Erasmus Medical Center
Rotterdam, The Netherlands

Prof.dr. TL Lerut
Department of Surgery
University Hospital Gasthuisberg
Leuven, Belgium

Dr BPL Wijnhoven
Department of Surgery
Royal Adelaide Hospital
Adelaide, Australia

Dr. H van Dekken
Department of Pathology
Erasmus Medical Center
Rotterdam, The Netherlands

Dr. M van Noort
Department of Urology
University Medical Center
Utrecht, The Netherlands

Dr. M van de Wetering
Hubrecht Laboratory, Centre for Biomedical Genetics
Utrecht, The Netherlands

Dr. AMW van den Ouweland
Department of Clinical Genetics
Erasmus Medical Center
Rotterdam, The Netherlands

Prof.dr. T. Stijnen
Dept. of Epidemiology and Biostatistics
Erasmus Medical Center
Rotterdam, The Netherlands

Dr. MLG Janssen-Heijnen, Drs. VEPP Lemmens, Dr. MWJ Louwman
Eindhoven Cancer Registry, Comprehensive Cancer Centre South (IKZ)
Eindhoven, The Netherlands

Prof.dr. JWW Coebergh
Eindhoven Cancer Registry, Comprehensive Cancer Centre South (IKZ)
Eindhoven, The Netherlands
And
Department of Public Health
Erasmus Medical Center
Rotterdam, The Netherlands

Dr. M Westerterp, Dr. CJ Buskens
Department of Surgery
Academic Medical Center
Amsterdam, The Netherlands

Prof.dr. FJW ten Kate
Department of Pathology
Academic Medical Center
Amsterdam, The Netherlands

Dr. JJHGM Bergman
Department of Gastroenterology
Academic Medical Center
Amsterdam, The Netherlands

Dr. PD Siersema
Dept. of Gastroenterology and Hepatology
Erasmus Medical Center
Rotterdam, The Netherlands

Prof.dr. JJB van Lanschot
Department of Surgery
Academic Medical Center
Amsterdam, The Netherlands

Dr. EW Steyerberg
Department of Public Health
Erasmus Medical Center
Rotterdam, The Netherlands

Prof.dr. CC Early, Dr. BA Neville, Dr. JC Weeks
Department of Medical Oncology
Dana-Farber Cancer Institute
Boston, USA

Dr. M Schutte
Department of Medical Oncology
Erasmus Medical Center
Rotterdam, The Netherlands

Dr. HFGM van den Ingh
Department of Pathology
Medisch Centrum Rijnmond Zuid
Rotterdam, The Netherlands

Dr. C van Krimpen
Department of Pathology
Reinier de Graaf Gasthuis
Delft, The Netherlands

Dr. A Driessen
Department of Pathology
Academic Medical Center
Maastricht, The Netherlands

Dr. AG Uitterlinden
Department of Internal Medicine
Erasmus Medical Center
Rotterdam, The Netherlands

AW van der Velden, M Abbou, DA Dezentjé, ES van Ballegooien, HFBM Sleddens, J Vermeulen, Drs. HWPC van de Meerendonk, MMCP Hoogmans, ECW Beerens
Department of Pathology
Erasmus Medical Center
Rotterdam, The Netherlands

ACKNOWLEDGMENTS/ DANKWOORD

Graag besluit ik mijn proefschrift met het bedanken van de mensen zonder wiens hulp en betrokkenheid mijn promotie niet tot stand was gekomen. Ik streef niet naar volledigheid, mijn dank gaat uit naar iedereen met wie ik op enig moment mocht samenwerken. Een aantal mensen wil ik echter bij name noemen.

Beste Winand Dinjens, "Chief", niemand kan een vriendelijker en kundiger copromotor hebben getroffen dan ik. Voor de manier waarop je in het leven staat, samen met Wil, heb ik diep respect. Het was en is een groot voorrecht met je te mogen samenwerken.
Beste Professor Tilanus, zeer gewaardeerde eerste promotor, als student kreeg ik de kans het studententeam Levertransplantaties te bemannen. De momenten, vaak in het holst van de nacht, waarop ik achter of naast u aan tafel mocht staan, maakten me enthousiast voor de chirurgie. U speelde de hoofdrol in het vormgeven van mijn wetenschappelijke ambities en u was niet teleurgesteld toen ik de mogelijkheid bij u op het leverlab te promoveren afsloeg… de oncologische chirurgie -prachtig!-, dáár had u ook nog een plaats vrij! Mijn dank is groot. Mag ik binnenkort weer van u leren aan tafel?
Geachte Professor Oosterhuis, tweede promotor, dank voor het onderdak dat u me bood op het Laboratorium Moleculaire Diagnostiek in het Josephine Nefkens Instituut. Ik vind het een eer bij u te promoveren en dank u voor de bereidheid mijn tweede promotor te willen zijn.

De kleine commissie
Dr. van Dekken, beste Herman, als coauteur op een aantal van mijn stukken gaf je me immer je eerlijke, kritische commentaar waarvoor veel dank. Het lijkt me leuk in de nabije toekomst casusgericht verder te discussiëren hóe moleculaire kennis te implementeren in de kliniek. Dank voor je zitting in de kleine commissie.
Hartelijk dank Professor Coebergh, beste Jan-Willem. Een voorrecht om met je te hebben mogen samenwerken en gebruik te mogen maken van de waardevolle schat aan data van de Kankerregistratie Eindhoven/IKZ. Aanstekelijk enthousiasme van jou en je team maakt hongerig naar epidemiologische antwoorden op klinische vraagstukken. Chirurgen kunnen niet zo goed registeren en jouw tijd is kostbaar…, maar de discussie gaat misschien toch verder?
Professor Kuipers, beste Ernst, dank voor je bereidheid plaats te nemen in de kleine commissie. Het inzetten van moleculaire kennis in de kliniek maakt nu al deel uit van je dagelijks werk en de toekomst wordt volgens mij des te interessanter. Leuk dat je er de 16e bij wilt zijn!

De grote commissie
Beste Professor Obertop, dank voor uw bereidheid plaats te nemen achter de tafel als Rotterdams lid van de grote commissie.

Dank Professor van Lanschot uit het AMC Amsterdam voor de prettige samenwerking met uw onderzoeksgroep (ik noem met name Marinke Westerterp en Christianne Buskens) en de mogelijkheid een grote serie materiaal te gebruiken voor de validatie van onze onderzoeksresultaten.
Geachte Professor Lerut, Universiteitsziekenhuis Gasthuisberg te Leuven, na met u een boekhoofdstuk te hebben geschreven bent u nu commissielid op mijn promotie. Hartelijk dank hiervoor en voor uw bereidheid naar Rotterdam te komen. Ik vind het een eer dat u wilt plaatsnemen in de commissie.
Dr. Mieke Schutte, wetenschapper Interne Oncologie pur sang, het was fantastisch het CHEK2 artikel met je te mogen schrijven. Ik ben onder de indruk van de manier waarop je wetenschap bedrijft en bediscussieert (en brieven schrijft!). Dank dat je als deskundige wilt opponeren.
Dr. Peter Siersema van de MDL, ik heb je leren kennen als zeer gedreven wetenschapper. Dank dat je zitting neemt in de kleine commissie!
Dr. Laurents Stassen, zeer gewaardeerde opleider te Delft, op 16 juni verruilen we de meester-gezel relatie eventjes voor die van opponent-promovendus, maar wel met een tafel ertussenin. Hartelijk dank voor je aanwezigheid.

Beste Bas Wijnhoven, eigenlijk baande jij voor dit proefschrift en daarmee voor mij de weg. Ik had het me niet beter kunnen wensen. Dank voor je betrokkenheid en de manier waarop je zowel in het hanteren van de pipet als van het mes voor mij een voorbeeld bent.

Mijn collega's van het Laboratorium Moleculaire Diagnostiek wil ik bedanken voor de gezellige samenwerking en opperbeste werksfeer. Jullie leerden me de verschillende technieken en wijdden me in in jullie jargon… Dank jullie wel Mustaffa Abbou (inmiddels commercieelga d'r voor Mus!), Albertina van der Velden (volg je hart lief mens), Monique Hoogmans (d'r komt weer een feessie aan…!), Hein Sleddens (gezelliger kan bijna niet), Erwin Beerens (wel ff checken of het in evenwicht is), Peter Riegman & Monique Oomen (tissue is the issue), Alex Nigg (mogen we flèèèètscreens Ááál ââh?), Frank van der Panne (kwaliteit is de naam), Ludo Uitdenwilligen (turbosnijder).
Tot ziens lieve collega's van het Josephine Nefkens Instituut (verdieping 2, 3 & 4), allemaal hartelijk bedankt voor de prettige samenwerking.
Op het lab heb ik veel mogen werken met stagiaires. Hartelijk dank voor jullie inzet Henk van de Meerendonk, Laura Piket, Taco van der Wel, Sita Ramcharan, Anke van Bruggen, Petra Lodder, Jolanda van Velzen, Laura Willemsteijn en Esther Hasper.

Dank aan de onderzoekers en het registratieteam van de Eindhoven Kankerregistratie/ IKZ. Beste Valery Lemmens, dank voor de zeer prettige samenwerking, op jou kon ik altijd bouwen. Ik wens je een succesvolle toekomst en een geweldige promotie! Beste Maryska Janssen-Heijnen, dank voor je intensieve begeleiding en je hartelijkheid. Bedankt voor de

prettige samenwerking Marieke Louwman. Conny Vollebregt wil ik hartelijk bedanken voor het datamanagement en het gezellige contact. Ewout Steyerberg, dankjewel voor je inzet op vele fronten! Jouw enthousiasme voor je vak en je werklust zijn aanstekelijk. Heel graag werk ik in de toekomst nog eens samen met je aan een project. Beste Khe Tran, n= heel veel buismagen inmiddels. Tussendoor misschien tijd voor nog een slokdarmcongres? Beste Marc van de Wetering, hartelijk dank voor je hulp bij de siRNA experimenten in de cellijnen. Ik voelde me erg welkom op het Utrechtse lab. Helaas waren we met de opzet van ons experiment uiteindelijk niet succesvol.

Ate van der Gaast, dank voor de prettige samenwerking bij het opzetten van de fase III trial. Lieve Marijke Smits-Schouten, dank voor al je hulp en gezelligheid!

Mijn interesse voor wetenschappelijk onderzoek kwam naar voren in de periode binnen het studententeam Levertransplantaties. Degenen die daar een rol bij speelden wil ik in dit dankwoord noemen: Gerda Bouma, Michiel Warlé, Pieter Zondervan (en Monica Seijbel, gezellig dat je inmiddels op de afdeling Heelkunde werkt!), Jaap Kwekkeboom, Herold Metselaar, Jan IJzermans, Geert Kazemier, Hans Brouwer, Rob de Man en Jeroen de Jonge.

Een aantal van de artikelen uit dit proefschrift heb ik geschreven tijdens mijn opleidingstijd in Delft. Van verschillende kanten werd regelmatig interesse getoond en- niet te vergeten- de 'herkenning' gedeeld. Bedankt voor de support en de prettige werksfeer chirurgen en collega arts-assistenten, verpleegkundigen van afdelingen 4.I GE, 4.I Vaat, en 4.II Trauma, collega's van de IC, de OK, de poli en de SEH en secretaresses Heelkunde.

Vanuit mijn vriendenkring was er de afgelopen tijd veel begrip voor mijn drukke agenda. Daar komt dus binnenkort verandering in… in de agenda bedoel ik. Dank voor al jullie belangstelling, support en bemoediging. Lieve Jan & Léanne, pappa en mamma van Sven, Roos en kleine Tess, bedankt voor jullie vriendschap, wat mij betreft 'for life'! Lieve Hester, praten over het leven is met jou zo leuk! Enneke, jij verstaat de kunst van vriendschap als geen ander. Stoere vriendin Marijke de Jong in Kaapstad, ik ben trots op je! Karo & Corné, pappa en mama van Roos en Stijn, Oud-Beijerland is inderdaad dichtbij, tot snel. Lieve Annemieke (& Marcel), dank voor warme vriendschap, … ooit samen aan tafel? Kinderdok Marijke (& Martin), binnenkort jij?! Andréa (& Michel), lieve chica, dank voor je meeleven... en niet alleen met de promotieperikelen! Beste Bart, komt mijn boek in 1 van jouw boekenkasten? Miriam & Erik, pappa en mamma van Sebastiaan en Olivier, dank voor jullie betrokkenheid en vriendschap. Doctor Hilde, je ging me voor maar we zijn tegelijk klaar met de opleiding.. zelfde maatschap misschien?.. Ik zeg <ja!>. Lieve Vera, jij leeft met mij mee, en ik met jou, zet hem op! Bedankt voor jullie warme betrokkenheid Ingrid & Michiel (Donna, Bo en Jim), Hans & Frieda, Merel, Jelle & Joke, Annemien, Ankie Meyran, Jannie & Leen Tanis, Olaf Schouten.

Bedankt lieve ouders van Niels, Thijs en Betty Kok voor jullie gastvrijheid en niet aflatende support in alle betekenissen van het woord. Bedankt Diederik, Eline en Roelof voor het toevoegen van die nieuwe dimensie: ADHD! Tijd voor nog een feestje?

Hartstikke trots ben ik op mijn paranimfen Madelon & Sabine. Lieve roommie, wat hadden we het gezellig op kamer Be332. Ruim tweeëneenhalf jaar lang gingen we dagelijks met elkaar om, ik mis het. Met jou als close friend is het leven aangenaam, dank voor wie je bent. Lieve sis, ondanks je drukke leventje ben je er altijd voor mij. Top dat ook jij naast me wilt staan op de 16e!

Sabine en Marjolein, zusjes, weer een hoogtepunt in ons leven, na jullie bruiloften nu mijn promotie. Wat een bijzondere tijd. Gelukkig weten we alledrie wie we mogen bedanken!... En dat geeft een hele bijzondere band. Dat we nog maar veel mooie momenten mogen beleven, samen met pap en mam én de mannen. Lieve Pim en Han, dank voor mannelijke gezelligheid in gezin Koppert én voor het feit dat jullie mijn zusjes gelukkig maken!

Lieve Niels, dank voor jouw liefde voor mij. Je hebt me ontzettend gesteund en bovenal aangemoedigd te genieten van deze unieke tijd. Jouw tomeloze energie is aanstekelijk. Ik heb er alle vertrouwen in dat jij binnenkort ook promoveert en zal je steunen waar ik kan. Op een mooie toekomst samen, ik hou van jou!

Lieve pappa en mamma, jullie hebben me geleerd te geloven, te hopen en lief te hebben. Jullie onvoorwaardelijke steun en liefde zijn overweldigend. Ik ben ontzettend trots op je pap, op de manier waarop je in het leven staat en dat ook volop uit in je vak. Jij bent mijn voorbeeld. Lieve mam, je bent hartsvriendin en moeder tegelijk… en tegenwoordig ook m'n golfmaatje! Ik ben jullie innig dankbaar. Mijn proefschrift draag ik dan ook aan jullie op.

Dank U Vader God, bron van levend water, dank voor wie U bent voor mij.

Zegen op jullie wegen,
Linetta

LIST OF PUBLICATIONS

Related to this thesis

Frequent loss of the AXIN1 locus but absence of AXIN1 gene mutations in adenocarcinomas of the gastro-oesophageal junction with nuclear ß-catenin expression.
LB Koppert, A van der Velden, M van de Wetering, M Abbou, A van den Ouweland, HW Tilanus, BPL Wijnhoven, WNM Dinjens, British Journal of Cancer, 2004;90(4):892-899

Neuroendocrine cells in Barrett's mucosa and adenocarcinomas of the gastroesophageal junction.
LB Koppert, BPL Wijnhoven, HW Tilanus, H van Dekken, WNM Dinjens, International Journal of Surgical Pathology, 2004;12(2):117-125

The CHEK2*1100delC mutation has no major contribution in oesophageal carcinogenesis.
LB Koppert, M Schutte, M Abbou, HW Tilanus, WNM Dinjens, British Journal of Cancer, 2004;90(4):888-891

Comparison of comorbidity prevalence in oesophageal and gastric carcinoma patients: a population-based study.
LB Koppert, MLG Janssen-Heijnen, MWJ Louwman, VEPP Lemmens, BPL Wijnhoven, HW Tilanus, JWW Coebergh, European Journal of Gastroenterology and Hepatology, 2004;16(7):681-688

Outcome of surgical treatment of early oesophageal cancer.
M Westerterp, LB Koppert, CJ Buskens, FJ ten Kate, HW Tilanus, H van Dekken, JJ van Lanschot, Virchows Archives, 2005;446(5):497-504

The molecular biology of esophageal adenocarcinoma.
LB Koppert, BPL Wijnhoven, H van Dekken, HW Tilanus, WNM Dinjens, Journal of Surgical Oncology, 2005;92(3):169-190

Identification of a 7.1 mega base pairs minimal deletion at 14q31.1-32.11 in adenocarcinomas of the gastro-esophageal junction**.**
WNM Dinjens, LB Koppert, DA Dezentjé, M Abbou, ES van Ballegooijen, HFBM Sleddens, H van Dekken, HW Tilanus, BPL Wijnhoven, Human Pathology, 2006;37(5):534-41

Genomic analysis of early adenocarcinoma of the esophagus or gastroesophageal junction: Tumor progression is associated with alteration of 1q and 8p sequences. H van Dekken, JC

Wink, KJ Vissers, R van Marion, LB Koppert, HW Tilanus, PD Siersema, HJ Tanke, K Szuhai, WC Hop. Genes Chromosomes Cancer, 2006;45(5):516-25

Surgical mortality in patients with esophageal cancer: development and validation of a simple risk score. EW Steyerberg, BA Neville, LB Koppert, VEPP Lemmens, HW Tilanus, JWW Coesbergh, JC Weeks, CC Early. Journal of Clinical Oncology, in press

Other publications

Regarding "3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors reduce the risk of perioperative stroke and mortality after carotid endarterectomy".
NFM Kok, LB Koppert. Journal of Vascular Surgery, 2006;43(3):643-4; author reply 644

Chapters in Books

HW Tilanus, LB Koppert, T Lerut. Oesophagus carcinoom. In: Oncologie. Eds. CJH van de Velde, JHJM van Krieken, PHM de Mulder, JB Vermorken, 2005

LB Koppert, HW Tilanus. Oesophagus en maag. In: Leidraad Chirurgie. Eds. HG Smeenk, NWL Schep, WMU van Grevenstein, 2005

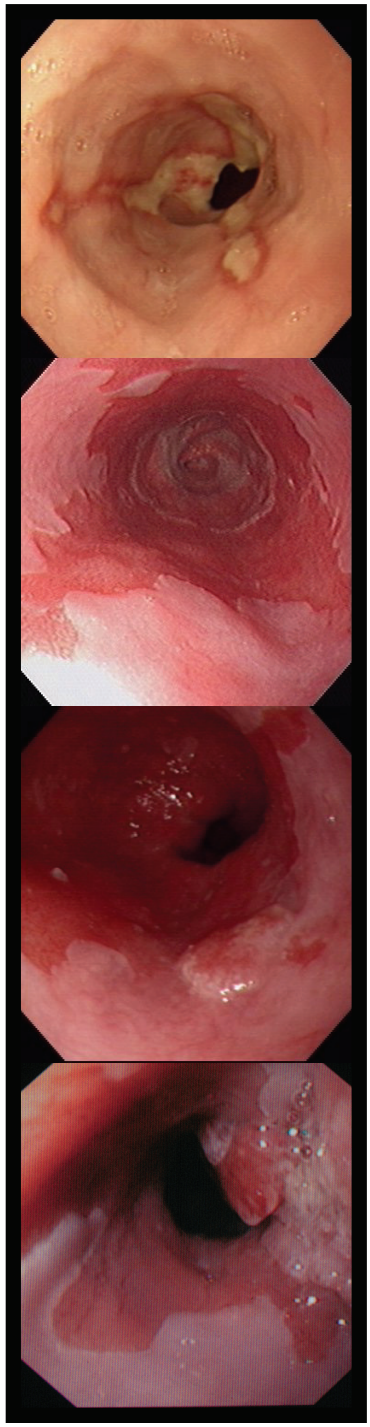


Figure 1.1 Endoscopy images

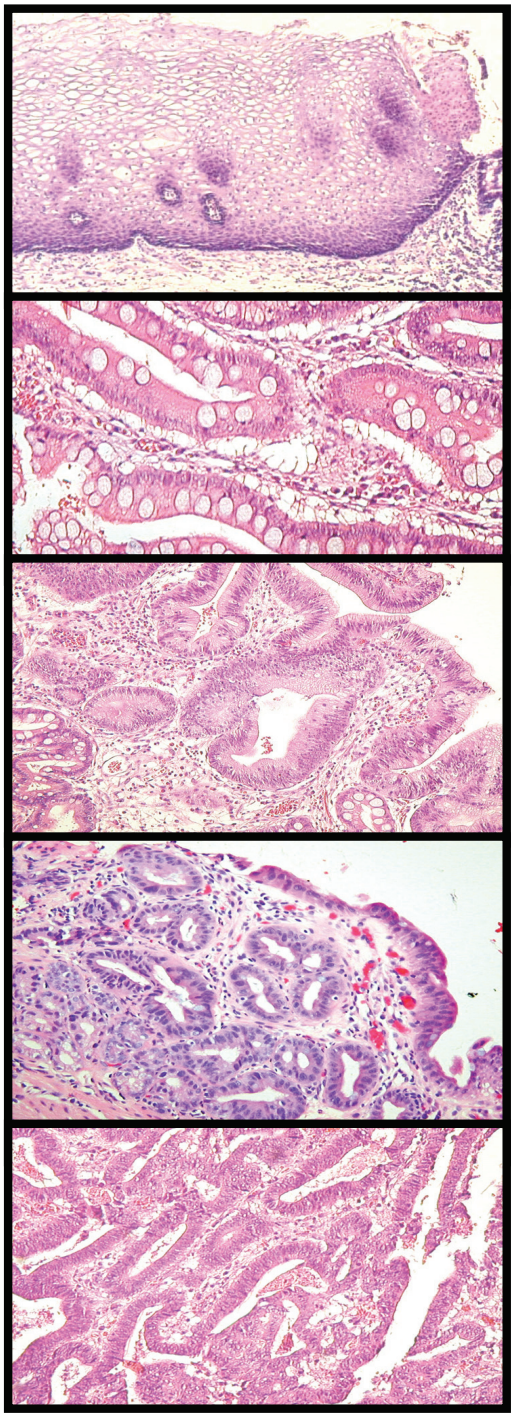


Figure 1.2 Histology of 'metaplasia-low grade dysplasia-high grade dysplasia-carcinoma sequence'

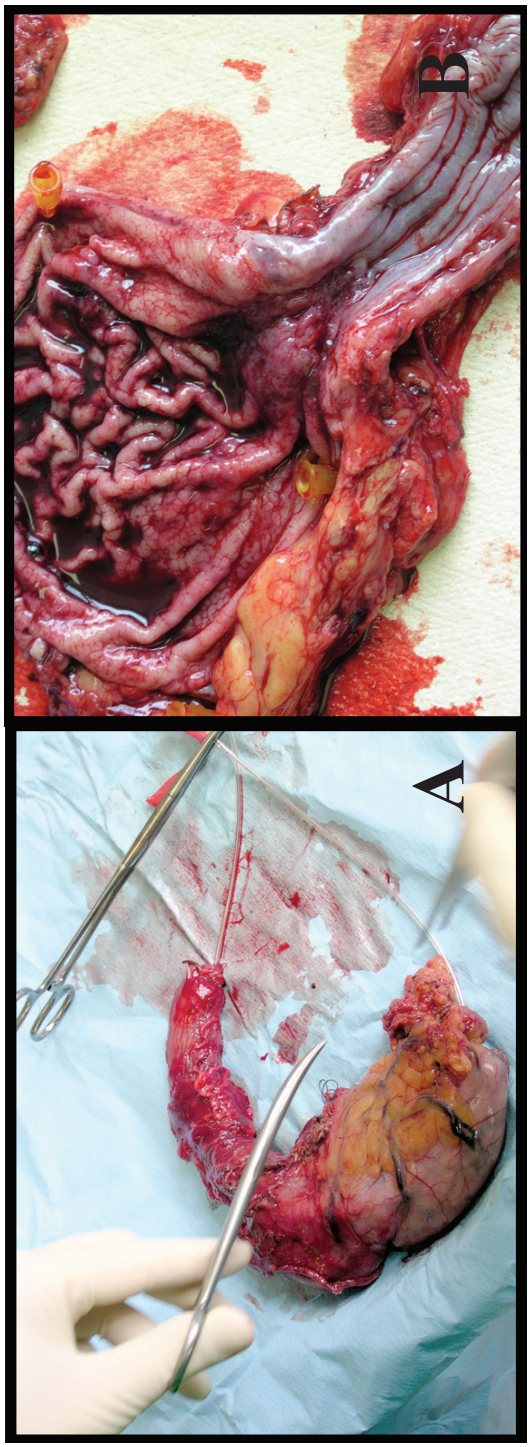


Figure 1.3 Resection specimen with GEJ adenocarcinoma

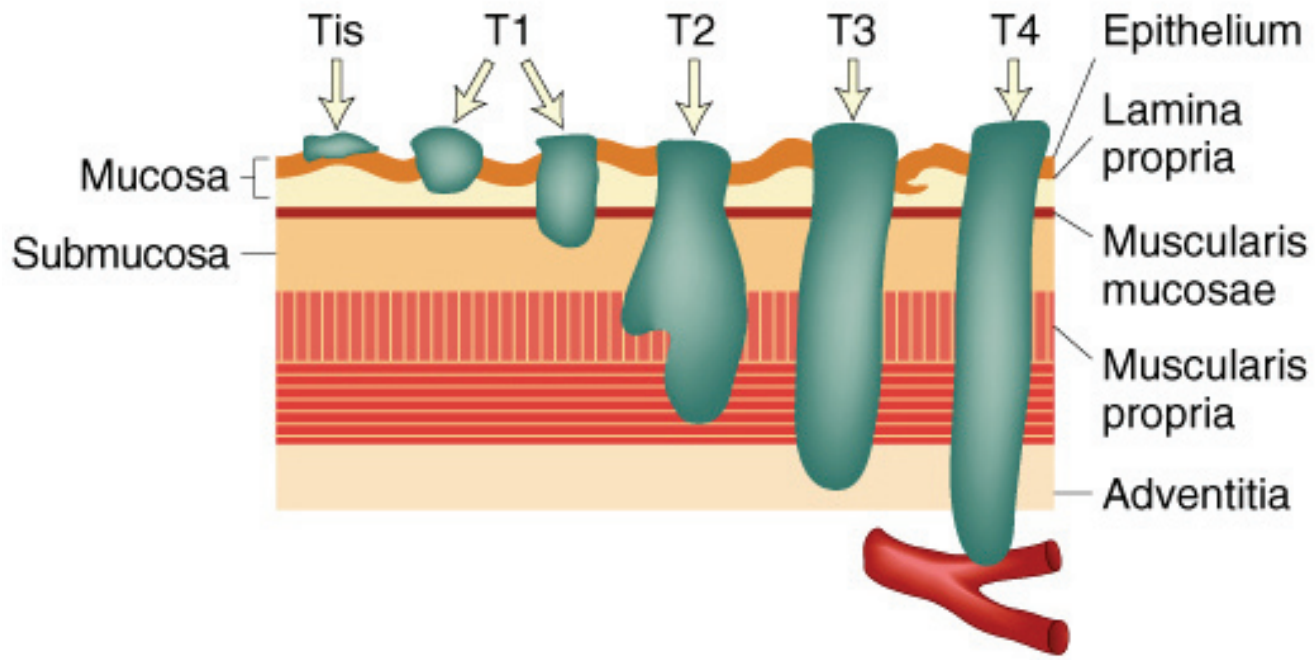


Figure 1.5 TNM classification (T-stage)

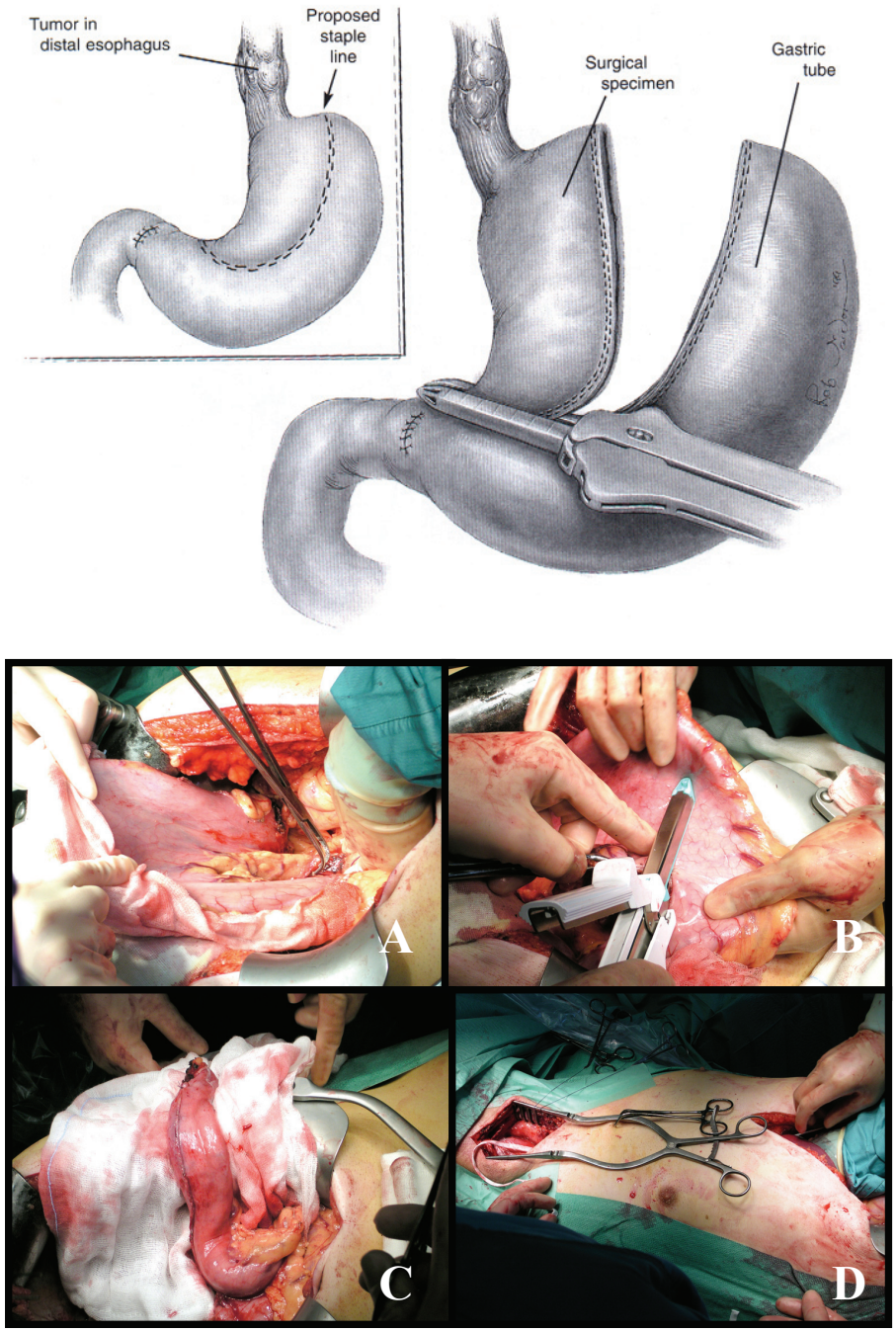


Figure 1.6 Schematic overview of surgical resection of GEJ adenocarcinoma. Photographs below the scheme show upper abdominal incision (A.), and creation of the gastric tube (A. B. C.) to restore continuation of the gastrointestinal tract after transhiatal en-bloc removal of esophagus and gastric cardia including tumor and adjacent lymph nodes (D.). Esophagogastrostomy will be performed in the neck (smaller incision left side of picture D.)

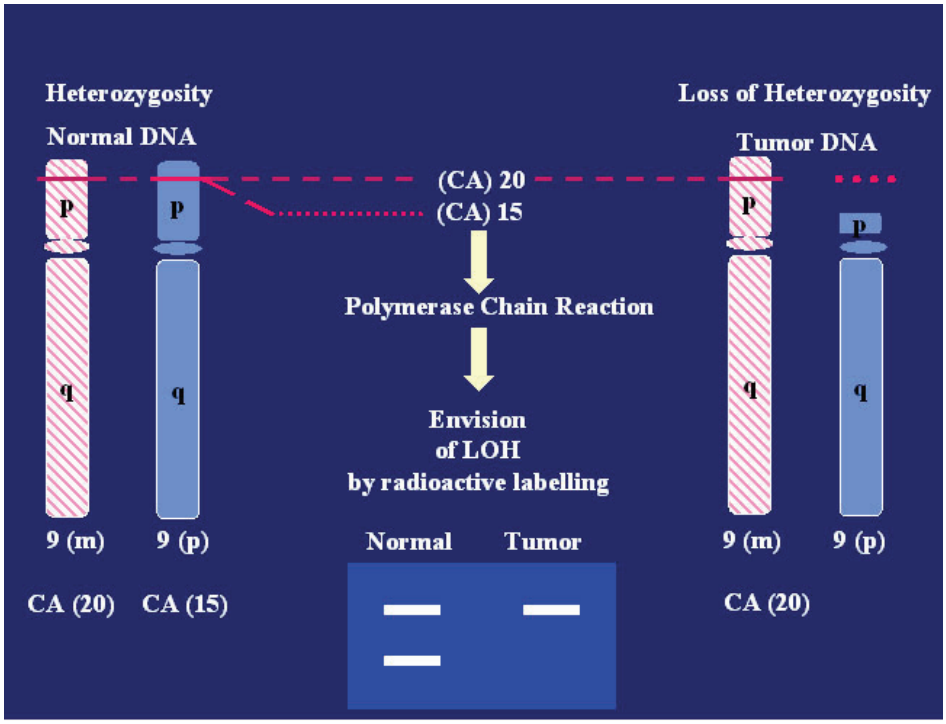


Figure 1.7 Schematic overview of Loss of Heterozygosity (LOH) analysis. Amplified fragments of maternal and paternal DNA of chromosome 9 are compared between normal and tumor DNA of a patient. Length of CA repeats within the fragments differs in the normal DNA, i.e. there is heterozygosity (maternal contains 20 CA repeats and paternal contains 15 CA repeats). In patients tumor DNA part of the short arm of paternal chromosome 9, containing the 15 CA repeat, is lost and therefore the heterozygosity is lost. This is visualized on a gel after radioactive labeling. Only the maternal 20 CA repeat is visualized in the tumor as compared to the normal DNA in which both fragments are shown and therefore this tumor shows LOH. Within LOH analysis, a separation of fragments takes place through difference in length, whereas Single Strand Conformation Polymorphism (SSCP) analysis separates fragments by a different conformation between normal and mutated DNA.

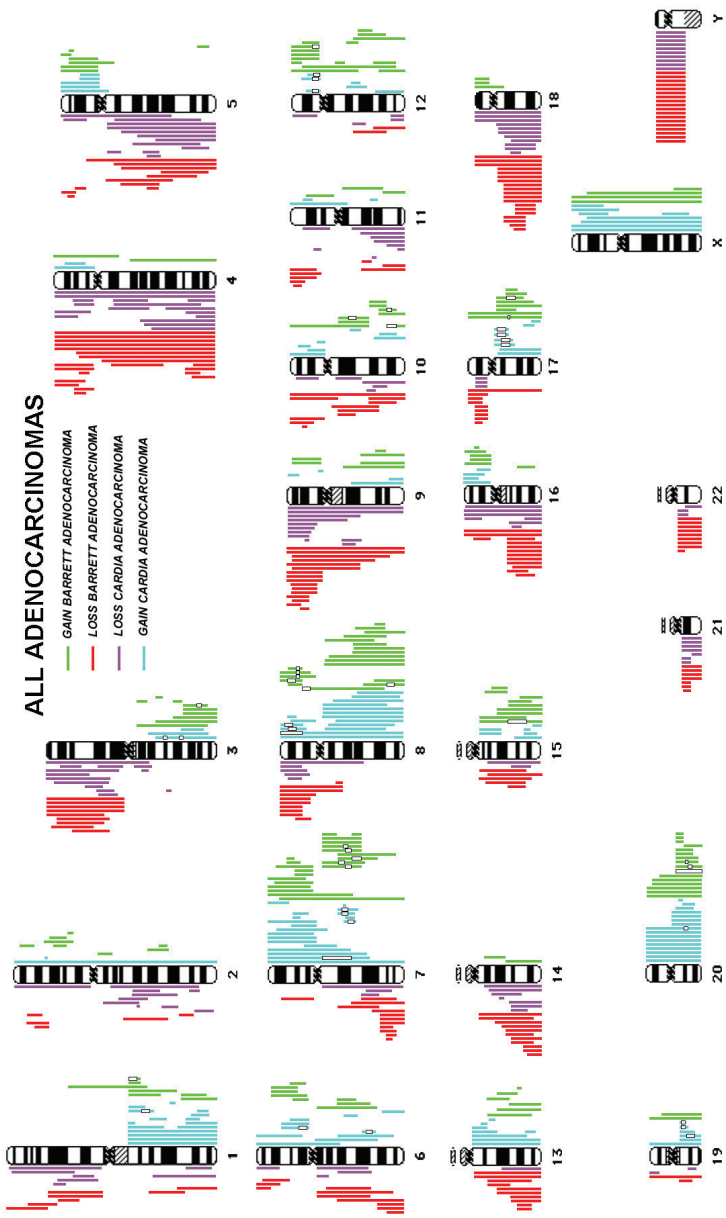


Figure 2.1 Combined CGH data from Van Dekken et al. (20 gastric cardia adenocarcinomas) and from Riegman et al. (30 Barrett adenocarcinomas) [167,171]. Boxes indicate regions of high-level amplification.

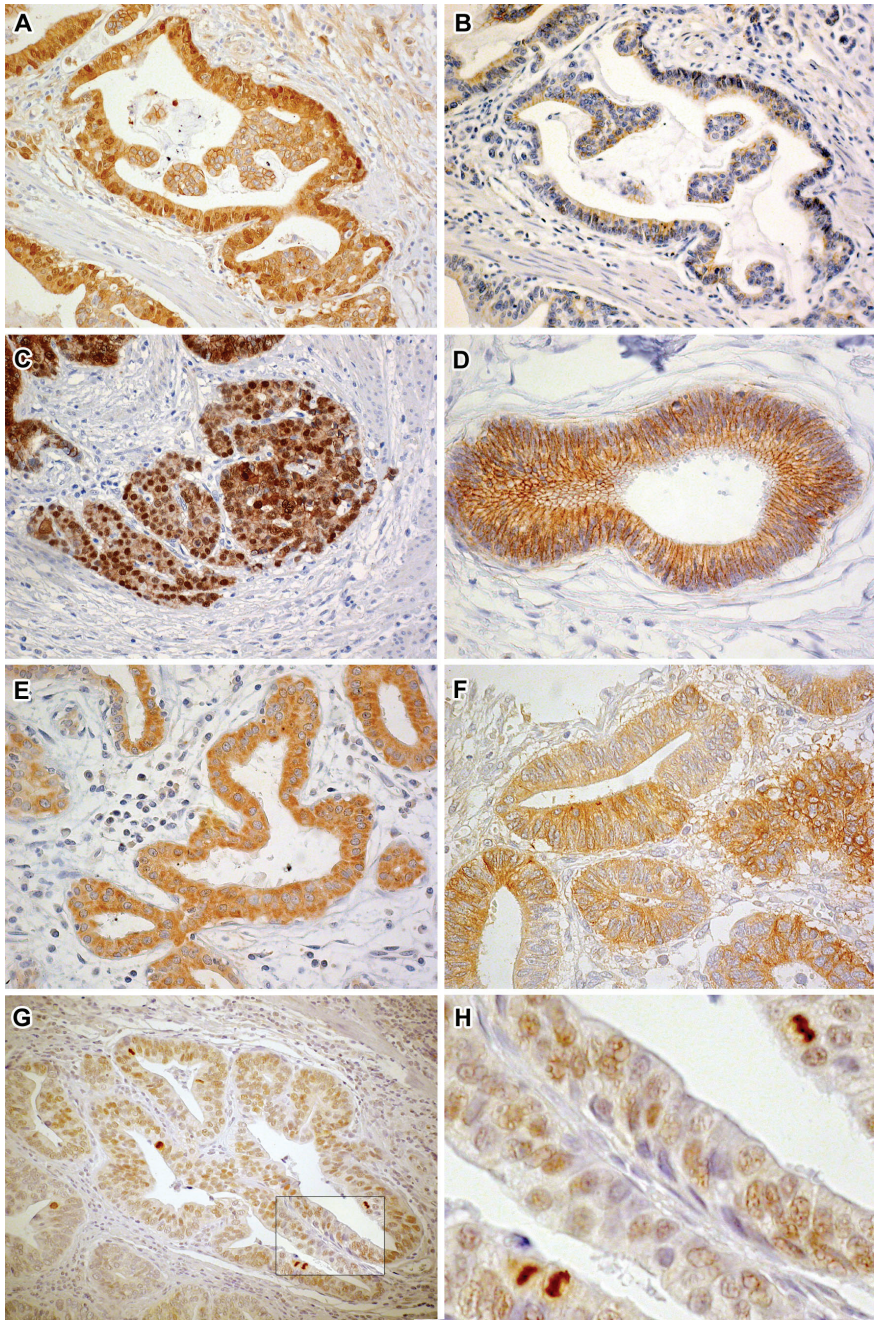


Figure 3.1. Immunohistochemistry of β-catenin in GEJ adenocarcinomas (Magnification x 200 (A., B., C., G.) and magnification x 400 (D., E., F., H.). A. Nuclear and cytoplasmic expression of β-catenin in the tumor cells. B. Nuclear active β-catenin staining in same GEJ tumor tissue as mentioned in A. C. Strong nuclear active β-catenin. D. Active β-catenin in membrane and cytoplasm of GEJ adenocarcinoma. E. EphB2 staining in cytoplasm of the tumor cells. F. Membranous EphB2 staining. G. Nuclear phospho β-catenin (Thr41/Ser45) with strong staining patterns in mitotic cells (insert H.).

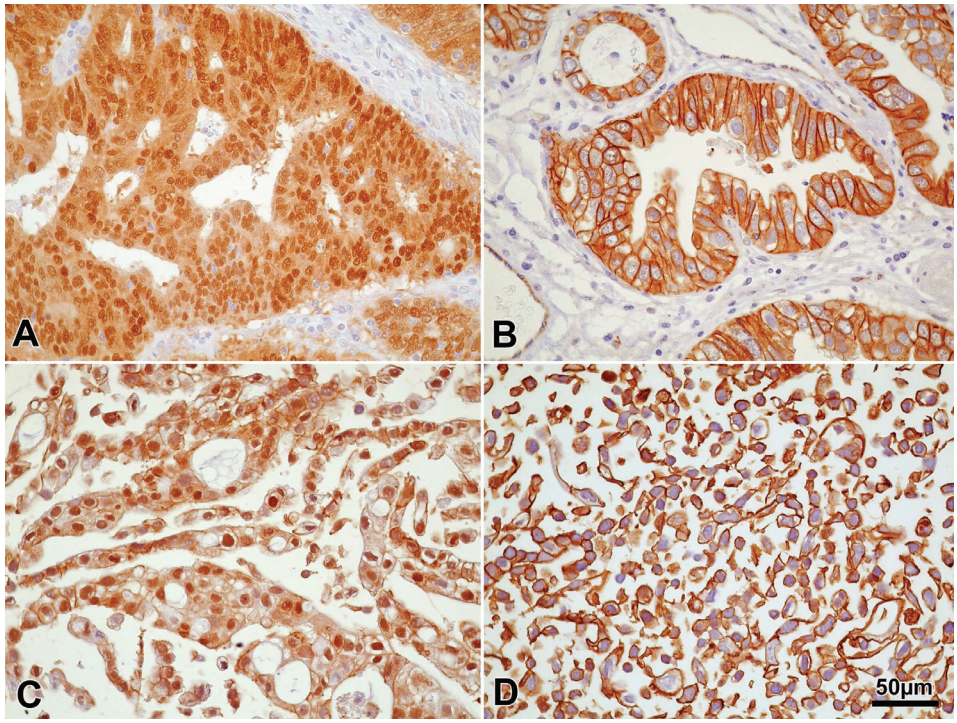


Figure 4.1 Immunohistochemistry of β-catenin in GEJ adenocarcinomas and cell lines. (β-catenin antibody, DAB and haematoxylin counterstain, magnification x 400). A. GEJ adenocarcinoma. Strong nuclear expression of β-catenin in the tumor cells. B. GEJ adenocarcinoma. Prominent membranous expression of β-catenin. C. Cell line JROECL19. Strong nuclear expression of β-catenin. D. Cell line TE-7. Membranous expression of β-catenin.

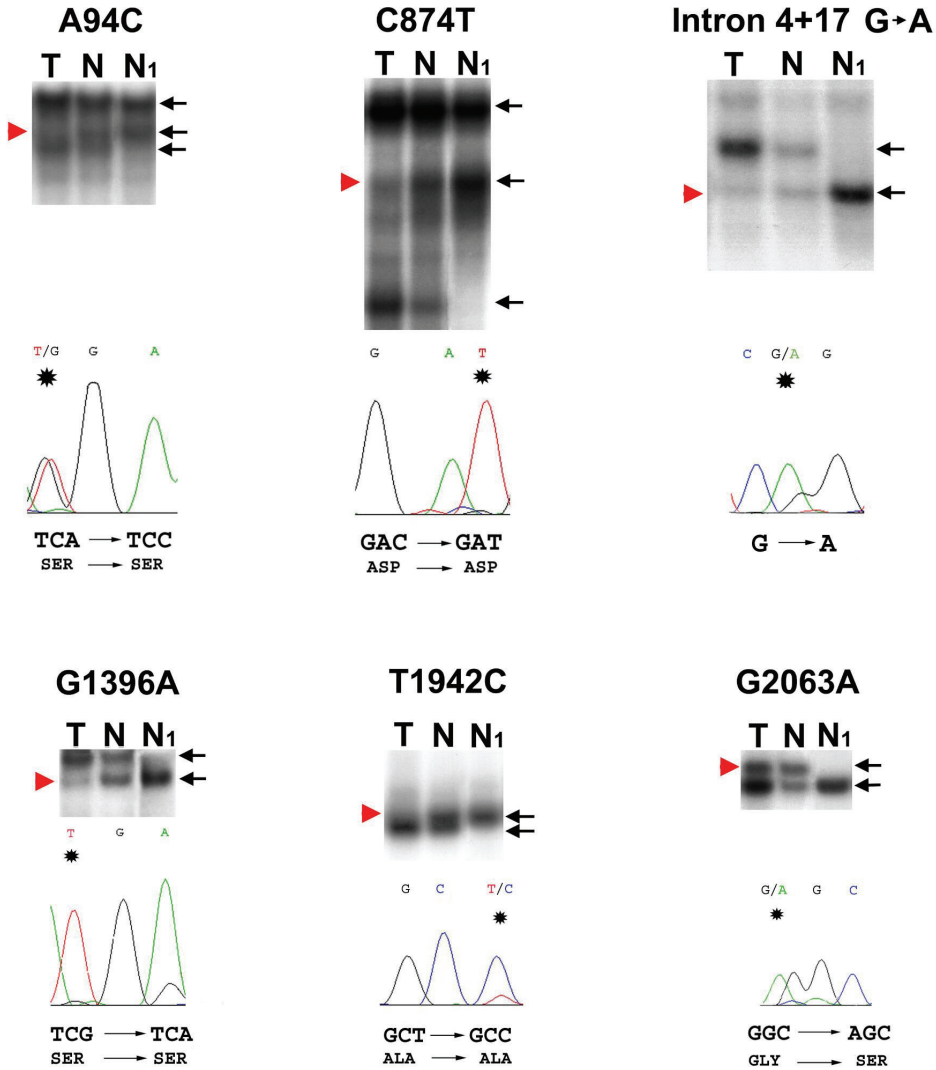


Figure 4.3 PCR-SSCP and sequencing analyses of the SNPs in tumors (T) and corresponding normal DNA (N), compared with DNA from individuals without SNPs (N1). Shown are informative cases with LOH. Black arrows point to allelic patterns. Red arrow heads point to deleted alleles in the tumor DNA. The sequencing chromatograms below each autoradiograph show the alterations (note the substituted nucleotide marked by an asterisk), which all represent SNPs. SNPs A94C and G1396A are annotated in the reverse complementary direction, whereas the SNPs C874, intron 4+17 G→A, T1942C and G2063A are annotated in forward direction.

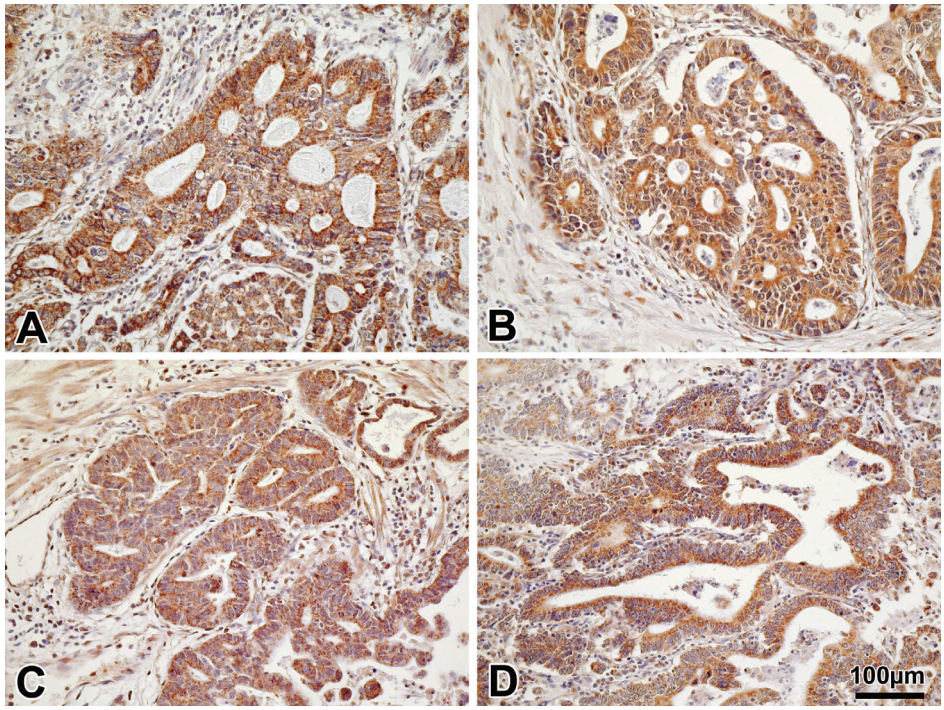


Figure 4.5 Immunohistochemistry of AXIN1 in GEJ adenocarcinomas. (AXIN1 antibody, DAB and haematoxylin counterstain, magnification x 200). A. and B. GEJ adenocarcinomas with nuclear β-catenin expression without (A.) and with (B.) AXIN1 locus LOH. C. and D. GEJ adenocarcinomas with membranous β-catenin expression without (C.) and with (D.) AXIN1 locus LOH. Note the strong cytoplasmic AXIN1 expression in the tumor cells in all 4 cases.

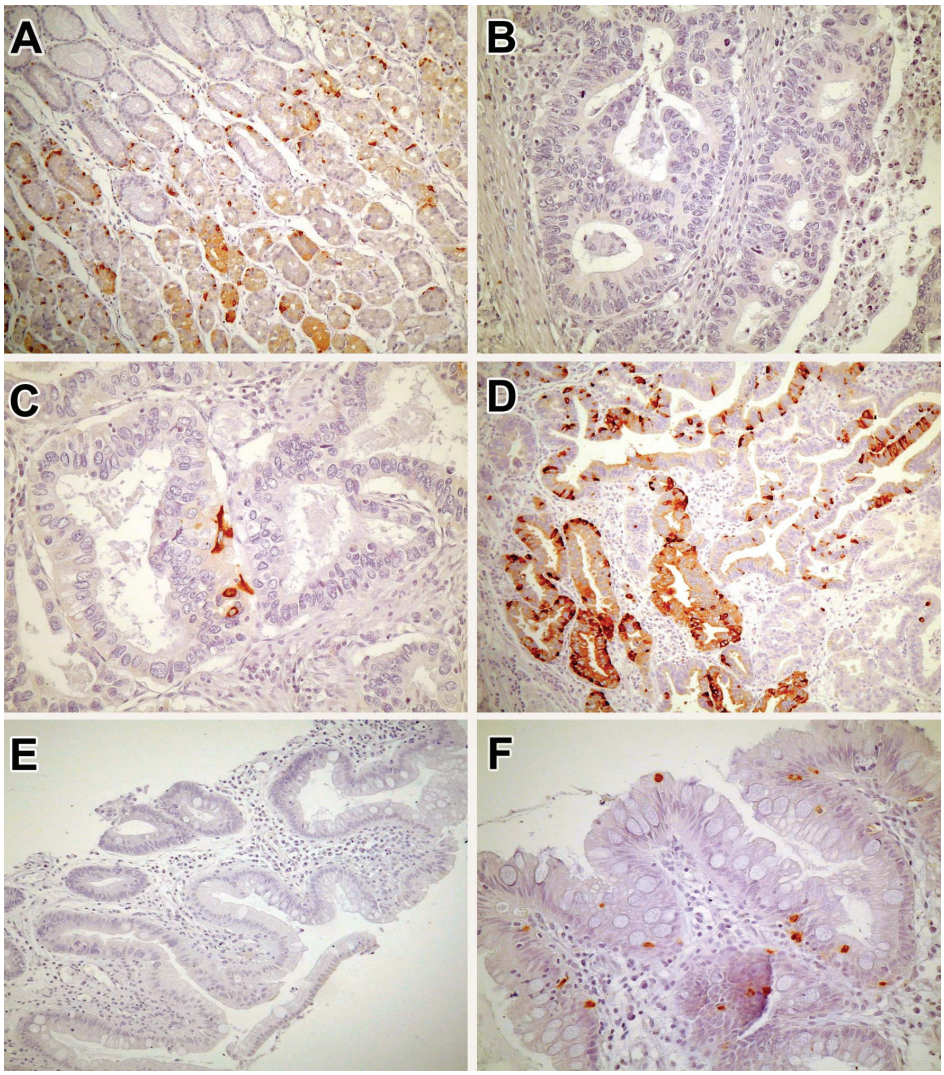


Fig. 6.1 CGA immunoreactivity in normal gastric epithelium (A), CGA negative adenocarcinoma (B). Scatters of individual tumor cells show CGA staining (1+) within a well-differentiated adenocarcinoma (C). Adenocarcinoma shows apparent cytoplasmic CGA staining in >20% (2+) of tumor cells (D). CGA negative Barrett's epithelium adjacent to adenocarcinoma (E), CGA positive cells in Barrett's epithelium most prominently located in the basal layer of the epithelium (F).

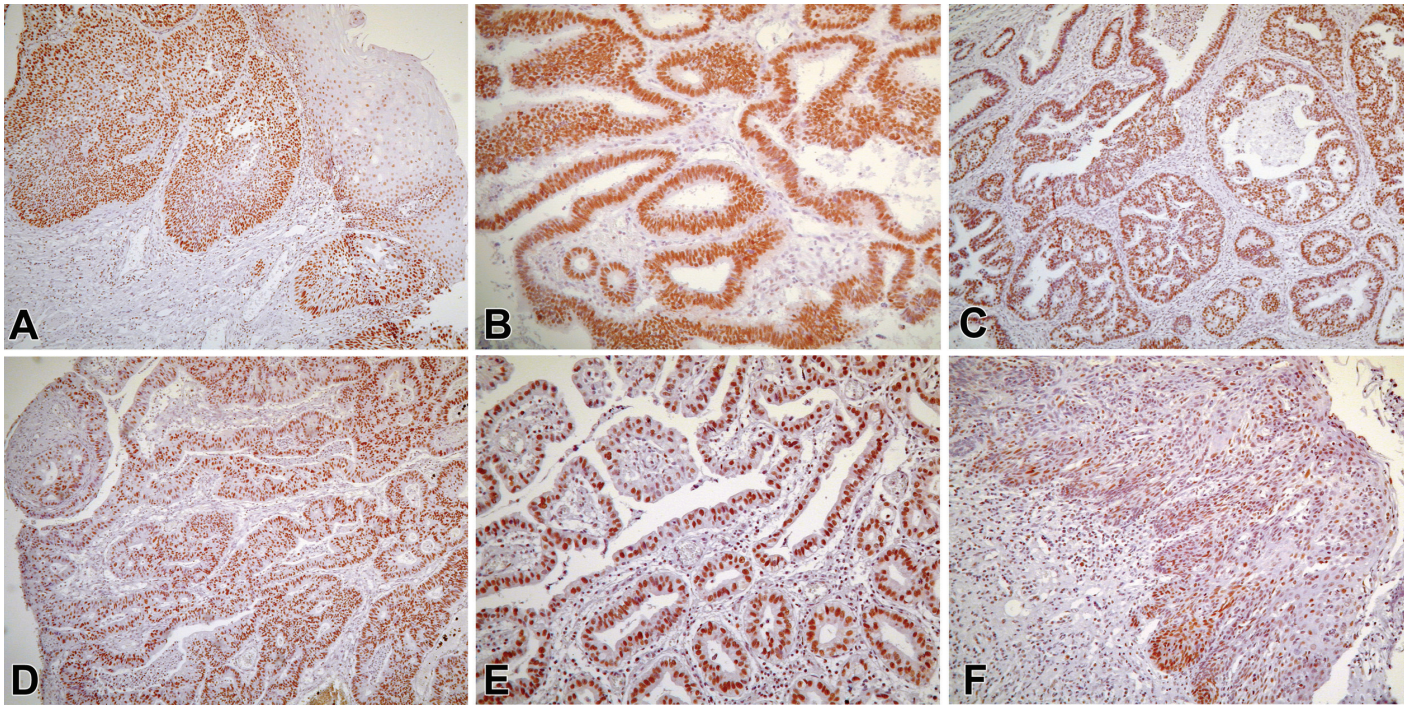


Figure 11.3 CHEK2 protein expression. Non-mutated squamous cell carcinoma in A, non-mutated adenocarcinoma in B. Remaining samples are from the 4 mutated tumors: adenocarcinomas T1, T2 and T3 shown in C, D, E, squamous cell carcinoma T4 shown in F. Magnification 100 x B, E 50 x A, C, D, F. Note: the strong nuclear CHEK2 immunoreactivity in the tumor cells.

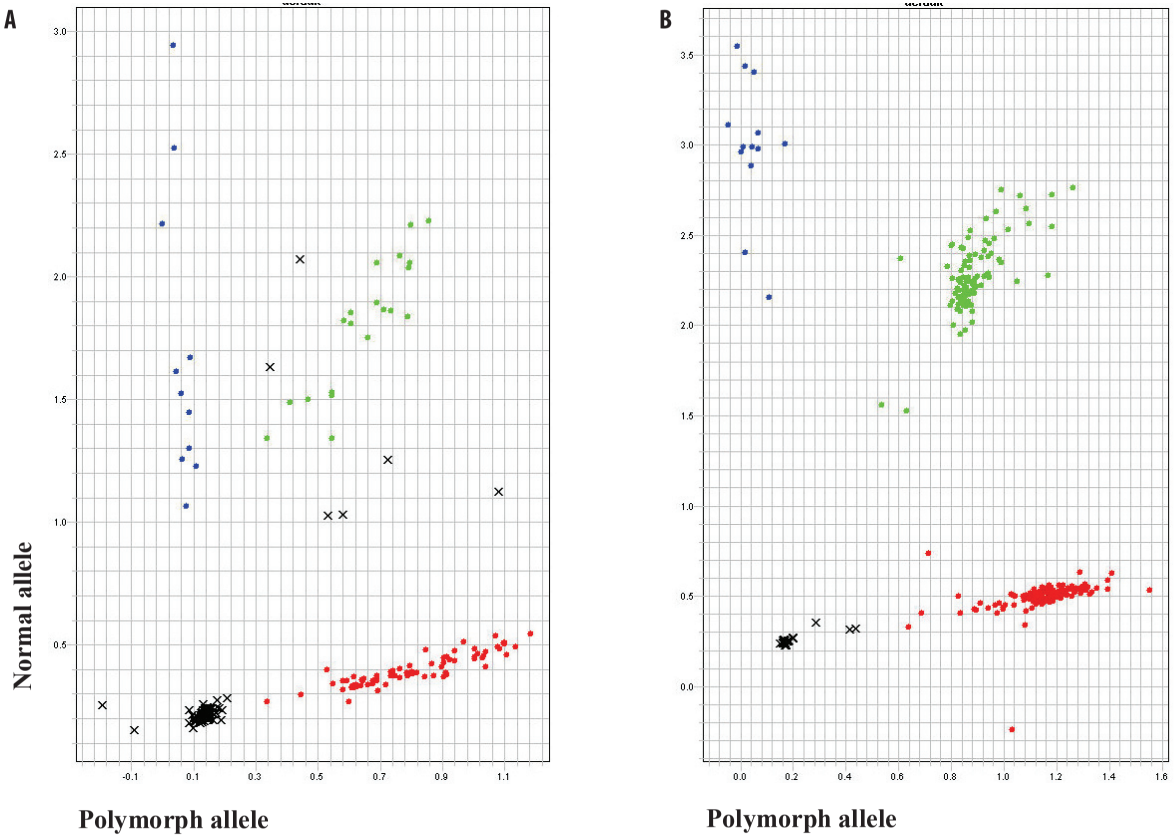


Figure 12.1. Taqman allelic discrimination assay concerning p73 exon 2 G4C14-A4T14 of 191 GEJ adenocarcinomas (A.) and 232 controls (B.). Distribution of homozygous normal (blue dots), heterozygous (green dots) and homozygous polymorphism (red dots).

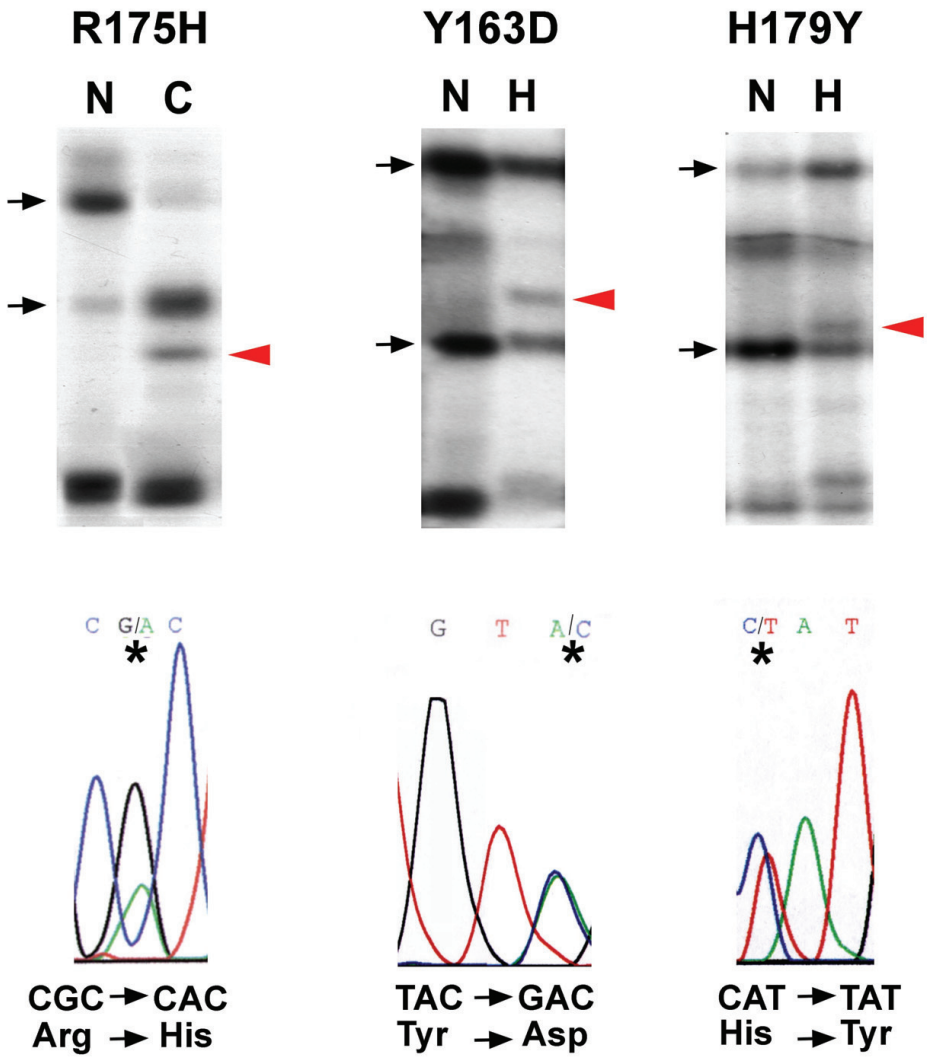


Figure 13.4 PCR-SSCP and sequencing analyses of three p53 mutations in High grade dysplasia (H), Carcinoma (C) and corresponding normal DNA (N). Black arrows point to allelic patterns. Red arrow heads point to mutated alleles. The sequencing chromatograms below each autoradiograph show the alterations (note the substituted nucleotide marked by an asterisk), which all represent p53 mutations.

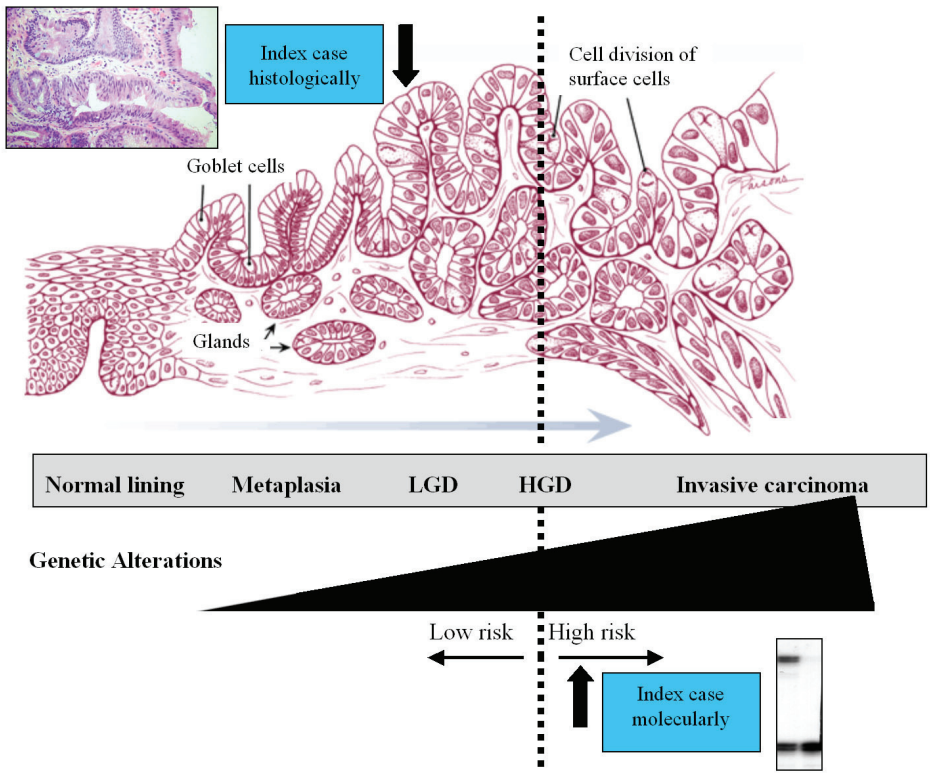


Figure 15.1 Metaplasia-Dysplasia-Carcinoma sequence. Index case is histologically at low risk for neoplastic progression, however genetic alterations are indicative for high risk profile.