FROM BARRETT'S ESOPHAGUS TO ADENOCARCINOMA AND METASTASIS

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FROM BARRETT'S ESOPHAGUS TO ADENOCARCINOMA AND METASTASIS

Van Barrett Oesofagus naar Adenocarcinoom en Metastase

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Aan mijn ouders,
aan Max

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Chapter 1

General Introduction

1.1 Historical perspective

The first description of islets of ectopic gastric mucosa in the esophagus was by Schmidt in 1805 (1). One century later, in 1906, Tileston described peptic ulcerations in columnar epithelium lining the distal esophagus (2). In 1950 Norman Barrett gave a detailed description of the columnar lined esophagus. He regarded the distal columnar lined esophagus a mediastinal extension of the stomach, as a result of a congenitally short esophagus. Barrett based his theory on the nature of the mucosa and mucosal secretions of the columnar lining, whereas absence of the musculature and peritoneal covering of the normal stomach were ignored (1). In that period Lortat-Jacob described the same condition, which he named endobrachyesophagus, a term still used in French literature (3). Barrett's observation was that of gastric mucosa, extending as a continuous sheet into the mediastinum. He observed that the columnar mucosa could extend for a varying distance and could reach as far as the aortic arch. Allison and Johnstone in 1953 showed that anatomically and functionally the segment of digestive tract described by Barrett is part of the esophagus. These authors suggested that the so-called Barrett's esophagus might be an acquired rather than a congenital condition. This implies that as a consequence of gastro-esophageal reflux, oesophageal squamous epithelium is converted to columnar epithelium through metaplasia (4). In several studies, authors noted "upward migration" of the squamo-columnar junction during follow-up of patients with gastro-esophageal reflux (5-7). Animal experiments proved that columnar epithelium in the esophagus is generated in the presence of gastro-oesophageal reflux (8). Through these observations it had become apparent that Barrett's esophagus is an acquired rather than a congenital condition. Around 1980 most authors appear to favor the view of the acquired origin of Barrett's esophagus (9-14). However, congenital islands of ectopic gastric mucosa do occur. They are found in up to 10% of individuals undergoing endoscopy (15). These so called "inlet patches" occur principally in the cervical esophagus and are mostly surrounded by normal squamous epithelium (16).

Different mechanism have been proposed for the development of columnar epithelium in the esophagus. One suggestion is upward extension of gastric epithelium, another is generation of columnar epithelium from superficial esophageal glands (heterotopic gastric mucosa). The theory proposing metaplastic change of squamous epithelium was already suggested by Allison in 1953. This theory was clinically supported by evidence of photographed ulcerations of

squamous mucosa which, while healing, were replaced by metaplastic columnar epithelium (17). Further evidence in favor of metaplasia was derived from the histology, mucin histochemistry and ultrastructural features of Barrett's esophagus. These features of multidirectional differentiation of epithelial cells, suggested that the columnar epithelium was derived from a mucosal stem cell (18-25). More evidence sustaining the theory of metaplasia from a multi potent stem cell was obtained from experiments in dogs. In these experiments, segments of esophagus from which the mucosa had been excised were re-epithelialized by columnar epithelium when reflux was induced. To prove that this re-epithelialization was not a process of upward migration of gastric mucosa, a strip of normal squamous epithelium between the excised area and stomach was left intact (26).

The epithelium which Barrett described in 1957, in the upper region contained flat cells in shallow tubular glands with mucus secreting units but little, if any, acid, pepsin or secretin secretion. In the lower esophagus a more typical gastric mucous membrane was seen (27). Over time, additional types of aberrant columnar epithelium with different secreting and non secreting cell types, such as mucous, parietal, chief, neuro-endocrine and goblet cells, were observed (12, 18-20, 22, 24, 28-30). Paull et al., distinguished three major types of columnar epithelium: an atrophic gastric fundic type with parietal and chief cells; a junctional type with cardiac mucous glands and a distinctive specialized columnar epithelium with villiform surface, mucous glands and goblet cells (13, 31). These authors noted that specialized columnar epithelium is the prevalent type of epithelium in Barrett's esophagus. This specialized columnar type appeared to be present most proximally in the esophagus, the gastric-fundic type being found most distally and the junctional type being interposed between the specialized columnar and gastric fundic type. This so called zonal expansion theory was challenged by Thompson et al., who described a mosaic pattern of different columnar cell types in Barrett's esophagus (22). Other authors confirmed the predominance of the specialized intestinal type. With respect to the two other types of epithelium the need for adequate sampling in the esophagus was pointed out. If sampling is performed blindly under manometric guidance, biopsies taken from the cardia could be mistakenly taken for Barrett's esophagus, for instance in cases of hiatal herniation of the cardia into the mediastinum (12, 32). Endoscopically, the exact gastro-oesophageal junction is difficult to locate. For instance, up to 2 cm of simple columnar lining may be present in the distal esophagus of healthy individuals. This led to the agreement that Barrett's

few studies reported regression of Barrett's epithelium after successful anti-reflux surgery (35, 36). In contrast, in several papers long-term treatment with H2 receptor blockers (cimetidin) and antacids showed decreased esophagitis and induced healing of Barrett's ulcers, but not regression of Barrett's esophagus (37-40). Nevertheless, more recent studies on treatment with proton pump inhibitors showed partial regression of Barrett's epithelium (41, 42).
Barrett already associated columnar lined esophagus with adenocarcinoma in his first description (1). Esophageal adenocarcinomas evidently associated with Barrett's esophagus were reported a few years later (43). This study was followed by numerous other reports, many of which also described dysplastic columnar epithelium adjacent to the adenocarcinomas (10, 44-52). Based on these observations Barrett's esophagus came to be regarded as a premalignant condition. It became clear that progression of Barrett's esophagus to adenocarcinoma is a gradual process going through different stages of dysplasia. In analogy to stomach, three stages of dysplasia (mild, moderate and severe dysplasia) in Barrett's esophagus were defined (53). Since esophageal adenocarcinomas are highly malignant, it was recommended that Barrett's esophagus patients should be under endoscopic surveillance. A consensus was reached that periodically biopsies taken from the mucosa should be screened for dysplastic changes or early carcinoma (54). In case of early carcinoma, the patient could be cured by surgical intervention.

1.2 Present definition

In Barrett's esophagus the normal stratified squamous mucosal lining of the esophagus is replaced by metaplastic columnar epithelium. Three types of metaplastic epithelium have been described (13), but careful examinations proved that fundic and gastric type of metaplasia are rarely seen in biopsies taken above the lower esophageal sphincter (33). Typical Barrett's epithelium shows incomplete intestinal metaplasia with goblet and columnar cells and a flat or villiform surface (55, 56).
Until recently, it was agreed that for diagnosing Barrett's esophagus, the columnar epithelium should extend at least three centimeters from the gastro-esophageal junction into the esophagus. This margin of three cm of columnar epithelium is debatable since endoscopic measurements of the length of columnar lining do not

columnar epithelium should extend at least three centimeters from the gastro-esophageal junction into the esophagus. This margin of three cm of columnar epithelium is debatable since endoscopic measurements of the length of columnar lining do not appear to be reproducible (57). Furthermore, intestinal metaplasia with dysplasia or adenocarcinoma may occur at the gastro-esophageal junction or in short segments of Barrett's esophagus (58, 59).
Regarding the three stages of dysplasia (53), there is limited inter- and intra observer agreement, particularly when diagnosing dysplasia less than high grade (60). This led to simplification of the dysplasia classification, in which "non dysplastic" lesions are distinguished from dysplastic and from unclassifiable lesions (mostly regenerative changes), which are marked as "indefinite for dysplasia" (61). In practice and for patient management indefinite for dysplasia and low grade dysplasia are grouped together.
When dysplasia occurs it may involve a considerable amount of the columnar mucosa or it may be limited in extent (62-64). Therefore, a thorough, systematic biopsy protocol is proposed to detect small areas of dysplasia and small carcinomas.
At present, the length of columnar epithelium extending in the esophagus may be ignored, four quadrant biopsies at intervals of 2 cm or less throughout the length of the Barrett's segment and biopsies of any suspect lesion should be taken. Only intestinal metaplasia, which is the prevalent type of Barrett's esophagus, is regarded as premalignant. Dysplasia, if present, should be classified as indefinite/low grade or high grade dysplasia.

1.3 Epidemiology, cancer risk and clinical outcome

Although it is generally accepted that Barrett's esophagus predisposes for esophageal adenocarcinoma, the prevalence of Barrett's esophagus and the frequency with which adenocarcinoma occurs in Barrett's esophagus are unknown. There is a discrepancy between data derived from clinical and that from population based autopsy series. For instance, in a population based study at the Mayo Clinic in Rochester USA, in the period of 1980-1986, a 21 fold higher prevalence of Barrett's esophagus was reported in autopsy series compared with the number of endoscopical diagnoses (65). This important discrepancy is mainly caused by the fact that many Barrett's esophagus patients have few symptoms or are asymptomatic. The prevalence of Barrett's esophagus is relatively low in

childhood and increases with age. A plateau is reached at the age of 60. Median age of developing Barrett's esophagus is 40 (66). Interestingly, the prevalence of Barrett's esophagus is highest in white middle aged males (67).

It has become clear that the high incidence of adenocarcinoma in Barrett's esophagus as published in the early studies is exaggerated (48, 68, 69). In endoscopic series of Barrett's esophagus patients the prevalence of esophageal adenocarcinoma is between 8 and 15%. Follow up series, however, shows that one case per 46 to 441 patient years of follow up will progress to adenocarcinoma. Thus the incidence of adenocarcinoma in Barrett's esophagus ranges between 0.2 and 2%. Compared to the general population, the risk for developing esophageal adenocarcinoma in Barrett's esophagus is between 30 and 125 fold increased (70-76). For many years, Barrett's esophagus patients only underwent major surgery in case of complete malignant transformation (carcinoma) had occurred. Decrease of postoperative mortality and morbidity by improved esophageal surgery (77-80), and the frequent coexistence of carcinoma with severe dysplasia led to a new approach. In this approach it is suggested that surgical intervention should be performed on all Barrett's esophagus patients with consistent high grade dysplasia (63, 81-83).

A striking fact is that in Western countries esophageal adenocarcinoma is the most rapidly increasing type of gastrointestinal malignancy (84-87). In the USA it shows probably the most rapidly rising incidence rate of all cancers (86). The increase in esophageal adenocarcinomas is most marked in higher social economic classes, in whites opposed to blacks, and is seven fold higher for males than for females (84, 86, 87). At present the incidence of esophageal adenocarcinoma lies between 0.6 and 0.8 per 100.000 per year (84-87). It is assumed that virtually all esophageal adenocarcinomas are associated with Barrett's esophagus. Since the vast majority of Barrett's esophagus is unrecognized (72), most esophageal adenocarcinoma associated with Barrett's esophagus are discovered when the disease has reached an advanced stage and patients already have symptoms of dysphagia and weight loss (69, 72, 88, 89). The only possible cure for these patients is esophagogastrectomy. Nevertheless, curation by surgery is only feasible in case the disease is limited to the esophageal wall and metastases are absent. Therefore, curative resection is only attempted after extensive preoperative staging of disease to exclude the presence of distal metastases (90-95). For patients with extensive disease and lymph node or organ metastases, palliative therapy is a more suitable option (96-104). In practice, local or distant lymph node metastases are seen in at least half of the

resected specimens. The cumulative five year recurrence rate for metastases in patients after esophagogastric resection is approximately 65% (105-107). Overall post operative long-term survival of patients with esophageal adenocarcinoma ranges between 15 and 60%. However, survival is strongly correlated with the stage of the disease. Five years survival of advanced stages, i.e., carcinomas with deep infiltration of the esophageal wall and lymph node metastasis, is as low as 0%, whereas survival rises to 100% in cases where patients are operated with disease limited to the mucosa (79, 108-111).

Chapter 2

Prognostic Factors in Barett's Esophagus and Esophageal Adenocarcinoma.

2.1 Biological markers

Biological markers in neoplastic disease are often factors involved in the regulation of normal and abnormal differentiation and proliferation of cells. In oncology, these markers can be used as parameters to diagnose at tissue level or to clinically monitor disease. In Barrett's esophagus the development of an adenocarcinoma involves many factors interacting in a complex manner rather than a simple transforming hit. Some factors can be detected early and others late during neoplastic progression, but an exact order of involvement of the various factors in malignant transformation in Barrett's esophagus has not been determined. Generally, markers which may be used in Barrett's esophagus include: Secretory factors, cell cycle regulating factors, onco- and tumor suppressor genes, cell surface proteins, genetic aberrations and ploidy status. Most of these have been extensively correlated with histopathological changes in Barrett's esophagus, but few have been evaluated in prospective follow-up series, in order to determine their prognostic significance.
Many markers evaluated in Barrett's esophagus have also been studied in esophageal adenocarcinomas. Some have even been correlated with clinico pathological parameters, such as tumor stage, grade and patient survival.

2.2 Secretory factors

Barrett's esophagus consists of a mixed population of secretory and non secretory cells, such as columnar, goblet, Paneth and endocrine cells. Most of the mucin secreted is of the neutral type, but sulphomucins and sialomucins can be found as well. The presence of sulphomucins, although previously reported to be a risk factor for adenocarcinoma (21, 23, 112) appears to be common in the intestinal type of Barrett's metaplasia and cannot be considered a marker for malignant transformation (55, 113). Decreased secretion of O-acetylated sialomucins was found in all dysplastic but also in a number of non-dysplastic cases of Barrett's esophagus with incomplete intestinal type of metaplasia (114). Likewise, sucrase-isomaltase expression was found in incomplete intestinal type of metaplasia and in esophageal adenocarcinomas (115). Glutathione and Glutathione-S transferase enzyme activity is low in Barrett's esophagus. These enzymes protect tissues from genetic damage, for instance, caused by oxygen radicals. Decreased levels

have been correlated with an increased risk of malignant transformation (116). Increasing levels of Ornithine decarboxylase (ODC) have been frequently found in metaplastic, dysplastic and neoplastic Barrett's esophagus and this may be regarded as a biochemical marker for malignant progression (117, 118). ODC catalyzes polyamine synthesis and is an important enzyme for cell proliferation and differentiation. Despite increased ODC levels in Barrett's esophagus, chemopreventive intervention trials with ODC inhibitors, for instance alpha-diflouromethylornithine, were inconclusive as to whether or not changes in the polyamine contents would be induced in Barrett's mucosa (119, 120). A complicating factor may be a defective ODC - polyamine pathway, which may also explain the poor correlation between ODC activity and polyamine levels (121, 122).

2.3 Proliferation

Proliferation of tissues can be assessed by several methods. Through metabolic labeling, tritiated thymidine or 5-Bromodeoxyuridine (BrdU), an analogue of thymidine, can be incorporated into the DNA during S-phase of the cell cycle, and proliferating cells can be identified. Early studies using these autoradiographic methods showed expansion of the proliferative compartment in Barrett's esophagus compared to other columnar epithelia (123, 124). Alternatively, proliferation can be assessed by immunohistochemistry using antibodies recognizing antigens which are expressed in the nucleus during proliferation. PCNA (Proliferating Cell Nuclear Antigen) and Ki-67 are such antigens. PCNA, a 36 kDa molecule, serves as a cofactor for DNA polymerase delta in both S-phase and unscheduled DNA synthesis associated with DNA repair. Since PCNA has a 20 hour half life, it can also be found in non cycling cells, i.e., G0 phase. The Ki-67 antigen is expressed in the nuclei during late G1, S, G2, and M phases of the cell cycle, but not in G0 phase and is therefore a more precise marker for estimating proliferation. Assessment of these markers in Barrett's esophagus showed that proliferation is increased in intestinal type of metaplasia even when dysplasia is absent. Interestingly, with increasing dysplasia the fraction of proliferating cells and the proliferative compartment increases further (125, 126). Eventually, in high grade dysplasia luminal epithelial cells show proliferative activity. (123, 125, 127, 128). Reid et al., used multi-parameter flow cytometry to simultaneously measure DNA content and cycling

cells, which allowed him to estimate the fractions of cells in G0, G1, S and G2M phase. In this study for instance an increased number of cells is found in G1 phase early on during the neoplastic progression (126).

2.4 Growth Factors

Growth factors are important for regulation of cell differentiation and proliferation. Abnormal expression of growth factor receptors, such as EGF-R (Epidermal Growth Factor) and ligands such as EGF (Epidermal Growth Factor) and TGF-Alpha (Transforming Growth Factor-Alpha) are associated with mitogenesis and carcinogenesis (129). In Barrett's esophagus expression of EGF-R and TGF-Alpha but not EGF is increased in intestinal type metaplasia compared to the other types of metaplasia. Both markers are also elevated in dysplasia and in esophageal adenocarcinomas (113, 130-134).

2.5 Oncogenes

Abnormal expression of (proto)oncogenes is involved in the transformation of normal tissues into malignancy (135). Many oncogenes studied in Barrett's esophagus did not yield specific data, while some need more extensive evaluation. For instance, in a small series of patients increased H-ras expression has been observed in high grade dysplasia and adenocarcinoma (136). The proto-oncogene product C-erbB2 is a glycoprotein which shows high homology with the epidermal growth factor receptor, EGF-R. Like EGF-R, C-erbB2 can be activated through ligands and in turn activate pathways in the cytoplasm involved in cell differentiation and proliferation (137, 138). Although, C-erbB2 expression has been found to be correlate with survival of patients with esophageal adenocarcinoma, the proportion of C-erbB2 expressing cases in the different studies varied between 10 to 73% and hence this protein needs to be further evaluated (139-142).

2.6 P53 and other tumor suppressor genes

In contrast to an oncogene, the function of a tumor suppressor gene is only

disrupted in case both alleles are defective. This condition is usually met by loss of one allele and functional inactivation of the other. Tumor suppressor gene alterations can be studied by a variety of techniques. Gene mutations can be studied by PCR (Polymerase chain reaction) and sequence analysis or SSCP (Single Strand Conformation Polymorphism) analysis, allelic loss by RFLP (Restriction Fragment Length Polymorphism) analysis.

The p53 tumor suppressor protein was detected in 1979 by Lane and Crawford (143). Though formerly regarded as an oncoprotein, the main function of p53 is that of a tumor suppressor (144, 145). Although p53 is a tumor suppressor, it can display oncogene-like behavior, because mutant p53 molecules may bind to and inhibit wild type p53 function. Under normal circumstances wild-type p53 plays a limited role in cell cycle control. In cells with genomic damage, accumulation of high levels of wild-type p53 in the cell nucleus results in a prolonged G1 phase of the cell cycle. The extended G1 phase consequently delays replication in damaged cells, which facilitates DNA repair (146, 147). Another unique property of this protein is that mutant forms acquire conformational changes which prolong half life, resulting in accumulation of mutated p53 protein in cells (148, 149). Wild type as well as mutated p53 protein are detectable by immunohistochemistry as long as a sufficient amount of protein is retained (150).

In Barrett's esophagus and esophageal adenocarcinoma p53 aberrations have been extensively documented. Although the applied methodologies vary, the findings show a certain consistency. The prevalence of p53 protein accumulation as determined by immunostaining in adenocarcinomas ranges between 53-87% (151-157). In esophageal adenocarcinomas p53 (17p) allelic loss is found in 55% to 100% of cases (152, 158-160), and p53 gene mutations in 8% to 89% of cases (159, 161-166). In esophageal adenocarcinomas no correlation was found between p53 protein accumulation and clinicopathological and prognostic factors (167). Nevertheless, in Barrett's esophagus the frequency of p53 alterations increases as the epithelium becomes more dysplastic (151, 154-156, 168). The prevalence of abnormal p53 reaches up to 89% in high grade dysplasia. Since aberrant p53 can be found in non dysplastic Barrett's esophagus and cases with low grade dysplasia (table 2), p53 mutations is considered one of the earliest genetic events during malignant transformation of Barrett's esophagus. Several studies have shown that in resection specimens mutations in the adenocarcinoma correspond with those in surrounding Barrett's epithelium (162, 164, 165). Interestingly, p53 assessment combined with DNA cell sorting showed that p53 mutations occur in diploid cells, suggesting that these precede aneuploidy. Only a few follow up studies

have been published. Younes et al., performed a follow-up study of 24 patients with Barrett's esophagus. After a follow-up period of 9-166 months, three cases of Barrett's esophagus had progressed into high grade dysplasia, two of which had low grade dysplasia but p53 immunoreactivity in their initial biopsies (155).

Table 1. P53 protein immunoreactivity in adenocarcinomas complicating Barrett's esophagus.

	Total # of adenocarcinomas	Cases with P53
Blount et al., 1992	13	8 (62%)
Ramel et al., 1992	15	8 (53%)
Younes et al., 1993	8	7 (87%)
Krishnadath et al., 1994	24	20 (83%)
Fléjou et al., 1994	62	41 (66%)
Moore et al., 1994	19	11 (58%)
Hardwick et. al., 1994	30	16 (53%)

Recently, Schneider et al, documented the prevalence p53 gene mutations in 50 esophageal adenocarcinomas, and in 48 cases of Barrett's esophagus, 32 without dysplasia, 13 indefinite for or with low grade dysplasia and 3 with high grade dysplasia. P53 gene mutations were found in 46% of the adenocarcinomas. Only in one case of Barrett's esophagus, which was highly dysplastic, was a p53 gene mutation found (165). After a mean follow-up period of 2.2 years non of the Barrett's esophagus patients developed adenocarcinoma. Although p53 seems to be a promising prognostic marker, malignant transformation occurred without p53 protein accumulation in one follow-up study which implies that in a subset of carcinomas malignant progression is initiated following alternative pathways.

Table 2. P53 protein immunoreactivity in Barrett's esophagus

	No dysplasia	Indef/low grade	high grade
Ramel et al	1/21 (5%)	2/13 (15%)	2/13 (15%)
Younes et al	0/53 (0%)	4/44 (9%)	5/9 (55%)
Krishnadath et al	3/50 (6%)	12/43 (28%)	8/9 (89%)
Rice et al		0/27 (0%)	18/26 (89%)

Another potentially important tumor suppressor is the APC gene. Allelic loss of the 5q chromosomal region harboring the APC gene, was found in 66-77% of esophageal adenocarcinomas (158, 169). Of interest is that during clonal evolution 17p was found to precede 5q allelic loss (170). Recent, investigations of APC allelic loss in esophagectomy specimens showed identical APC gene alterations in adenocarcinoma and in Barrett's epithelium surrounding the

adenocarcinoma (171). Despite the high frequency of 5q allelic loss in the APC region as observed several studies, APC gene mutations were only sporadically found in esophageal adenocarcinomas (172). Possibly another gene or genes on 5q, distinct from APC, are involved.
Other allelic losses of familiar tumor suppressor genes have been found for the mutated colon carcinoma (MCC) gene in 63%, for deleted in colon carcinoma (DCC) gene in 24% and for the retinoblastoma (Rb) gene in 48% (158, 169).

2.7 Numerical and structural chromosomal aberrations

A variety of cytogenetic and molecular biological techniques is available for detecting numerical and structural chromosomal abnormalities. The general idea is that these methods will lead to the detection of chromosomal regions which may harbor genes involved in cell cycle regulation and carcinogenesis. Such genes may be onco- and tumor suppressor genes, cell cycle and cell death (apoptosis) regulating genes, growth factors, genes coding for transcription and signal transduction factors and cell adhesion genes. In esophageal adenocarcinomas many aberrant chromosomes and chromosomal regions have been identified, whereas the number of genetic aberrations in Barrett's esophagus is limited. The most frequently documented numerical chromosomal aberration in Barrett's esophagus and esophageal adenocarcinomas is loss of the Y-Chromosome (173-179). In esophageal adenocarcinoma Y-chromosome loss was found in 31 to 93 % of cases. In Barrett's esophagus, for instance Garewal et al., noticed Y-loss in 7/10 cases. In chapter 5 of this thesis the frequency of Y-chromosome loss in Barrett's increased along with grade of dysplasia and all cases of high grade dysplasia showed Y- chromosome loss. Although Barrett's esophagus and esophageal adenocarcinoma occur more frequently in men, no specific onco- or tumor suppressor genes have been assigned to the Y-chromosome. Perhaps, as genetic instability increases during malignant transformation of Barrett's esophagus, Y-chromosome loss randomly occurs. Nonetheless, the Y-chromosome may harbor genes involved in signal transduction or cell cycle regulation, which in a more subtle fashion may influence cell transformation.
Other frequent numerical aberrations in esophageal adenocarcinomas are over representation of chromosomes 8, 14 and 20 and loss of chromosomes 4, 17, 18

and 21 (174, 175, 177, 179). Karyotyping revealed frequent structural rearrangements in esophageal adenocarcinomas in the 1p, 3q, 11p13-15, and 22p regions (174, 179). Loss of heterozygosity studies showed high frequencies of 17p (100%) and 5q (80%) allelic loss in adenocarcinomas (152, 160, 170). 17p and 5q loss were also seen in Barrett's esophagus with high grade dysplasia. Interestingly in Barrett's esophagus 17p preceded 5q loss (170). In approximately 60% of esophageal adenocarcinomas structural loss of 9p and in 40% of 1p, 13q, and 18q was observed (160).

2.8 Aneuploidy

DNA flow cytometry (DNA-FCM) is the method of choice for measuring DNA cell content. In addition, DNA-FCM may serve to determine the fraction of cells in different phases of the cell cycle, such as resting cells in G0 and G1, cells with active DNA replication in S-phase and cells with doubled DNA content and in mitosis in G2M phase. Karyotyping and DNA in situ hybridization may also provide information on cell ploidy. Aneuploidy is found in esophageal adenocarcinomas with a frequency ranging from 79% to 100% (126, 141, 178, 180-184). In several studies aneuploidy of esophageal adenocarcinoma was found to correlate with the presence of lymph node metastases and poor survival (141, 184).

Although data on aneuploidy in Barrett's esophagus have been inconsistent, aneuploidy and increased G2M/tetraploid populations seem to increase along with increasing dysplasia (126, 178, 180-183, 185-189). Most discordance between several studies can be explained by methodological differences. For instance, interpretation of a DNA FCM histogram depends on parameters chosen by the investigator. Other confounding factors are contamination with non-epithelial (diploid) cells and differences between fresh and paraffin embedded tissues. Convincing results have been published by Reid et al (126, 180, 182, 187). Aneuploidy and increased tetraploidy were not found in esophagitis, but in a low percentage of cases with intestinal type of metaplasia without dysplasia or indefinite for dysplasia, in a high percentage of cases with dysplasia and in all cases of adenocarcinoma (180). These findings were confirmed by others by DNA -flow or - image cytometry (181, 183), and DNA in situ hybridization with (peri-) centromeric DNA probes (178). Aneuploidy and increased tetraploidy have also been correlated with ultrastructural abnormalities (186). In a prospective

follow-up study most (70%) patients with aneuploidy or increased tetraploidy in their initial biopsy specimens progressed toward high grade dysplasia or carcinoma. In contrast patients without DNA-FCM abnormalities did not develop high grade dysplasia or carcinoma (187).

2.9 The E-Cadherin-Catenin Complex.

The transmembrane 120 kDa glycoprotein E-Cadherin belongs to the family of calcium dependent adhesion molecules (190, 191). The E-Cadherin gene has been mapped on chromosome 16q22.1 (192). Cadherins play an important role in morphogenesis of tissues during embryogenesis (193). E-Cadherin is part of a larger system, and in addition to Catenins also interacts with Plakoglobin and a tyrosine kinase substrate, p120cas (194-196). As E-Cadherin is responsible for tight cellular connections in mature cells which arrange epithelial architecture, expression of E-Cadherin between epithelial cells is concentrated at tight junctions, adherens junctions and desmosomal regions (197). It has become apparent that homotypic E-Cadherin cell-cell mediated adhesion is structurally organized as a zipper (198). In malignancies, dissolution of cell-cell adhesions is the first step in invasion. There is substantial evidence that reduced expression or inhibited function of E-Cadherin leads to detachment of cells and increased invasiveness. For instance in vitro, monoclonal antibodies against E-Cadherin or blocking of E-Cadherin expression in canine kidney cells leads to transformation into an invasive phenotype (199). In the other direction, induction of E-Cadherin expression inhibits the invasive phenotype (195). In addition to its role in cellular adhesion, E-Cadherin is also involved in signal transduction via connections with Catenins and the actin cytoskeleton (200). Furthermore, aberrant Catenin expression may impair normal E-Cadherin function (201). The Catenin anchorage complex, which interacts with the cytoplasmic domain of E-Cadherin, consists of a heteromere of at least three proteins: Alpha-, Beta- and Gamma-Catenin. Alpha-Catenin a 102 kDa protein, homologous to Vinculin, is ubiquitously expressed in cells and mediates E-Cadherin connection to actin via Beta-Catenin (200-202). At least two isoforms of Alpha-Catenin have been cloned (Alpha-Neuro and Alpha- Epith-Catenin) (201). In turn, Beta-Catenin, a 92 kDa protein, homologous to the Drosophila armadillo gene and human plakoglobin, is directly linked to E-Cadherin (203, 204). The role and organization of Gamma-Catenin is not well understood, it appears that Gamma-Catenin has high homology to or

might be identical with plakoglobin (194, 205).
Normal squamous epithelial cells, except in the most superficial keratinizing layer, intensely express E-Cadherin as well as Alpha- and Beta-Catenin on all cell-cell boundaries (206-208). Likewise, strong membrane bound expression of E-Cadherin and Catenins can be found at cell-cell boundaries of metaplastic columnar epithelia such as Barrett's esophagus (207-210).
In esophageal adenocarcinomas aberrant E-Cadherin expression has been observed in 88% (45/51) of the cases, 47% (24/51) of which showed disorganized E-Cadherin, whereas 41% (21/51) showed reduced expression (209). Aberrant expression correlated with poor differentiation and advanced stages of disease. Interestingly, northern blot analysis revealed in terms of size normal E-Cadherin and Alpha-Catenin mRNA expression in adenocarcinomas with normal but also in those with disorganized E-Cadherin expression. These findings imply that disorganized E-Cadherin expression with impaired E-Cadherin function might be indirectly induced by factors which interact with E-Cadherin. Beta-Catenin is such a potential factor. However, no literature data is available regarding Alpha- and Beta-Catenin expression in esophageal adenocarcinomas. In chapter 7, E-Cadherin, Alpha- and Beta-Catenin is investigated in 65 esophageal adenocarcinomas. Reduced E-Cadherin, Alpha- and Beta-Catenin expression was observed in between 60 and 75% of cases. Reduced expression of E-Cadherin and Alpha-and Beta-Catenin correlated with unfavorable clinico-pathological parameters, such as poor differentiation grade, advanced stage disease and poor patient survival.

2.10 CD44 and CD44 splice variants

CD44 is a family of cell surface molecules, involved in intercellular interactions such as lymphocyte homing, lymphocyte activation, hemopoiesis, cell migration and metastasis, but also in binding of cytokines, hyaluronate and collagen (211, 212). The CD44 gene has been mapped on chromosome 11p13 (213). The gene consists of at least 20 exons, 10 of which (exons 1-5 and 16-20) encode for the standard form of CD44 (CDst). At least ten remaining exons are subjected to alternative splicing. Differential expression of these exons give rise to several CD44 isoforms (214-216). The structure of the CD44 glycoprotein is complex. Its standard form (CDST), a 85-90 kDa glycoprotein, is involved in the functions mentioned above. So far, specific ligands which interact only with the variable

regions, have not been identified. In the first experiments which led to the discovery of splice variants, monoclonal antibodies raised against membrane epitopes of a metastasizing rat pancreatic cell line transiently inhibited the metastatic potential of these cells (217, 218). The cDNA sequence encoding the epitope recognized by one of these antibodies, appeared to be part of the exon encoding the v6 variant (216). Transfection experiments with cDNA partly coding for v6 conferred metastatic potential to a non metastatic rat pancreatic cancer cell line (219). The application of monoclonal antibodies raised against the transfected epitope blocked metastatic potential of the transfected cell line (220). In various malignancies expression of several CD44 splice variants, such as 5v, 6v, 7v, 8v and 9v, has been related with increased tumor invasiveness, metastatic potential and patient survival (221-228). Nonetheless, splice variants are not exclusively expressed in metastatic cells. The epithelial CD44 version (CD44E) besides standard CD44 also includes the 8-9 and 10 variable exons (229-231). CD44 isoforms are typically expressed by cells that participate in dynamic processes, such as normal tissue development, regenerative processes, inflammation and wound healing. Thus normal expression of CD44 isoforms can be found in embryonic cells, activated T and B lymphocytes, and in proliferative zones of both squamous and columnar epithelium (232). Although the exact mechanisms are not yet known, regulated CD44 isoform expression presumably plays a major role in cell movement and cell-cell and cell-extra cellular matrix contacts (233). In metastatic cells genetic events may initiate expression of CD44-isoforms, rendering cancer cells metastatic.

Little literature data is available concerning CD44 and CD44 isoform expression in Barrett's esophagus and esophageal adenocarcinomas. In chapter 8, a panel of tissues from Barrett's esophagus, esophageal adenocarcinomas and lymph node metastases was investigated for CDST and CD44v6 expression. Expression of CDST in Barrett's esophagus correlated with grade of dysplasia and proliferation, whereas increased CD44V6 expression appeared to occur early during neoplastic progression. Interestingly, in the esophageal adenocarcinomas increased CD44V6 expression tended to be correlated with worse five year survival.

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Chapter 3

Aim of the Study and Introduction to the Papers

3.1 From Barrett's esophagus to esophageal adenocarcinoma and metastasis.

A major difficulty concerning Barrett's esophagus is whether to screen or not to screen (70). Only a fraction of Barrett's esophagus cases will progress to malignancy. Consequently, screening of all cases of Barrett's esophagus' for malignant degeneration is not efficient. However, patients with early stage disease as discovered in screening programs have significantly better prognosis than patients with advanced stage disease. Therefore, a screening approach, which can predict in an early phase which Barrett's esophagus will progress to malignancy is of paramount importance. At present biological parameters with this potential has not been found. It should be realized that, malignant progression in Barrett's esophagus may follow several pathways in which many different factors are involved. Histopathological assessment of dysplasia, although to a certain degree subjective, seems to give the best reflection of the level of progression in a lesion. Any expectation to be able to replace histological assessment with a single biological parameter is, given the complexity of the process involved, not realistic. As important as identifying Barrett's esophagus patients at risk for neoplastic transformation is improving treatment of patients with esophageal adenocarcinoma. As the incidence of esophageal adenocarcinomas is rapidly rising, optimal care and treatment of this group of patients is of increasing importance. In general, patients with esophageal adenocarcinoma have a poor prognosis. The extent and aggressiveness of disease are important parameters for selecting treatment strategies. The tendency of esophageal adenocarcinomas to metastasize in early phase of disease, sometimes with only limited invasion of the esophageal wall, is poorly understood. Histopathologically, esophageal adenocarcinomas are heterogeneous, varying from well differentiated to undifferentiated phenotypes. Nevertheless, grade of differentiation seems not to be a good parameter for behavior of these malignancies. Although stage indirectly determines the aggressiveness of disease and accurately predicts long term survival of patients, it does not yield specific information about the intrinsic mechanisms which are involved in tumor behavior.

3.2 Aim of the study

The aim of this study was to assess the potential role of a number of events,

related to carcinogenesis in the evolution of Barrett's esophagus towards esophageal adenocarcinoma and metastasis. The information would help us to understand how this cancer develops and how metastases occur, and pointly at an early stage to detect which benign lesions might be prone for malignant progression, and which carcinomas tend to metastasize. Parameters were chosen which are related to molecular genetic events, cellular proliferation, and intercellular adhesion. Each of these factors plays a role in neoplastic progression. As the progression of Barrett's esophagus towards adenocarcinoma may take up to several decades, not a longitudinal but a transsectional approach was used. For such an approach archival material collected from endoscopically surveyed Barrett's patients and surgical resection specimens of patients with esophageal adenocarcinoma provides an ideal basis.

3.3 Introduction to the papers

Chapter 4:
"Detection of genetic changes in Barrett's adenocarcinoma and Barrett's esophagus by DNA in situ hybridization and immunohistochemistry."
The main goal of this paper was to screen a pilot of archival resected esophageal adenocarcinomas for common numerical chromosomal aberrations, aneuploidy, proliferation and p53 protein accumulation. By immunohistochemistry and the application of in situ hybridization with DNA probes on tissue sections, both the adenocarcinoma as the adjacent Barrett's esophagus in the resection specimens could be evaluated. The findings in this paper formed the basis for the next studies.

Chapter 5:
"Accumulation of p53 protein in normal, dysplastic and neoplastic Barrett's esophagus."
In this paper the intention was to determine the frequency of p53 protein accumulation in esophageal adenocarcinoma and to investigate whether aberrant p53 is a prognostic parameter for neoplastic progression in Barrett's esophagus. By immunohistochemistry on paraffin sections, p53 protein accumulation was studied in a large penal of Barrett's esophagus with varying degrees of dysplasia and in a number of esophageal adenocarcinomas.

Chapter 6:

"Accumulation of genetic abnormalities during neoplastic progression in Barrett's esophagus."

In an earlier study (chapter 4), Y-chromosome loss and aneuploidy were found in a subset of esophageal adenocarcinomas and in Barrett's esophagus adjacent to these carcinomas. In this study the meaning of these aberrations was further investigated. In the adenocarcinomas the frequencies of Y-loss and aneuploidy were determined in archival resection specimens. In cases of Barrett's esophagus not related to adenocarcinoma the prognostic value of Y-loss and aneuploidy was studied. In situ hybridization with DNA probes on archival tissues allowed us to evaluate these genetic aberrations with respect to grade of dysplasia in a series of biopsy specimens of patients with Barrett's esophagus.

Chapter 7:

"E-Cadherin, Alpha- and Beta Catenin expression in Barrett's adenocarcinoma."

The goal of this study was to investigate expression of several members of the E-Cadherin-Catenin complex in esophageal adenocarcinoma and to evaluate the results against clinico-pathological criteria and patient survival. Immunohistochemistry was applied to a series of archival resection specimens and patient follow-up data was analyzed.

Chapter 8:

"CD44st and CD44v6 splice variant expression in Barrett's esophagus and Barrett's adenocarcinoma."

In this paper upregulated expression of CD44st and CD44v6 was investigated in esophageal adenocarcinomas to determine whether these parameters are prognostic for aggressive tumor behavior. In Barrett's esophagus the prognostic value of upregulated CD44st and CD44v6 expression for malignant progression was evaluated. Immunohistochemistry was applied to archival material, consisting of esophageal resection specimens with adenocarcinomas and a series of archival biopsied material of patients with Barrett's esophagus. Data was evaluated against clinico-pathological parameters of the adenocarcinomas, and against dysplasia and proliferation rate in Barrett's esophagus.

Chapter 4

Detection of Genetic Changes in Barrett's Adenocarcinoma and Barrett's Esophagus by DNA in Situ Hybridization and Immunohistochemistry.

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4.1 Abstract

We have investigated the occurrence of chromosomal DNA and cell cycle related protein changes in Barrett's epithelium and adenocarcinoma. The presence of numerical chromosomal aberrations was studied by applying non-isotopic in situ hybridization (ISH) with (peri-)centromeric DNA probes, specific for chromosomes 7, 8, 17, and Y, to routinely processed tissue sections of five cases (4 male , 1 female) of Barrett's adenocarcinoma and adjacent Barrett's epithelium. Cell cycle related protein expression was studied by immunohistochemistry (IHC) for p53 protein and the Ki-67 antigen (Mib-1) in subsequent sections. P53 protein overexpression was found in 3 of the 5 tumors. Over representation of chromosome 8 and loss of chromosome 17 were found in two adenocarcinomas, both also negative for p53 protein overexpression. Y-loss, mostly clonal, was detected in 3 of the 4 male adenocarcinomas and 2 cases of adjacent Barrett's epithelium. One tumor had both areas of over representation and loss of the Y chromosome. All Barrett's adenocarcinomas appeared to contain aneuploid cell populations. No relation was found between cell proliferation characteristics and chromosomal aberrations. We conclude that ISH with chromosome specific DNA probes can be applied for the assessment of potentially important numerical chromosome changes in Barrett's esophagus. Further, the combination of IHC and ISH is useful for evaluation of specific genetic events.

4.2 Introduction

In Barrett's disease the stratified squamous epithelium of the distal part of the esophagus is replaced by columnar epithelium, a process called metaplasia(1, 2). This condition is associated with a 30 to 40 times increased risk of developing esophageal adenocarcinoma (3, 4). Even after surgical resection and/ or radiotherapy and chemotherapy, Barrett's adenocarcinoma has a poor prognosis(5). Early diagnosis of malignancy in Barrett's esophagus is essential for optimal treatment and better prospects for the patient. Therefore a better understanding of the underlying mechanisms of carcinogenesis in Barrett's epithelium is of great importance.

The progression to malignancy in Barrett's epithelium is a multistep process: Metaplasia progresses to dysplasia and eventually to adenocarcinoma. This

neoplastic transformation is related to major genetic changes ultimately progressing into malignancy (6). The correlation between aneuploidy and histological diagnosis of dysplasia or carcinoma has been well demonstrated in prospective studies by flow cytometric techniques (7, 8). However, little is known about the specific chromosomal aberrations. Karyotyping revealed frequent loss of the Y-chromosome in Barrett's epithelium (9), while structural rearrangements were found in the 11p13-15 region in Barrett's adenocarcinoma (10).

P53 gene mutations and protein overexpression has been demonstrated in Barrett's adenocarcinomas, and in Barrett's esophagus (11, 12). Genetic analysis demonstrated a high incidence of loss of heterozygosity (LOH) of multiple tumor suppressor genes, including p53, in Barrett's adenocarcinomas (13). Cytogenetic studies of other gastrointestinal malignancies, i.e., gastric adenocarcinomas revealed frequent loss of the Y-chromosome and over representation of chromosomes 7, 8, 9 and 12 (14, 15). In colon carcinomas a frequent loss of chromosomes 18, 22, Y and gain of the chromosomes 7, 8, 12, 13, 20 was shown (16).

The cytogenetic study of solid tumors by karyotyping is often unprofitable and strenuous (17). In contrast, analysis of numerical chromosomal aberrations by non-isotopic in situ hybridization (ISH) to isolated nuclei of solid tumors has proved to be efficient and successful (18-22). ISH on formalin-fixed paraffin-embedded tissue sections created the possibility of chromosomal analysis in archival material (23-26). The latter ISH technique has the great advantage of preserved morphology leaving different cell types distinguishable and enabling analysis of the tumorigenic cells only (27).

In this study we analyzed the presence of numerical chromosomal aberrations by applying non-isotopic in situ hybridization with (peri-)centromeric DNA probes, specific for chromosomes 7, 8, 17 and Y, to five archival cases of Barrett's adenocarcinoma and adjacent Barrett's epithelium. In each case cardia tissue and leucocytes were used as controls. Karyotyping of all five specimens had failed to produce analyzable metaphases of Barrett's epithelium or adenocarcinoma cells. Cell proliferation was estimated by immunolabeling of the Ki-67 antigen through it's reactivity with Mib-1 (28). Immunohistochemistry was also used to detect overexpression of the p53 protein.

4.3 Materials and methods

4.3.1 Tissue preparation

Paraffin-embedded material, obtained between 1990 and 1991, of five esophageal-cardia resection specimens containing adenocarcinoma in Barrett's epithelium was used. In situ hybridization (ISH), immunohistochemistry (IHC) and flow cytometry were performed on subsequent tissue sections. For ISH and IHC, 4 μm sections were sliced from paraffin blocks containing tumor and/or Barrett's epithelium. Sections were adhered to amino alkyl-coated slides (Sigma, St Louis, MO) for better preservation of tissue architecture.

Table 1: Tumor/Patient information

Case	Age/Sex	Tissue	Stage[1]	Grade[2,3]
#1	75/F	Adenocarcinoma Epithelium	T1N0M0 High	G1
#2	69/M	Adenocarcinoma Epithelium	T3N1M0 High	G3
#3	65/M	Adenocarcinoma Epithelium	T3N1M1 Moderate	G3
#4	61/M	Adenocarcinoma Epithelium	T3N1M1 Low	G3
#5	70/M	Adenocarcinoma Epithelium	T3N2M1 Moderate	G3

[1] *Tumor staging by TNM classification; T1: Tumor infiltration in submucosa, T2: in muscularis propriae, T3: in adventitia and beyond. N0: lymphe node metastasis are absent, N1: regional lymph node metastasis, N2: distant lymphe node metastasis, M0: no organic metastasis, M1: organ metastasis.*

[2] *Histopathological grade of differentiation in tumor: G1-G2-G3 = good-moderate-poor differentiation.*

[3] *Histopathological grade of dysplasia in Barrett's epithelium: low-moderate-high.*

4.3.2 Probe and probe labeling

A probe set specific for chromosomes 7, 8, 17, and Y was selected. Selection criteria were based on literature data considering numerical (and structural) aberrations in esophageal and other gastro-intestinal tumors (2,10,11,12,41). The (peri)centromeric repetitive satellite DNA probes were labeled with biotin-14-dATP by nick translation of complete plasmid DNA according to manufacturer's directions (BRL, Gaithersburg, MD). DNA probes were stored at -20 °C.

4.3.3 In Situ Hybridization

The in situ hybridization procedure was performed as described by van Dekken et al. (27). Briefly, after deparaffinizing of the tissue sections in xylene, endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol. Enhancement of tissue permeability was achieved by proteolytic enzyme digestion (pepsin: Sigma, 4 mg/ml in 0.2 M HCl) for 15-20 minutes at 37 °C. Before applying the probe set, the optimal digestion time for each tumor was determined by a pepsin time series (5, 10, 15 and 20 minutes).

Tissue DNA was heat denatured for 2 minutes in 70% formamide in 2xSCC (pH 7.0), followed by dehydration in an ethanol series. Chromosome specific repetitive DNA probes were denatured for 5 minutes at 70 °C in a hybridization mixture, containing: 1-2 μg/ml probe DNA, 500 μg/ml sonicated herring sperm DNA (Sigma), 0.1% Tween-20, 10% dextran sulphate, and 60% formamide in 2xSSC at pH 7. The probe mixture was then applied to the sections and incubated overnight at 37 °C. Then the slides were washed in 60% formamide in 2xSSC (pH 7.0) at 42 °C.

Histochemical detection was performed by immunoperoxidase staining. Slides were subsequently incubated for 30 minutes at 37 °C with mouse-anti-biotin (Dakopatts, Glostrup, Denmark), biotin-labeled horse-anti-mouse (Vector, Burlingame, CA) and with avidin-biotin-complex (Vectastain Elite ABC Kit). Visualisation was achieved with 0.05% H_2O_2, 0.5 g/l DAB, 0.1 M imidazole in PBS (Phosphate Buffered Saline). The signal was amplified with 0.5% $CuSO_4$ in 0.9% NaCl. Slides were rinsed in distilled water and counterstained with hematoxylin. Finally, slides were dehydrated in ethanol, cleared in xylene and mounted in Malinol (Chroma-Geselschaft, Köngen, Germany).

4.3.4 Immunohistochemistry

For immunostaining of p53, DO-7 (Dakopatts) was applied, diluted 1/25 in PBS for overnight incubation. This monoclonal antibody recognizes an epitope in the N-terminus, residing between amino acids 35 and 45 of the human p53 protein. Primary labeling of the Ki-67 antigen was performed with a monoclonal antibody, Mib-1 (Immunotech, Marseille, France), diluted 1/25 in PBS. The monoclonal Mib-1 reacts with native Ki-67 antigen and recombinant fragments of the Ki-67 molecule (28). When compared with staining of Ki-67 on fresh (frozen) tissues, Mib-1 shows corresponding staining of proliferating cells in formalin-fixed paraffin embedded tissues (29). For negative controls the primary antibodies were replaced by PBS.

Before immunolabeling enhancement of p53 protein staining by DO-7 was achieved by heating slides submerged in distilled water in a microwave (700 W) at 95 °C for 2x5 minutes with an interval of 1 minute. During this interval fluid level was checked and restored. The same procedure was followed for Ki-67 staining by Mib-1, using citrate buffer (10 mM citric acid monohydrate, pH 6.0) instead of distilled water. Immunohistochemistry was performed using the routine ABC-immunoperoxidase method (Vector).

4.3.5 DNA flow cytometry

DNA content of the paraffin material was measured, basically as described by Hedley et al. (30). Tumor, dysplastic and cardia regions were selectively cut out of the paraffin blocks. Flow cytometry and analysis of the ethidium bromide (Sigma) stained nuclei from these areas was carried out using a Facscan (Becton Dickinson, Mountain View, Ca).

4.3.6 Analysis

Results of ISH with probes for chromosomes 7, 8, 17, and Y were evaluated on consecutive 4 μm sections in previously defined areas. This approach was applied to reduce artefacts due to tumor heterogeneity and section thickness. Leucocytes on the same sections served as controls for hybridization quality, cardia cells were used as a tissue specific control. For each probe the number of solid DAB spots was scored (0, 1, 2, 3, 4, >4 spots) of 100 distinct, non-overlapping, single or side by side 4 μm nuclear slices. Scoring was performed by two independent investigators. The counted spot distributions were compared,

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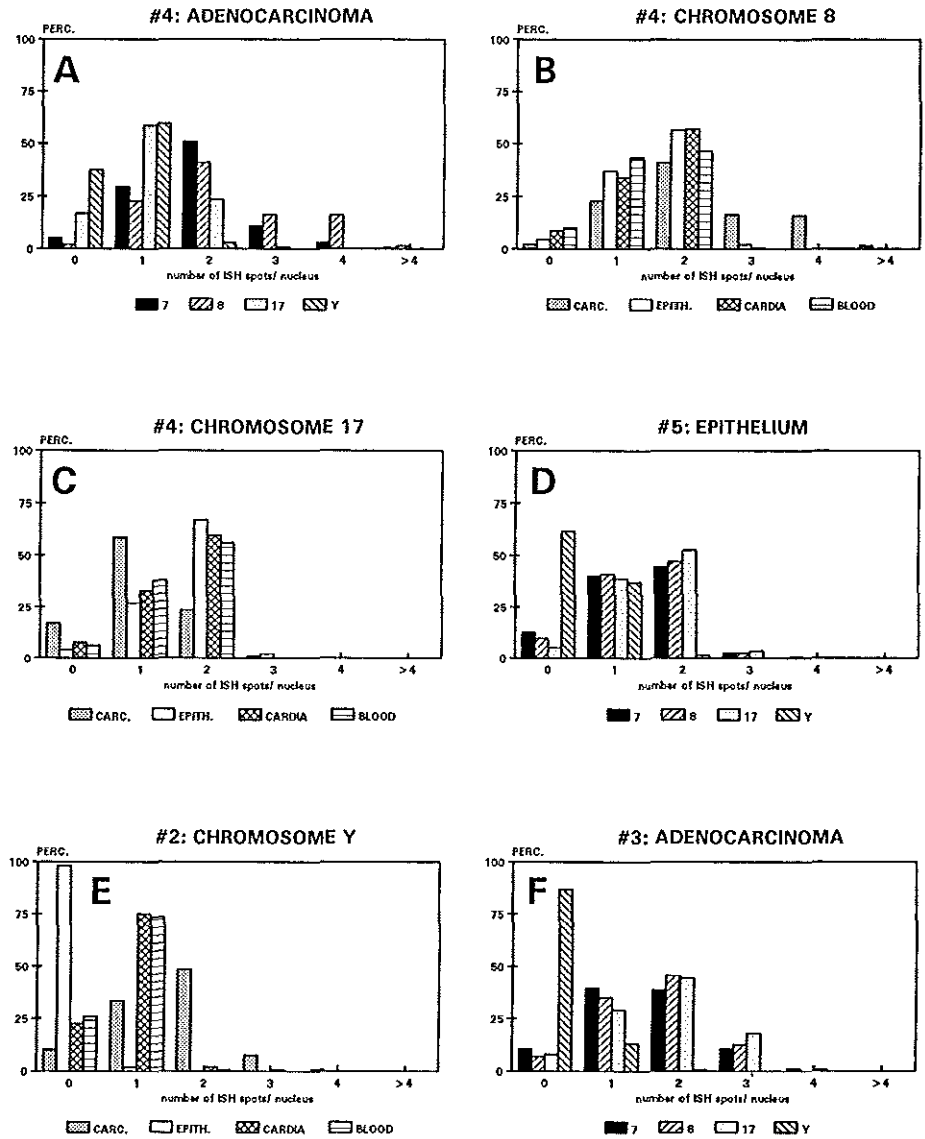
Figure 1: (A) Frequency distributions of the hybridization spots, obtained by in situ hybridization (ISH) on 4 μm tissue sections with (peri-)centromeric DNA probes, specific for chromosomes 7, 8, 17 and Y, in Barrett's adenocarcinoma of patient #4. For each DNA probe the number of hybridization spots (0,1,2,3,4,>4) was determined in 200 nuclei and expressed as a percentage. For each of the DNA probes a different spot distribution was found. The chromosome 7 profile was taken "modal", based on the flow cytometric DNA index. Then a gain can be seen for chromosome 8, whereas loss is found of 17. (B) Chromosome 8 DNA probe distribution in Barrett's adenocarcinoma (carc.), Barrett's epithelium (epith.), normal cardia tissue and leucocytes (blood) of patient #4. Strong hyperdiploidy (gain) of chromosome 8 is seen in the adenocarcinoma. (C) In the same case the chromosome 17 DNA probe distribution shows loss of chromosome 17 in the adenocarcinoma. (D) Spot distribution of DNA probes 7, 8, 17 and Y in Barrett's epithelium of case #5 reveals non-complete loss of chromosome Y. (E) Spot pattern of the Y probe in patient #2 shows complete loss in Barrett's epithelium, but not in the tumor. Note the presence of nuclei with more than one copy of Y in this tumor. (F) Spot distributions of the DNA probes in the adenocarcinoma of patient #3 shows loss of the Y chromosome.

Figure 1


#4: ADENOCARCINOMA
PERC.
A
number of ISH spots/ nucleus
7
8
17
Y
#4: CHROMOSOME 8
B
CARC.
EPITH.
CARDIA
BLOOD
#4: CHROMOSOME 17
C
#5: EPITHELIUM
D
#2: CHROMOSOME Y
E
#3: ADENOCARCINOMA
F

averaged and plotted in bar histograms. Hyperdiploidy was estimated by determining the mean percentage of cells with more than 2 spots per nucleus for the autosomal probes (7, 8, and 17). In general, ISH hyperploidy was closely associated with the results of DNA flow cytometry. Therefore, in case multiple ISH aberrations occurred, the DNA probe distribution(s) that followed the flow cytometric profile most closely, was assigned "modal". For statistical analysis of the spot distributions the Kolmogorov-Smirnov test was used (27).
Cellular proliferation was estimated by determining the percentage of Ki-67 positive cells. The percentages of both p53 and Ki-67 positive cells were established by serial counting of 200 cells in the same areas. Again scoring was performed by two independent investigators.

Table 2: Results of in situ hybridization (ISH) with a set of chromosome specific repetitive DNA probes (7, 8, 17 and Y), DNA flow cytometry, and immunohistochemistry with DO-7 (P53) and Mib-1 (Ki-67) of five cases of Barrett's adenocarcinoma (A) and epithelium (E).

Case	Tissue	Ki-67[1] (%)	P53[2] (%)	DI[3] (p2%)	ISH[4] aneuploidy	ISH[5] aberrations
#1	A	23%	78%	1.6/51	18%	
	E	27	48	1.6/51	7	
#2	A	46	80	1.7/63	54	
	E	15	21	1.7/40	19	-Y
#3	A	46	89	1.6/16	14	-Y
	E	17	0	1.0/-	3	
#4	A	55	0	1.9/16	16	+8,-17,-Y
	E	21	0	1.0/-	2	
#5	A	40	0	2.0+2.7/10[6]	17	+8,-17,+Y/-Y
	E	15	0	1.0/-	3	-Y

[1] *Ki-67: proliferation estimated by the percentage of Ki-67 positive cells.*
[2] *Percentage of p53 positive cells.*
[3] *DNA Index by flow cytometry, (p2%) is percentage of cells in aneuploid peak.*
[4] *Percentage of hyperdiploid cells (> 2 spots per nucleus) measured by ISH with centromeric DNA probes for autosomal chromosomes 7, 8 and 17.*
[5] *Kolmogorov-Smirnov test: $p < 0.01$.* [6] *Percentage of cells in two aneuploid peaks.*

4.4 Results

We have used in situ hybridization (ISH) with a set of (peri-)centromeric DNA probes and immunohistochemistry (IHC) for cell cycle related proteins of five Barrett's adenocarcinomas and adjacent Barrett's epithelium. Normal cardia and

blood cells within the same specimen served as controls. One (female) tumor was well differentiated (G1), while infiltration was limited to the submucosa (T1). Four (male) poorly differentiated (G3) tumors infiltrated the adventitia (T3) and were metastatic. Grade of dysplasia in Barrett's esophagus was determined following a standardized classification (31). Two of the five adjacent Barrett's epithelia showed high grade dysplasia, two showed moderate and one low grade dysplasia. Surgical intervention was at a mean age of 68 (tab 1). ISH with specific centromeric probes for chromosomes 7, 8, 17 and Y, revealed a number of abnormalities in Barrett's adenocarcinoma as well as in Barrett's epithelium (tab 2). Specific chromosomal aberrations were found for chromosomes 8, 17 and Y (Figs 1 and 2 ; P values <0.01). Over representation of chromosome 8 and loss of chromosome 17 were found in the adenocarcinoma of patient #4 (figs 1A-C and 2A-C) and of patient #5. Both cases were negative for immunoreactivity of p53. The ISH aberrations could not be distinguished in the control cells (figs 1B-C). P53 overexpression was found in the other 3 cases of adenocarcinomas, two of which also had p53 immunoreactivity in the adjacent Barrett's epithelium, i.e. patients #1 and #2. These two cases of Barrett's epithelium also contained significant amounts of aneuploid nuclei (tab 2). Loss of chromosome Y was seen in 3 of the 4 male tumors, and in the Barrett's epithelia of case #2 and #5 (figs 1D-F and 2D-F; tab 2). This loss appeared to be clonal in both Barrett's epithelium and tumor of patient #5 (figs 1D and 2E-F), and in the tumor of case #3 (fig 1F). Tumor #5 also showed loss and over representation of the Y-chromosome in distinct areas (fig 2F).

In cardia specimens the proliferation index (PI), as measured by Mib-1 staining (Ki-67 antigen expression), was approximately 12%, while staining was limited to parabasal crypt cells (fig 3A). Slightly elevated Mib-1 staining was found in Barrett's epithelium (fig 3B; tab 2).

Figure 2(A) In situ hybridization to a 4 μm section of Barrett's adenocarcinoma (arrows) of patient #4 with the chromosome 7 specific DNA probe. The ISH-related spots were visualized with immunoperoxidase/DAB (black); hematoxylin was used as a counterstain (grey). (B) In the same tumor ISH with the chromosome 8 specific DNA probe shows many hyperdiploid (>2 spots) nuclei in strings of tumor cells (arrows). (C) ISH with the chromosome 17 specific DNA probe reveals numerous nuclei with 0 or 1 spot, indicating loss of this chromosome (arrows: tumor cells). (D) Barrett's epithelium of patient #2 demonstrates Y-loss in the epithelial cells (arrows), but not in the stromal cells (arrowheads). (E) ISH with the chromosome Y probe in Barrett's epithelium of case #5 shows clonal loss in the epithelial cells: Glands with (o) and without (*) this chromosome can be distinguished. (F) Cell nuclei with and without Y-loss are also seen in the adenocarcinoma of patient #5. A 40x objective was used in A-F.

Figure 2
(A,B,C)

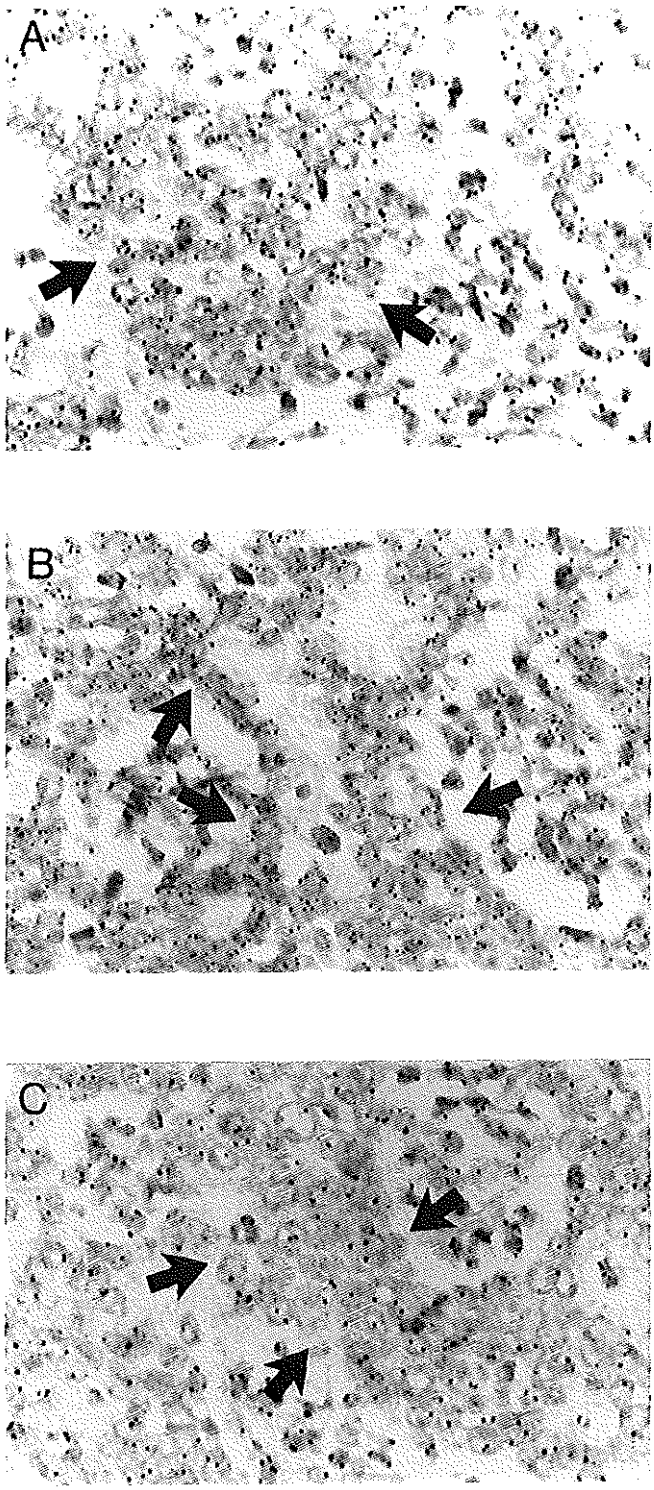
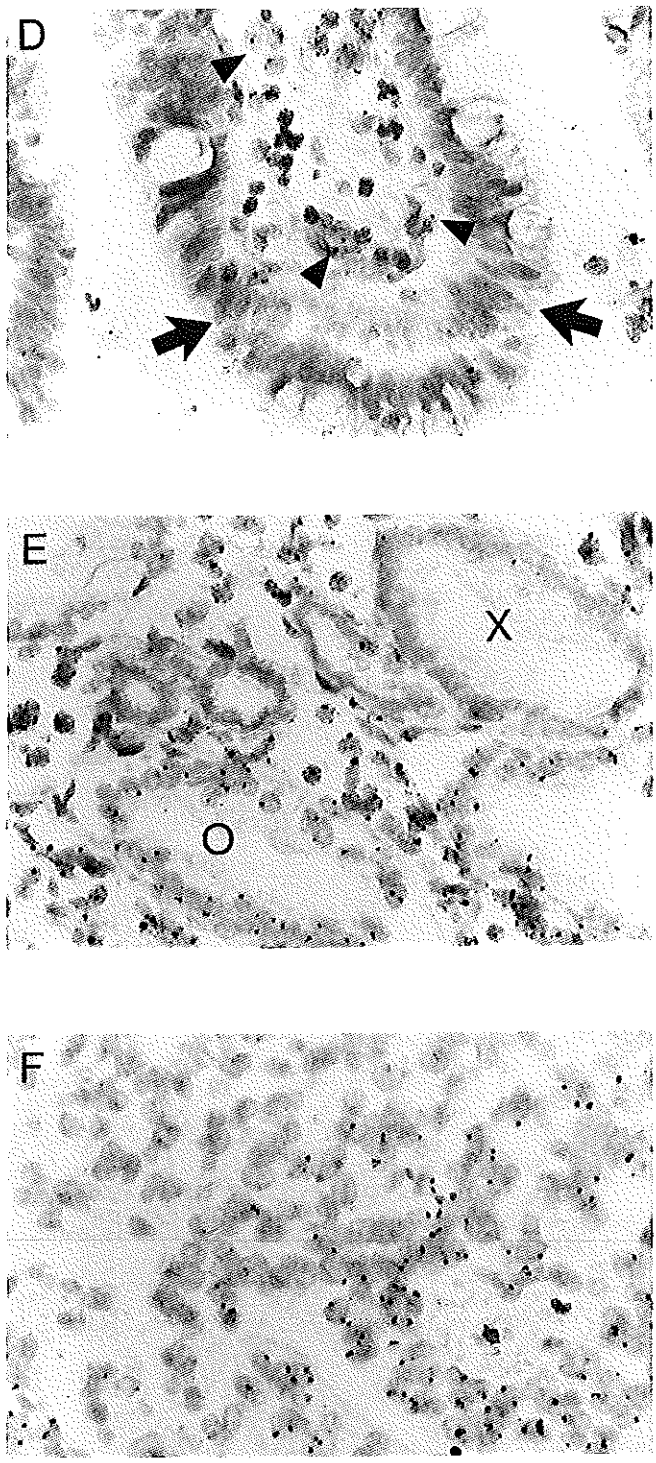


Figure 2
(D,E,F)



In the low stage and grade carcinoma of patient #1 PI was 23% (fig 3C; tab 2). In 4 cases of adenocarcinoma, all four poorly differentiated and metastatic, PI was >30%. No significant correlation was found between Y-loss or other chromosomal changes, and the PI. By means of ISH we were able to distinguish aneuploidy (>2 spots/nucleus) in all adenocarcinomas (range: 14-54%) and in two adjacent Barrett's epithelia (tab 2). In contrast, no significant percentages of ISH aneuploid cells were seen in cardia and leucocytes (<1%).

4.5 Discussion

The analysis of numerical chromosomal aberrations by ISH in five Barrett's adenocarcinomas and adjacent Barrett's epithelium revealed specific changes for chromosomes 8, 17 and Y. Loss of the Y chromosome has been described in different gastro-intestinal malignancies(14, 15, 32). Garewal found Y-loss in seven out of ten cases of Barrett's disease (9). Some investigators related Y-loss to a high proliferation rate of tissues, e.g. bone marrow cells (33). This is not supported by our study, since we did not see a correlation between proliferation rate (Ki-67) and loss of the Y chromosome. In a previous experiment combining proliferation markers and ISH no relationship was either found between loss of a chromosome (monosomy 9) and growth characteristics in bladder cancer cells (34). In our study proliferation rates in Barrett's esophagus were somewhat low, when compared with previous data(35). Our series however, is too small to draw conclusions. High copy numbers of chromosome 8 were found in two cases of Barrett's adenocarcinoma. Over representation of chromosome 8 has been reported in one case (out of four) of Barrett's adenocarcinoma (10). +8 is also seen in other malignancies of the gastrointestinal tract. In our set of tumors we did not detect the more common +7 abnormality.

Figure 3(A) Immunohistochemical staining of the Ki-67 antigen with the monoclonal antibody Mib-1 in normal cardia tissue, counterstained with hematoxylin. Proliferation (Ki-67 antigen expression) is limited to parabasal crypt cells (arrows). (B) Barrett's epithelium of patient #5 shows a slightly higher number of Mib-1 positive cells. (C) Mib-1 staining in Barrett's adenocarcinoma of patient #1 displays immunoreactivity in a high number of tumor cells. (D) P53 immunohistochemistry with monoclonal DO-7 in the same case shows positive reaction in a corresponding pattern. Tissue was counterstained with Light Green. A 10x obj. was used in A-D.

Figure 3
(A, B)

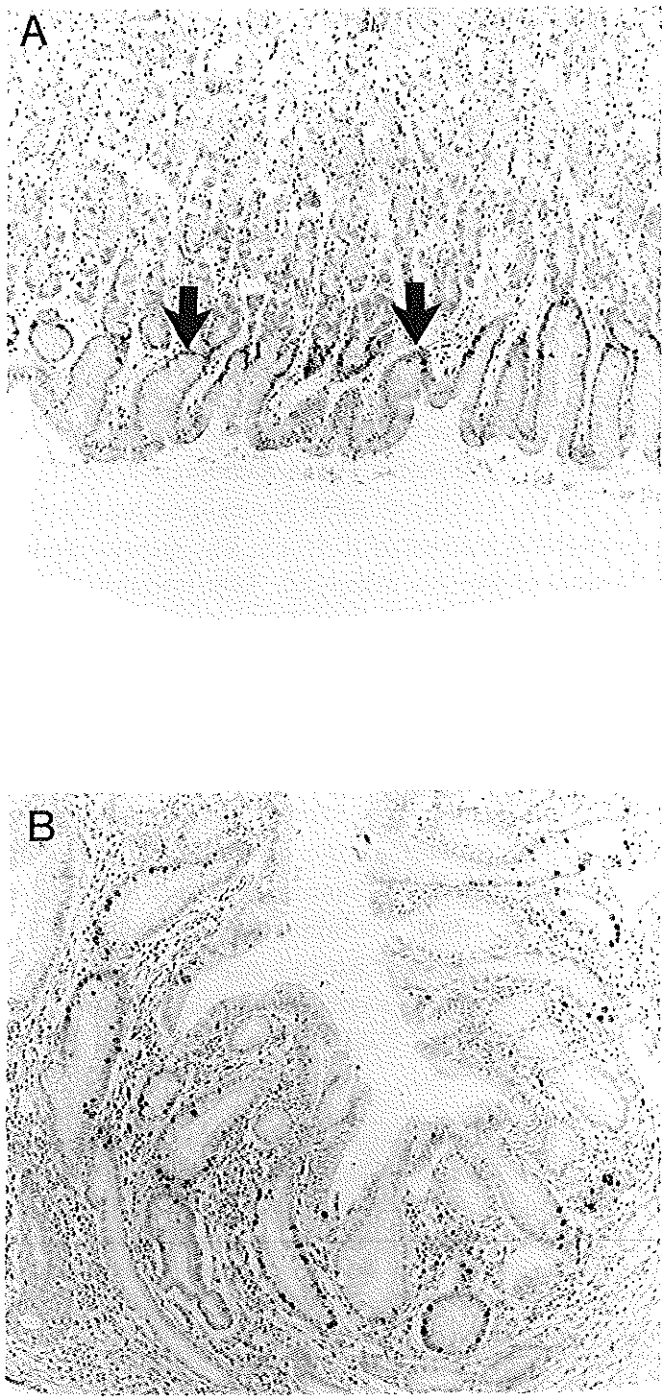
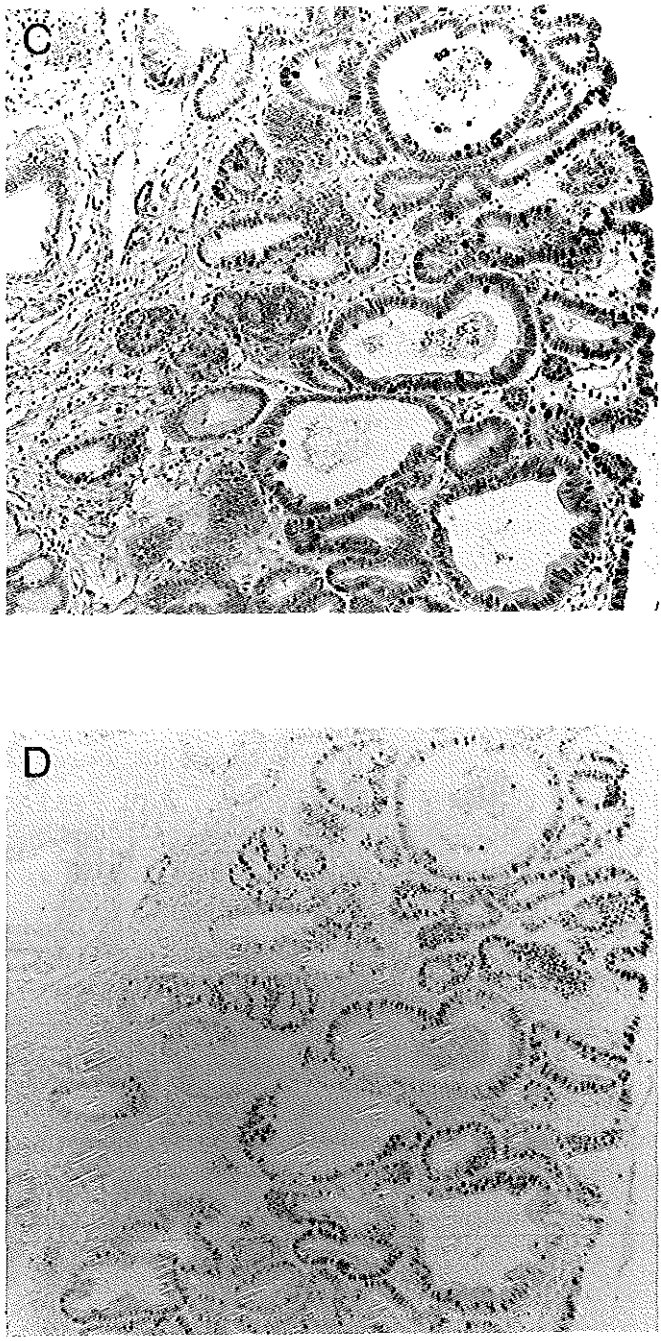


Figure 3
(C, D)



The numerical changes of the Y chromosome appeared to be clonal in most cases. These cytogenetically different areas did not contain simultaneous clonal aberrations of the autosomal chromosomes. In one patient (#2: tab 2) chromosome Y was lost in the epithelium, while it was present in the tumor. This apparently indicates that Y-loss is not a subsequent event in carcinogenesis of Barrett's esophagus. Further, Barrett's adenocarcinomas tend to be heterogeneous (36), and clonal Y-loss might not be detected in this case due to tissue selection. These data suggest that presence of the Y chromosome has no relationship with tumor viability, as defined by proliferative and genomic features (21).

Blount et al. found p53 overexpression combined with LOH of the 17p (p53 gene) region in a high number of Barrett's adenocarcinomas (11). Our study demonstrates loss of chromosome 17 in two p53 negative tumors that contained multiple numerical chromosome aberrations (tab 2). We assume that in these two tumors one copy of chromosome 17 is lost while the p53 allele in the remaining chromosome is not expressed due to deletion, non-sense mutations or stop codons in the gene (37, 38). Since p53 allelic deletions have been observed in 92% of Barrett's adenocarcinomas (11), the presence of immunohistochemically undetectable levels of normal p53 protein is less likely. Therefore, future experiments with DNA probes specific for the p53 region are needed to gain more insight in this matter.

Barrett's epithelium is a unique model for studying carcinogenesis (39). Surveillance of patients with this disorder is by periodic endoscopy and biopsies. Surgical intervention is executed only in case of severe dysplasia or carcinoma. Unfortunately, cytogenetic study by karyotyping of surgical specimens of epithelial and cancerous tissue is often unsuccessful. Only one study is available about karyotyping in Barrett's epithelium (9). Karyotyping in our five cases yielded poor results. Interphase techniques such as in situ hybridization and immunohistochemistry demonstrate the possibility to study genetic events in Barrett's epithelium and adenocarcinoma. Future investigations with more refined DNA probe sets and larger panels of protein markers will possibly enable us to further identify genetic changes that are involved in tumorigenesis of Barrett's esophagus.

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Chapter 5

Accumulation of P53 Protein in Normal, Dysplastic and Neoplastic Barrett's Esophagus.

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5.1 Abstract

Accumulation of p53 protein was determined by immunohistochemistry in archival material of biopsy specimens from 102 patients with Barrett's esophagus with different grades of dysplasia, in 24 esophageal adenocarcinomas associated with Barrett's esophagus and in 23 cases of metaplastic epithelium adjacent to these carcinomas. Immunostaining for the p53 protein was found in 23/102 (23%) cases of the Barrett's esophagus biopsies and in 12/23 (52%) cases of Barrett's esophagus adjacent to adenocarcinoma. Significant correlations were found between grade of dysplasia and p53 immunoreactivity in both Barrett's biopsies without adenocarcinoma ($p<0.001$) and Barrett esophagus adjacent to adenocarcinoma ($p<0.05$). In the adenocarcinomas intense nuclear immunohistochemical staining for p53 was diffusely or focally present in 20/24 (83%) of the specimens. In Barrett's esophagus p53 is a progression marker with high expression in high grade dysplasia (89%) and adenocarcinoma (83%).

5.2 Introduction

In Barrett's esophagus the stratified squamous epithelium of the distal esophagus is replaced by metaplastic columnar epithelium (1,2). Patients with Barrett's esophagus have a 30-40 fold increased risk of developing esophageal adenocarcinoma (3,4). Although adenocarcinoma in Barrett's esophagus is preceded by epithelial dysplasia, time course of progression of simple metaplasia into dysplastic Barrett's esophagus and ultimately into infiltrating adenocarcinoma is unknown. Furthermore, grading of dysplasia is to a certain extent subjective, leading to significant interobserver variation. Therefore, objective prognostic criteria predicting which cases of Barrett's esophagus will progress towards malignancy are of major importance. Previous immunohistochemical studies with proliferation markers already showed higher proliferation rates in dysplastic Barrett's esophagus (5) and abnormalities measured by DNA flow cytometry may precede malignancy (6).

During the past years interest in tumor suppressor genes and proteins in (pre)malignant lesions has intensified. The appearance of the p53 tumor suppressor protein has been reported in a wide variety of malignant en premalignant conditions (7-14). P53 immunoreactivity has been reported in 62%-87% of esophageal adenocarcinomas, and in non dysplastic and dysplastic

Barrett's esophagus (15-18).
Aim of the present study was to determine whether immunohistochemically detectable p53 protein accumulation occurs along with the development of dysplasia in Barrett's esophagus.

5.3 Materials and methods

5.3.1 Tissues

P53 immunoreactivity was studied in archival biopsies taken between 1980 and 1992 from 102 patients with Barrett's esophagus with various degrees of dysplasia but without adenocarcinoma. The number of biopsies per patient varied from one to twelve. Furthermore, paraffin-embedded tissue blocks from 24 esophagus-cardia resection specimens with adenocarcinoma in Barrett's esophagus encountered between 1990 and 1992 were investigated. All patients had undergone cardiac-esophageal resection with gastric tube interposition. Surgery was performed at a mean age of 62 (range: 45-80). Non of the patients with adenocarcinoma had received chemo- or radiotherapy prior to surgery. One paraffin block containing adenocarcinoma was selected in each case. To determine the efficacy of p53 immunostaining in paraffin embedded tissues, immunohistochemistry for the detection of p53 protein was performed on corresponding fresh frozen samples of 20 of the adenocarcinomas. Barrett's esophagus adjacent to the tumor was found in 23 cases. In one case Barrett's esophagus was diagnosed in biopsy specimens prior to esophageal resection. Normal gastric-cardiac mucosa was used for control purposes.

5.3.2 Histopathological criteria

The histological sections were reviewed by two pathologists (A.H.M. and F.T.B.) Tumor staging was performed according to pTNM criteria. Barrett's esophagus was defined as columnar epithelial lining of the esophagus of at least three cm proximal to the gastroesophageal junction. All cases had specialized columnar type of metaplasia characterized by the presence of intestinal type of absorptive cells e.g., goblet cells. Dysplasia was graded into three categories following criteria revised by Reid et al. (19), i.e.; negative, indefinite or low grade dysplasia and high grade dysplasia. Slides were coded and scoring of p53 immunoreactivity was performed independent from grading sections.

5.3.3 Immunohistochemistry

Cryostat sections (5 μm) of fresh tissues were airdried and fixed in acetone (-20°C). After blocking of endogenous peroxidase activity and rinsing in phosphate buffered saline (PBS, pH 7.4), p53 protein labeling was performed by standard peroxidase-anti-peroxidase method (Dakopatts Z 259; Sigma P 2416, St Louis, MO).

DO-7 (Dakopatts, Glostrup, Denmark), an Ig2b kappa monoclonal antibody recognizing an epitope in the N-terminus between amino acids 35 and 45 of human p53 protein, was used on formalin-fixed paraffin-embedded tissues.

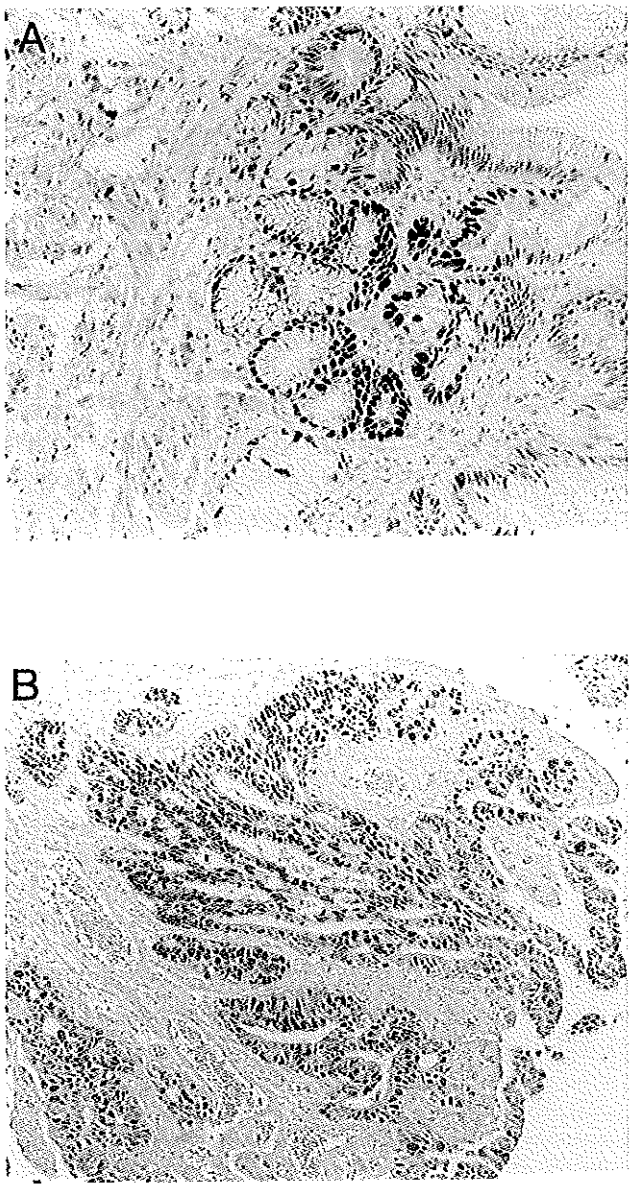
For paraffin sections an antigen retrieval method was applied (20). After blocking of endogenous peroxidase P53 labeling with DO-7 was performed by the streptavidin-biotin-complex procedure. The sections were preincubated for 15 minutes at 37°C with 10% normal goat serum, diluted in PBS. DO-7, diluted 1/25 in PBS, was added for overnight incubation at 4°C. The negative controls were incubated with PBS only. The sections were subsequently incubated with biotinylated goat-anti-mouse IgG (1/400 in PBS, Dakopatts), rinsed and finally incubated with a Streptavidin-biotinylated horseradish peroxidase complex (Dakopatts). Sections were developed for seven minutes in 3.3' diaminobenzidine tetrachloride.

5.3.4 Microscopical evaluation

Positive immunohistochemistry with the DO-7 p53 antibody was considered by moderate or intense brown nuclear staining. Cytoplasmic staining was not found. In a semi-quantitative scoring approach, two staining patterns were distinguished: A focal pattern, with >10% of nuclei positive in clusters was mostly found in non dysplastic and low grade dysplastic Barrett's esophagus. The focal staining was mostly confined to epithelial glands (figure 1A). A diffuse staining pattern with >10% of positive nuclei scattered through the tissue was seen in most adenocarcinomas and high grade dysplasia (figure 1B).

Figure 1 A: Focal immunohistochemical staining of the p53 protein with the monoclonal antibody DO-7 in a paraffin embedded section of a Barrett's esophagus biopsy with low grade dysplasia. B: Moderately differentiated esophageal adenocarcinoma showing a diffuse immunostaining pattern of tumor cells. Both sections were counterstained by hematoxylin. A 10x objective was used.

Figure 1
(A, B)



5.4 Results

P53 labeling by standard immunohistochemistry on unfixed frozen sections of 20 Barrett's adenocarcinomas gave similar results as immunohistochemistry after antigen retrieval method on corresponding paraffin embedded formalin-fixed sections of these tumors.

5.4.1 Normal cardia and esophageal mucosa

Normal cardia tissue taken from the resection material was used for control purposes. All 24 cases were negative for p53. Weak nuclear staining with DO-7 was sporadically seen in the basal layer of normal squamous epithelium.

Table 1: P53 immunostaining in biopsies from Barrett's esophagus

dysplasia	N	Positive p53	
No	n=50	3 (6%)	
Indef/low	n=43	12 (28%)	
High	n=9	8 (89%)	
			$p<0.001^{1)}$
Total	N=102	23 (23%)	

1) P-value as calculated by Trend test (21).

5.4.2 Barrett's esophagus

Immunohistochemical assessment of biopsy material from 102 patients with - Barrett's esophagus without carcinoma revealed intense nuclear p53 staining in 23 of 102 (23%) biopsies. A significant correlation was found between grade of dysplasia and p53 staining (Trend test: $p<0,001$, table I). Staining for p53 was found in eight of nine cases with high grade dysplasia, 12 of 43 (28%) of cases with indefinite or low grade dysplasia, and in three of 50 (6%) cases of simple metaplasia, (table 1). Staining was focal in all non dysplastic, in eight indefinite/low grade dysplastic and in two high grade dysplastic cases (figure 1A). In ten cases, 4 indefinite/low grade and six high grade, staining was diffuse.

Table 2: P53 immunostaining in Barrett's esophagus adjacent to adenocarcinoma.

Dysplasia	N	Positive p53	
No	n= 3	0 (0%)	
Indef/low	n=11	4 (36%)	
High	n= 9	8 (89%)	
			$p<0.05^{1)}$
Total	N=23	12 (52%)	

1) P-value as calculated by Trend test[21].

5.4.3 Barrett's esophagus adjacent to adenocarcinoma.

Staining for p53 in Barrett's esophagus adjacent to the infiltrating tumor was performed in 23 cases. P53 staining correlated significantly with grade of dysplasia (Trend test $p<0.05$, table II). Eight of nine (89%) of the severely dysplastic cases showed diffuse staining. In four out of 11 (36%) cases with indefinite for or low grade dysplasia focal p53 staining was observed. P53 immunostaining was not observed in the three non dysplastic epithelia adjacent to tumor. In a total of 11 cases of adjacent Barrett's esophagus p53 immunoreactivity was not seen, four of which were adjacent to adenocarcinomas lacking p53 staining as well.

5.4.4 Adenocarcinoma in Barrett's esophagus

The 24 cases of adenocarcinoma in Barrett's esophagus were distributed according to tumor stage as shown in table three (table III). In 20 of 24 cases (83%) diffuse (19 cases) or focal (1 case) nuclear immunohistochemical staining for p53 was found (figure 1B). Staining was similar in the three stage groups, with a slight preponderance of negative cases in the advanced tumors. Also no significant correlation was found between p53 staining and grade of differentiation.

Table 3: P53 immunostaining in adenocarcinomas in Barrett's esophagus.

Tumor stage[1)]	N	Positive p53[2)]
T1	n= 6	6 (100%)
T2	n= 6	5 (83%)
T3/4	n=12	9 (75%)
Total	N=24	20 (83%)

[1)] Tumor stage according to TNM classification; T1: tumor infiltration in submucosa, T2: in muscularis propria, T3/T4: in adventitia and beyond.
[2)] Focal or diffuse nuclear immunostaining.

5.5 Discussion

The study of different stages in carcinogenesis in a model which includes early stages of cancer development has important clinical implications. Barrett's esophagus might serve as a unique model in which early changes preceding full blown malignancies are encountered. Patients suffering from Barrett's changes are monitored by periodic endoscopies and biopsies, allowing longitudinal follow up studies. Although Barrett's esophagus may lead to malignancy, the course of the development of carcinoma in the individual can hardly be predicted by histopathology. Therefore tracing more objective parameters associated with dysplasia and reflecting the progression of malignancy is of paramount importance. P53 mutation, a common genetic event in many human malignancies

(7-14), might be such a parameter. Positive p53 protein immunostaining is generally assumed to occur in case of gene mutations, which results in increased stability and consequently accumulation of mutated p53 protein (22). Intense immunostaining with the DO-7 anti p53 antibody has proved to be highly specific for mutated p53 (23).

In the present study nuclear p53 protein staining was shown in 23 of 102 (23%) biopsies from Barrett's esophagus with different grades of dysplasia and in 12 of 23 (52%) Barrett's esophagus adjacent to tumor. In these specimens p53 staining correlated significantly with grade of dysplasia ($p<0,001$ and $p<0,05$ respectively), with a high prevalence (89%) in high grade dysplasia. The number of cases with nuclear p53 immunostaining in Barrett's esophagus in our study is somewhat higher than previously reported (16,17,18). Differences in the applied monoclonal antibodies and/or in the histochemical techniques used, may account for the discrepancies.

Positive p53 immunostaining in Barrett's esophagus with no or low grade dysplasia (16,17,18), may indicate that p53 protein accumulation is an early event during carcinogenesis in Barrett's esophagus. Focally staining areas in these Barrett's esophagus may be associated with p53 gene mutations, moreover these clones may be precursors of progressive lesions with ultimately diffuse p53 staining. P53 gene mutations in non dysplastic and minimal dysplastic Barrett's esophagus were demonstrated by PCR and sequence analysis by Casson et al (24). Alternatively, increased amounts of wild type p53 protein, resulting in positive immunostaining, were noticed in irradiated and highly proliferative tissues (25,26). Since the DO-7 monoclonal also reacts with wild type p53 protein and proliferation rates are high in Barrett's esophagus (5), we cannot rule out that in a subset of cases immunostaining was a result of immunoreaction of DO-7 with wild type p53. Furthermore, using monoclonal antibodies, certain conditions may give false negative results, e.g., when the epitope which is recognized by the DO-7 monoclonal antibody is mutated or inaccessible as a consequence of complex formation of p53 with other proteins (27). On the other hand complete absence of p53 protein (due to loss of both p53 alleles, non-sense mutations or stop codons in the gene) would not be distinguishable from normal levels of wild type p53 by immunohistochemical techniques; in both conditions staining would be negative.

In the present study p53 immunostaining was observed in 20 out of 24 (83%) adenocarcinomas in Barrett's esophagus. In esophageal adenocarcinomas associated with Barrett's esophagus, Younes et al., reported p53 immunostaining in 87% (7 of 8) of cases. Additionally, in a follow-up series of 24 Barrett's esophagus patients three cases progressed to high grade dysplasia, two of which had p53 immunostaining and low grade dysplasia in initial biopsy specimens (16). In the study of Blount et al., p53 immunostaining was found in 62% (8 of 13) of esophageal adenocarcinomas, whereas allelic loss of the p53 gene (17p) was detected in 92% of cases (15). Although we did not find a correlation between tumor grade and p53 protein accumulation, a trend between increasing p53

expression with reduced tumor differentiation was recently seen in a larger series (18). The absence of p53 staining in four tumors and in two cases of highly dysplastic Barrett's esophagus may indicate that malignant progression is possible without involvement of the p53 gene (28).

P53 staining in tumor and in adjacent Barrett's esophagus do not necessarily correspond. In seven out of 19 patients Barrett's esophagus adjacent to p53 positive tumor tissue showed no p53 immunostaining. None of these cases showed severe dysplasia. A similar observation was reported by Flejou et al., who found that p53 immunostaining in Barrett's esophagus adjacent to p53 positive adenocarcinomas was only present in case the epithelium was severely dysplastic (29). Recently Hardwick et al., found positive p53 immunostaining in 10 cases of dysplastic Barrett's esophagus, all adjoining p53 positive esophageal adenocarcinomas (30). In the present study four carcinomas did not show p53 immunostaining. In all these cases adjacent Barrett's esophagus, regardless of degree of dysplasia, were negative as well.

In conclusion, we found that p53 protein accumulates in Barrett's adenocarcinoma and in premalignant lesions, correlating with the degree of dysplasia. P53 immunostaining might therefore constitute a useful diagnostic tool. However, the value of p53 immunostaining in biopsies without high grade dysplasia in predicting the chance of development of carcinoma should be determined in prospective follow-up studies.

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Chapter 6

Accumulation of Genetic Abnormalities During Neoplastic Progression in Barrett's Esophagus.

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6.1 Abstract

Sex chromosome status, ploidy and proliferation rate were evaluated in archival material of 73 Barrett's esophagus patients (48 male, 25 female). Diagnosis in esophageal mucosa samples ranged from intestinal metaplasia with no dysplasia to invasive esophageal adenocarcinoma; also four lymph node metastases were studied. Chromosomal and ploidy aberrations were determined by in situ hybridization with repetitive DNA probes specific for chromosomes Y, X and 1. Proliferation index (Ki-67 protein expression) was assessed by immunohistochemistry. Proliferation rate was elevated in all stages of dysplasia and in the adenocarcinomas. Aneuploidy (hyperdiploidy) and loss of the Y chromosome correlated with the advancing stages towards neoplasia ($p<0.001$), and reached high prevalences (70-100%) in high grade dysplasia and adenocarcinoma. Abnormalities of the X chromosome were not seen. These data suggest that in Barrett's esophagus genetic perturbations may be generated in relation to a high proliferation rate.

6.2 Introduction

Barrett's esophagus is a condition in which the stratified squamous epithelium of the distal esophagus is replaced by columnar epithelium (metaplasia) as a result of chronic esophageal reflux (1-4). Barrett's esophagus is associated with dysplasia and, compared to the general population, with a 30 to 40 fold increased incidence of esophageal adenocarcinoma (5,6). Patients with esophageal adenocarcinoma have a poor prognosis, even after surgical intervention. However, when surgical treatment is performed at an early stage, five year survival rates improve up to 100% (7,8). Surveillance of Barrett's esophagus patients is conducted by periodic endoscopy and histopathological examination of biopsy specimens. Although high grade dysplasia is considered an indication for surgical treatment (9-12), Barrett's esophagus with no or low grade dysplasia generally does not progress, but in individual cases is unpredictable. Therefore surveillance programs are unprofitable (13). Clearly, dysplasia cannot be the only criterium to recognize Barrett's esophagus patients with an increased risk for acquiring esophageal adenocarcinoma. To identify this subgroup prognostic parameters which can reliably predict malignant progression in Barrett's esophagus are required.

Along with the development of neoplasia in Barrett's esophagus, cell cycle abnormalities and a broad spectrum of genetic events, ranging from point mutations to gross numerical and structural chromosomal rearrangements, have been observed (14-27). Increased proliferation rate in Barrett's esophagus were found in PCNA and Ki-67 protein immunolabeling studies (14-16). Aberrations of the p53 gene have been found along with the development of neoplasia in Barrett's esophagus (17-20), as were aneuploidy and increased tetraploid cell

populations identified by flow cytometry (28-32). The most consistent numerical chromosomal aberration found in karyotyping studies is loss of the Y chromosome (21-24). By non-isotopic in situ hybridization (ISH), our group found Y-loss in gastric (25), and esophageal adenocarcinomas (26). In the latter study, this aberration was also observed in Barrett's esophagus adjacent to adenocarcinoma. Recently, Hunter et al., using a similar ISH technique, found Y-loss in 92% of esophageal adenocarcinomas (27).
Objective of the present study was to investigate ploidy, sex chromosome status and proliferation rate in non-dysplastic, dysplastic and neoplastic Barrett's esophagus. Ploidy and sex chromosome abnormalities were determined by non-isotopic in situ hybridization with (peri-) centromeric DNA probes specific for chromosomes 1, X and Y. Ploidy in the esophageal adenocarcinomas was also measured by means of DNA flow cytometry. Accumulation of the Ki-67 antigen was assessed through immunohistochemistry.

6.3 Materials and methods

6.3.1 Tissue preparation

Routinely formalin fixed paraffin-embedded tissues, obtained between 1987 and 1993 from patients at our hospital, were used. The material consisted of endoscopic biopsies of 50 patients (32 male, 18 female) with Barrett's esophagus, 23 esophagus-cardia resection specimens (16 male, 7 female) with adenocarcinoma in Barrett's esophagus and 4 lymph node metastases. All specimens were derived from different patients. In situ hybridization (ISH), immunohistochemistry (IHC) and flow cytometry were performed on consecutive tissue sections. For ISH and IHC, 4 μm sections were cut from paraffin blocks containing tumor and/or Barrett's epithelium. Tissue sections were adhered to coated slides (ITK, Uithoorn, The Netherlands) and stored overnight at 60 °C.

6.3.2 Histopathological criteria

Hematoxylin and eosin stained sections from all tissue blocks were reviewed by two pathologists (A.H.M. and F.T.B.). Barrett's esophagus was defined as columnar epithelial lining of the esophagus at least three centimeters proximal to the gastro-esophageal junction. All cases had specialized columnar type of metaplasia characterized by the presence of intestinal type of absorptive cells, e.g., goblet cells. Grading of dysplasia was performed following the criteria modified by Reid et al. (33), which distinguish three categories, i.e., no dysplasia, indefinite/low grade dysplasia and high grade dysplasia. Eighteen biopsy specimens (11 male, 7 female) with Barrett's esophagus had no dysplasia, 25 (16 male, 9 female) showed indefinite/low grade dysplasia, and seven (5 male, 2 female) showed high grade dysplasia. Tumor staging was performed according to pTNM criteria. In nine (7 male, 2 female) of the adenocarcinomas infiltration was limited to the submucosa (pT1), in three (2 male, 1 female) to the

muscularis propria (pT2) and in 11 (7 male, 4 female) the serosa was invaded (pT3/4). Nine of the latter cases had lymph node metastases.

6.3.3 Probe and probe labeling

A probe set specific for chromosomes 1, X and Y was selected. The (peri-) centromeric repetitive satellite DNA probes were labeled with biotin-14-dATP by nick translation of complete plasmid DNA according to manufacturer's directions (BRL, Gaithersburg, MD). DNA probes were stored at -20 °C.

Table 1:Results of in situ hybridization (ISH) with a set of chromosome specific repetitive DNA probes (1, X and Y), DNA flow cytometry and immunohistochemistry (IHC) with an antibody to Ki-67 of 50 biopsy specimens from Barrett's esophagus, 23 cases of adenocarcinoma in Barrett's esophagus, and 4 lymph node metastases.

	Cardia Normal Control[a]	Barrett's esophagus Grade of dysplasia No	I/L	High[b]	Adenocarcinoma Stage pT1	pT2	pT3[c]	Metastasis Lymph noded
Total:	5	18	25	7	9	3	11	4
Sex:								
Female	1	7	9	2	2	1	4	0
Male	4	11	16	5	7	2	7	4
IHC:								
PI>20%[e]	0	0	16	24	7	3	11	
ISH:								
Y loss[f]	0	1	6	5	6	2	6	3
Hyperdiploid[g]	0	3	10	5	7	3	10	4
Flow cytometry:								
Total:					9	2	10	
Anuploide/ tetraploid[h]					5	2	9	

[a] *Normal cardia control tissue.*
[b] *Barrett's esophagus with no (No), indefinite/low (I/L) and high (High) grade dysplasia (34).*
[c] *pT-Stages of esophageal adenocarcinoma according to pTNM classification: pT1: Tumor in submucosa, pT2: in muscularis propriae, pT3: in adventitia.*
[d] *Lymphnode metastases of 4 male adenocarcinomas.*
[e] *Proliferation index (PI), defined as the percentage of cells with Ki-67 antigen expression (Mib-1 immunostaining).*
[f] *The number of cases of Y chromosome-loss in males.*
[g] *The number of cases with ISH aneuploidy defined as >4% hyperdiploid nuclei (>2 ISH spots/nucleus) estimated by ISH with a chromosome 1 specific centromeric DNA probe.*
[h] *DNA flow cytometry of 21 adenocarcinomas: The number of carcinomas with aneuploid peaks and/or increased G_2M/tetraploid fraction.*

6.3.4 In Situ Hybridization

The in situ hybridization procedure was performed as described earlier (26, 34),

with some slight modifications. Briefly, after deparaffinizing of the tissue sections in xylene, endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol. Prior to enzymatic digestion slides were heated in a microwave oven (Biorad, UK), submerged in 2XSSC at 85 °C for 25 min with an interval of 1 min. During this interval fluid level was checked and restored. Further enhancement of tissue permeability was achieved by proteolytic enzyme digestion (pepsin: Sigma, 1 mg/ml in 0.2 M HCl) for 5-10 min at 37 °C. Before applying the probe set, the optimal digestion time for each tumor was determined by a pepsin time series (5-10 min). Tissue DNA was heat denatured for 2 min in 70% formamide in 2xSSC (pH 7.0), followed by dehydration in an ethanol series. Chromosome specific repetitive DNA probes were denatured for 7 min at 70 °C in a hybridization mixture, containing 1-2 μg/ml probe DNA, 500 μg/ml sonicated herring sperm DNA (Sigma), 0.1% Tween-20, 10% dextran sulphate, and 60% formamide in 2xSSC at pH 7. The probe mixture was then applied to the sections and incubated overnight at 37 °C. Then slides were rinsed in 60% formamide in 2xSSC (pH 7.0) at 42 °C.
Histochemical detection of probe binding was performed by immunoperoxidase staining. Slides were incubated for 30 min at 37 °C with mouse-anti-biotin (Dakopatts, Glostrup, Denmark), biotin-labeled horse-anti-mouse (Vector, Burlingame, CA) and with avidin-biotin-complex (Vectastain Elite ABC Kit). Visualization was achieved with 0.05% H_2O_2, 0.5 g/l DAB, 0.1 M imidazole in PBS (Phosphate Buffered Saline). The signal was amplified with 0.5% $CuSO_4$ in 0.9% NaCl. Slides were rinsed in distilled water and counterstained with hematoxylin. Finally, slides were dehydrated in ethanol, cleared in xylene and mounted in Malinol (Chroma-Geselschaft, Köngen, Germany).

6.3.5 Immunohistochemistry

Before immunolabeling enhancement of Ki-67 antigen staining was achieved by heating slides submerged in citrate buffer (10 mM citric acid monohydrate, pH 6.0) in a microwave oven (700 W) at 95 °C for 2x5 min with an interval of 1 min. During this interval fluid level was checked and restored. Labeling of the Ki-67 antigen was performed with a monoclonal antibody, Mib-1 (Immunotech, Marseille, France), diluted 1/25 in PBS. The monoclonal Mib-1 reacts with the Ki-67 molecule as detectable in late G1, S and G2 phase, but not in G0 phase of the cell cycle (35). For negative controls the primary antibodies were replaced by PBS. Immunohistochemistry was performed using the routine ABC-immunoperoxidase method (Vector).

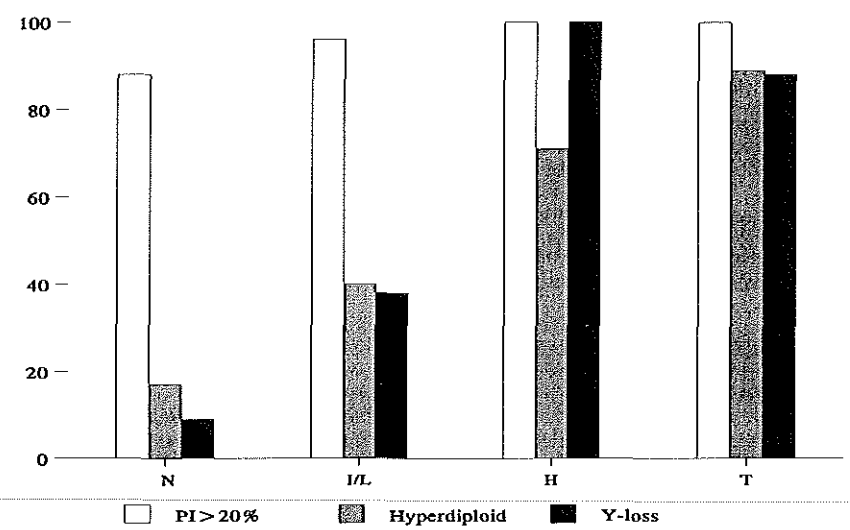
6.3.6 DNA Flow Cytometry

DNA content of the paraffin material of 21 carcinomas was measured, as described by Hedley et al. (36), using 50 μm paraffin tissue sections. Flow cytometry was carried out using a Facscan (Becton Dickinson, Mountain View, Ca).

6.3.7 Analysis

Results of ISH with probes for chromosomes 1, X and Y were evaluated on consecutive 4 μm sections in previously defined areas (26,34). This approach was applied to reduce artifacts due to biological heterogeneity and section thickness. Leukocytes on the same sections and stromal cells served as controls for hybridization quality. For each probe the number of solid DAB spots was scored (0, 1, 2, 3, 4, >4 spots) of 2x100 distinct, non-overlapping, single or side by side 4 μm nuclear slices. Scoring was performed by two independent investigators. The counted spot distributions were compared and averaged. Loss of the Y chromosome appeared as small focal areas or extensive fields with absence of hybridization of chromosome Y in epithelial or cancer cell nuclei. Conversely, ISH spots had to be present in surrounding stromal cells and leukocytes. Aneuploidy (hyperdiploidy) was estimated by determining the percentage of cells with more than 2 hybridization spots per nucleus after ISH with the chromosome 1 specific (peri-) centromeric DNA probe.

Figure. 1. Graphic representation of the results of in situ hybridization with chromosome 1 and Y, and immunohistochemistry with and antibody for the KI-67 antigen of: Barrett's esophagus with no (N), indefinite/low (I/L), high grade dysplasia (H) and adenocarcinoma (T). The Y axis represents the percentage of cases with elevated proliferation index (PI>20), hyperdiploidy and chromosome Y-loss, respectively. Increased PI appears to be an early event during carcinogenesis in Barrett's esophagus. Hyperdiploidy and Y chromosome loss increase along with advancing dysplasia and carcinoma.



Tissues were regarded hyperdiploid when >4% of the nuclei showed more than 2 ISH spots. The sensitivity of this approach is high, since the detection of hyperdiploidy is not biased by G_2M cells (34,37). In previous studies we have

also observed good correlations between ploidy as defined by ISH, and DNA flow cytometric measurements (26, 37). In control tissues, such as cardia, leukocytes and stromal cells, hyperdiploidy was $<1\%$.
Proliferation index (PI) was estimated by determining the percentage of Mib-1 immunostained nuclei (2x200 cells; ref. 26). In Barrett's esophagus the percentage of positive cells was determined in longitudinally sectioned crypts and villi. In the adenocarcinomas this was established by scoring of immunostained cancer cells. For control purposes PI was also determined in five cases of normal cardia tissue. The mean proliferative index (PI), in normal cardia specimens was 15%. In Barrett's esophagus and adenocarcinomas a PI >20 was regarded as increased.

6.3.8 Statistics

The relation between dichotomous parameters was investigated using Fisher's exact test. The relation between the presence of a characteristic, i.e., hyperdiploidy or Y chromosome loss and histopathological grading was assessed using the Chi-square test for trend (38). Correlation coefficients given are Spearman's; $p=0.05$ (two sided) was considered the limit of significance.

6.4 Results

Proliferation rates were elevated ($PI>20\%$) in all grades of dysplasia in Barrett's esophagus and in the adenocarcinomas (Table 1; Figs. 1, 2B, 3B). Furthermore, mean PI gradually increased ($r=0.65$, $p<0.001$) along with increasing dysplasia in Barrett's esophagus from 40% to 51%, reaching 63% in adenocarcinomas.
Ploidy as measured by DNA flow cytometry (FCM) correlated ($p<0.05$) with aneuploidy (hyperdiploidy) as found by ISH for chromosome 1. In 19 of 21 adenocarcinomas both outcomes were concordant. The remaining 2 adenocarcinomas were found diploid by FCM, whereas low percentages of hyperdiploid cells (range 5%-6%) were seen with ISH.

Figure 2. Adjacent 4μm tissue sections of a biopsy specimen from a male patient with Barrett's esophagus. A: Hematoxylin-Eosin stained section of Barrett's esophagus with specialized columnar type of metaplasia and high grade dysplasia (20x obj.). B: Immunohistochemical staining of the Ki-67 antigen with the monoclonal antibody Mib-1 in Barrett's esophagus counterstained with hematoxylin. (20x obj.). C: In situ hybridization (ISH) with a chromosome 1 specific DNA probe on a 4μm tissue section. The ISH-related spots were visualized with immunoperoxidase/DAB (black); hematoxylin was used as a counterstain (grey; 100x obj.). Note the presence of hyperdiploid cells (arrows). D: ISH for chromosome Y shows focal loss in epithelial cells: A gland with (o) and without (*) this chromosome can be distinguished. Also stromal cells contain Y chromosome spots (40x obj.). E: ISH with a chromosome X specific DNA probe shows clear hybridization for chromosome X in the gland that showed loss of Y in D (40x obj.).

Figure 2
(A,B)

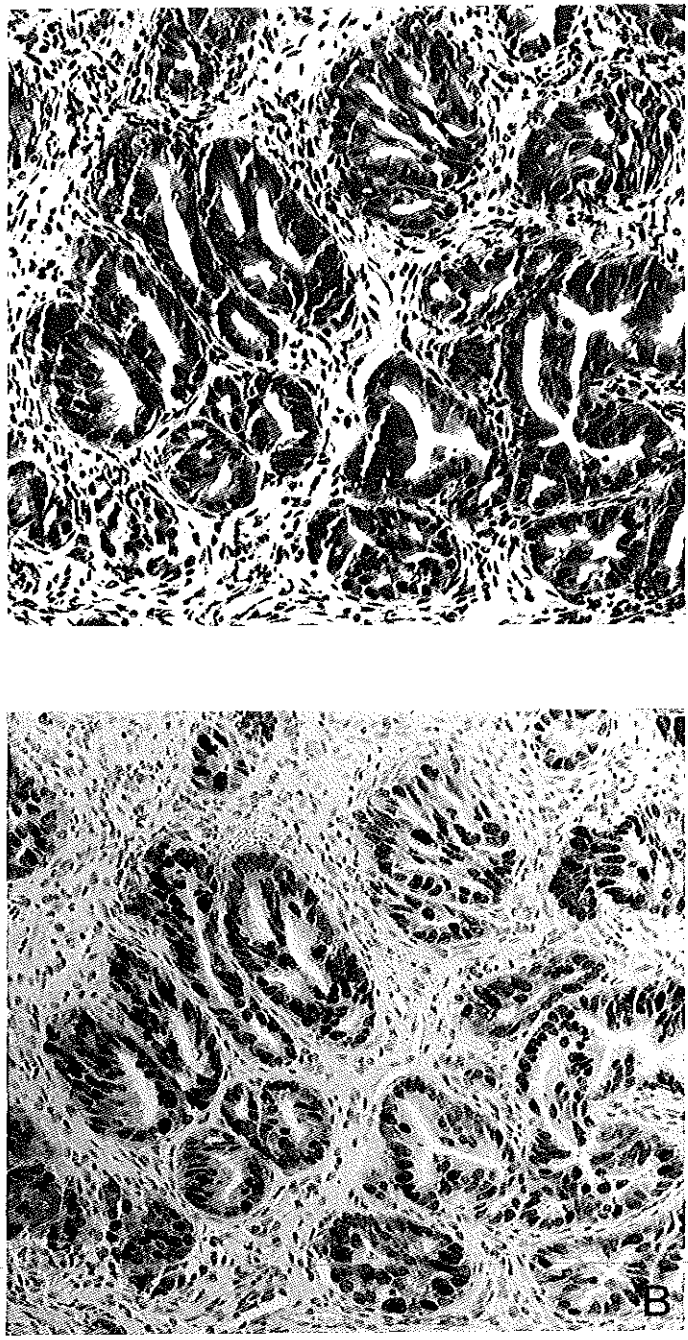


Figure 2
(C)

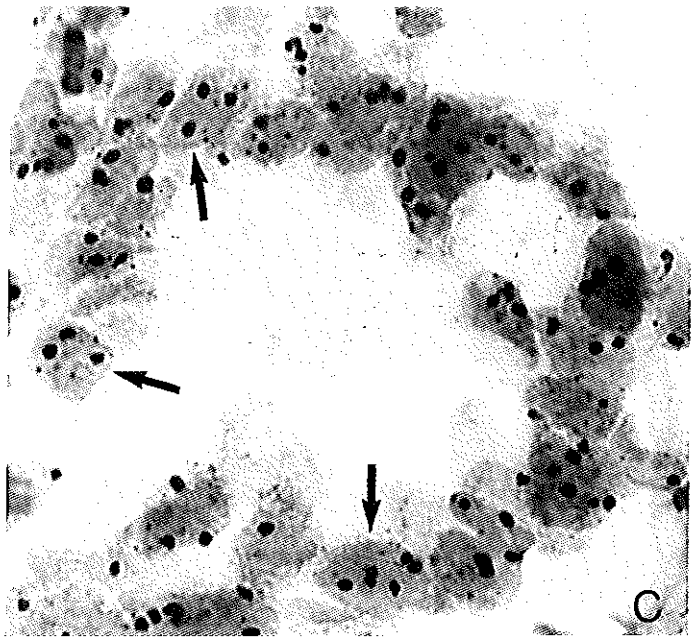
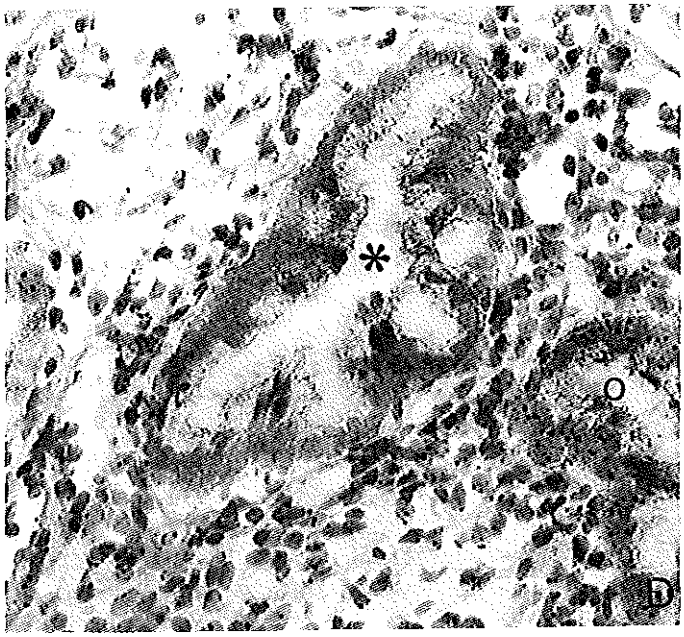
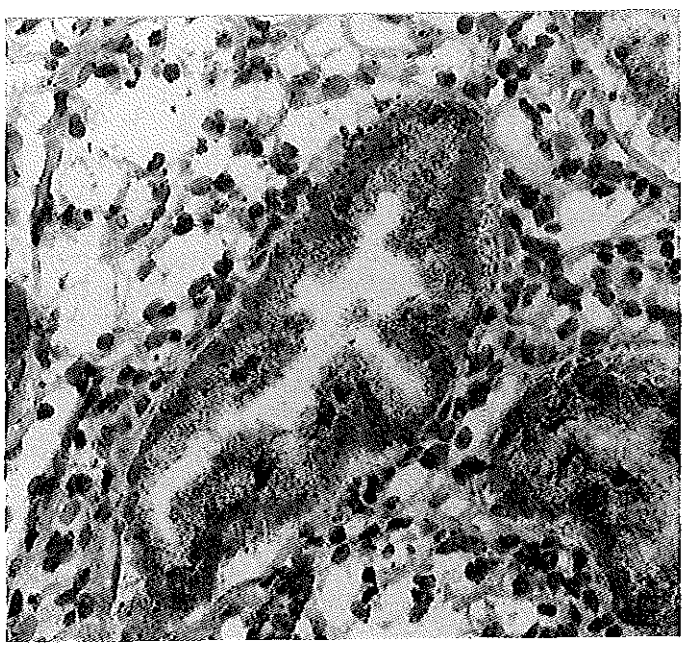


Figure 2
(D,E)





The percentage of cases with hyperdiploid cells gradually increased ($p<0.001$) from 17% in Barrett's esophagus biopsies without dysplasia, to 40% in cases with indefinite/low grade dysplasia, to 71% with high grade dysplasia; 89% of carcinomas were found hyperdiploid (Table 1; Figs. 1, 2C, 3C). The percentage of aberrant cells in the cases with hyperdiploidy ranged from 5% to 30%. The mean percentage of cells with abnormal ploidy increased ($r=0.75$, $p<0.001$) from 7% in cases with no/indefinite/low grade dysplasia to 20% in Barrett's esophagus with high grade dysplasia or adenocarcinoma (Table 1).

Nine percent of Barrett's esophagus cases without dysplasia, 38% of cases with indefinite/low grade dysplasia, 100% of cases with high grade dysplasia and 88% of the adenocarcinomas showed loss of the Y chromosome ($p<0.001$; Table 1; Fig. 1). Y chromosome loss in Barrett's esophagus was focal, but extensive or total in the adenocarcinomas (Figs. 2D, 3D). In three out of four lymph node metastases Y chromosome loss was found (Fig. 3G). In the four lymph node metastases, Y chromosome status agreed with that of the primary adenocarcinoma. Numerical aberrations of the X chromosome were not found in any of the groups (Fig. 2E).

In summary, a high proliferation rate was observed in all stages, whereas hyperdiploidy and Y-loss correlated with increasing degrees of dysplasia and carcinoma. Further, these parameters were not correlated within the different histopathological stages. In high grade dysplasia and adenocarcinomas prevalence of all 4 parameters was high, range 70-100% (Fig. 1).

6.5 Discussion

Optimal treatment of Barrett's esophagus patients requires early detection of malignancy. This has encouraged investigators to study biological parameters for use as prognostic markers. In this context we have investigated ploidy, numerical chromosomal abnormalities and proliferation rate in archival tissue sections of Barrett's esophagus patients with lesions ranging from simple intestinal type of metaplasia to metastasizing adenocarcinoma. We found that all these parameters were associated with progression of the lesions towards neoplasia. An elevated proliferation rate appeared to be an early change in Barrett's esophagus (Fig. 1). Furthermore, the proliferation index gradually increased with advancing stages of development towards neoplasia. Although the latter observation was not always found by other investigators, proliferative activity is assumed to be elevated in both non-dysplastic and dysplastic intestinal type of Barrett's esophagus, and in esophageal adenocarcinomas (14-16).

Hyperdiploidy and Y chromosome loss appeared to increase with advancing degrees of dysplasia (Fig. 1). The percentage of hyperdiploid nuclei in no to low grade dysplasia was relatively low. In contrast to flow cytometry, non-isotopic in situ hybridization on tissue sections is highly efficient for the detection of (few)

Figure 3
(A-D)

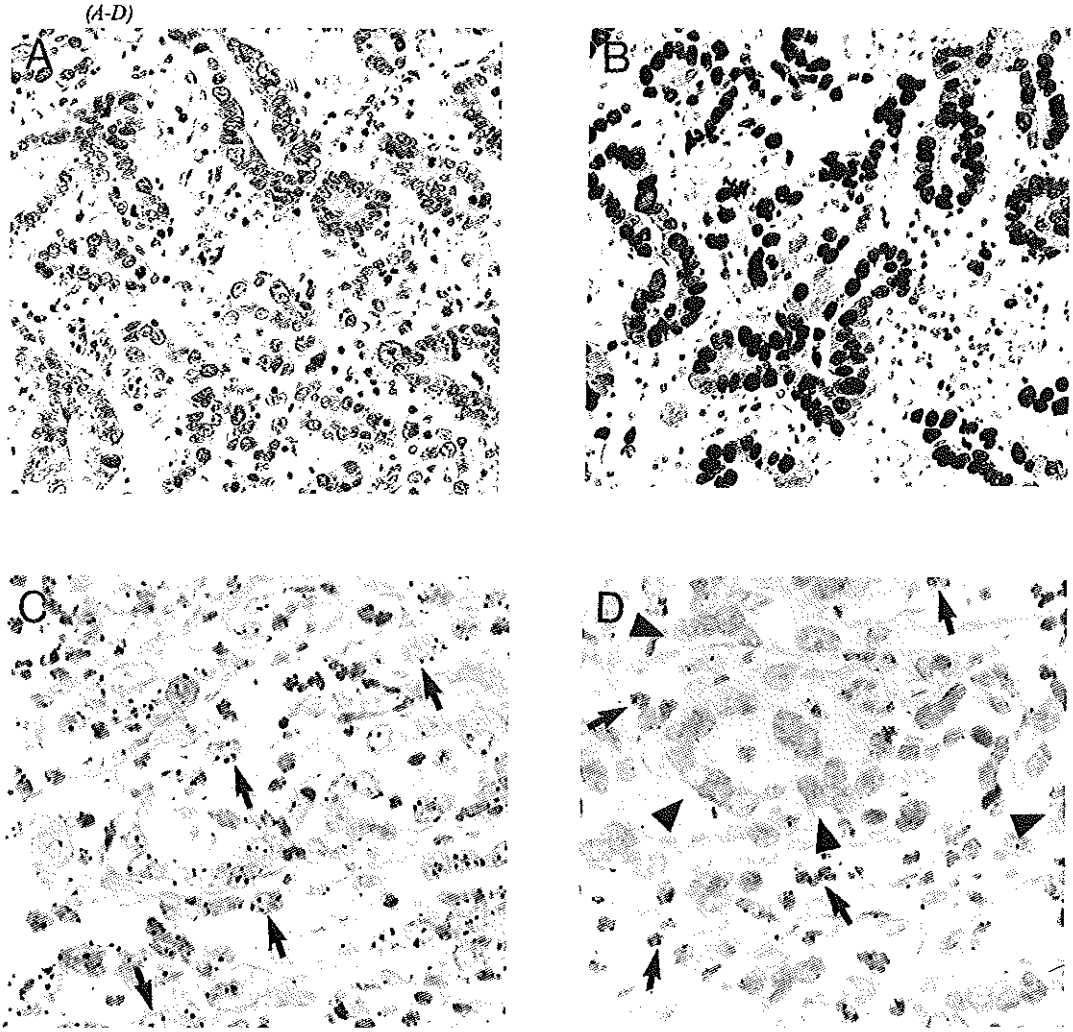
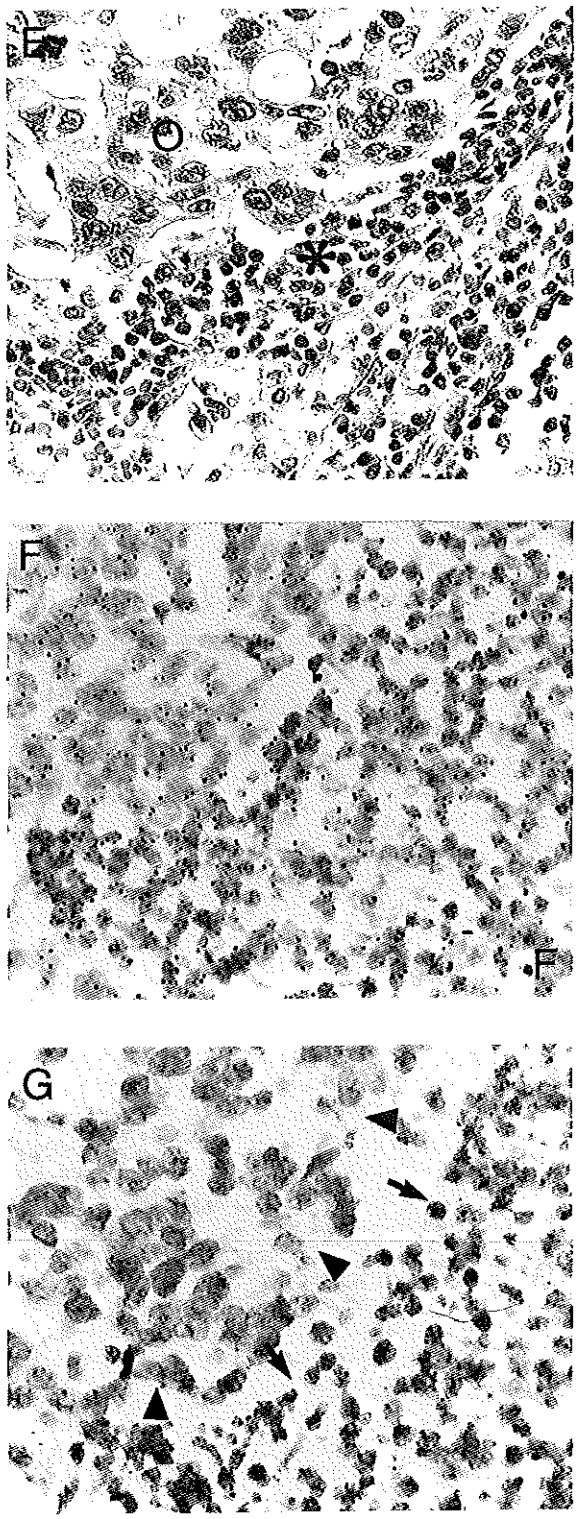


Figure 3. Adjacent 4μm tissue sections of an esophageal adenocarcinoma and lymph node metastasis. A: Hematoxylin-Eosin stained section of moderately differentiated, esophageal adenocarcinoma (20x obj.). B: Immunohistochemical staining of the Ki-67 antigen with the monoclonal antibody Mib-1 in esophageal adenocarcinoma. (20x obj.). C: In situ hybridization (ISH) with a chromosome 1 specific DNA probe on a 4μm tissue section. ISH shows many hyperdiploid (> 2 spots) nuclei (arrows; 40x obj.). D: ISH with a chromosome Y specific DNA probe shows Y-loss in carcinoma cells (arrowheads), but not in stromal tissue (arrows; 40x obj.). E: Same patient: Hematoxylin-Eosin stained 4μm tissue sections of a lymph node metastasis with leukocytes (*) and adenocarcinoma (o). F: ISH with a chromosome 1 specific DNA probe, revealing ISH signals in both type of cells. G: ISH with a chromosome Y specific DNA probe shows Y-loss in tumor nuclei (arrowheads), but not in leukocytes (arrows). A 40x objective was used in E-G.

Figure 3
(E-G)



dispersed cells with abnormal ploidy status (37). In the present study this was demonstrated by discordance of ploidy measured by FCM and ISH in two (of 21) tumor samples. Both samples were diploid by FCM, but were found to contain a low number of hyperdiploid nuclei by ISH. Y-loss was rarely observed in non-dysplastic Barrett's esophagus, yet increased significantly in most advanced lesions with a high prevalence in high grade dysplasia and adenocarcinoma (Fig. 1). Loss of the Y chromosome appears to be the most consistent chromosomal abnormality in Barrett's esophagus and esophageal adenocarcinoma. It was found previously in several karyotyping and ISH studies (21, 23-27). However, its association with dysplastic changes in Barrett's esophagus had not been observed previously. The role of Y-loss in neoplastic transformation in Barrett's esophagus is uncertain. Barrett's esophagus and esophageal adenocarcinoma have a three and seven times increased incidence in males (39, 40). Since as yet no genes involved in tumorigenesis have been linked to the Y chromosome, Y-loss may be the result of genomic instability in transformed cells. In contrast to Y chromosome aberrations, X chromosome changes were not observed. The X chromosome is therefore not an informative numerical marker in (female) Barrett's esophagus patients.

Our results suggest that during the development of dysplasia and carcinoma in Barrett's esophagus a high cell turnover is an initial event. The resulting high proliferative activity may induce genomic instability. The latter leads to cytogenetic alterations, and transformation of cells, which may further expand into cell clones with malignant behavior.

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Chapter 7

Reduced Expression of the Cadherin-Catenin Complex in Esophageal Adenocarcinoma Correlates with Poor Prognosis.

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7.1 Abstract

The E-Cadherin-Catenin complex is important for cell-cell adhesion of epithelial cells. Impairment of one or more components of this complex is associated with poor differentiation and increased invasiveness of carcinomas. Esophageal adenocarcinomas cause early metastases, progress rapidly and consequently have a poor prognosis. By means of immunohistochemistry, we studied the expression of E-Cadherin, Alpha- and Beta-Catenin in 65 esophageal adenocarcinomas and 15 lymph node metastases. Expression of these proteins was evaluated with respect to clinico-pathological parameters and patient survival.
Expression of the proteins was strongly correlated. In carcinomas reduced expression of E-Cadherin, Alpha-Catenin and Beta-Catenin were found in 74%, 60%, and 72%, respectively. Expression of E-Cadherin and Alpha-Catenin correlated significantly with stage and grade of the carcinomas, whereas expression of Beta-Catenin only correlated with grade of the carcinomas. Reduced expression of all three proteins correlated with shorter patient survival. In contrast to grade, E-Cadherin and Beta-Catenin were significant prognosticators for survival, independent of stage of disease. We conclude that in esophageal adenocarcinomas decreased expression of E-Cadherin, Alpha- and Beta-Catenin are related events. Furthermore, expression of at least E-Cadherin and Beta-Catenin, is significantly correlated with poor patients' prognosis.

7.2 Introduction

For several decades the incidence of esophageal adenocarcinoma in Western countries is rapidly increasing (1). The predisposing condition for developing esophageal adenocarcinoma is Barrett's esophagus (2-3). Progression of Barrett's esophagus to adenocarcinoma occurs through gradually increasing dysplasia (4-5). The survival of patients with esophageal adenocarcinoma is poor, even after seemingly radical surgical intervention. Stage of disease is the best parameter for predicting survival (6, 7). Patients with limited tumor invasion of the esophageal wall and without metastases at time of resection have significantly better survival than those with lymph node metastases or organ infiltration (6, 7). Understanding the mechanisms of metastatic behavior of carcinomas, and recognition of cases running higher risk for developing metastases, might help to design new strategies for diagnosis and treatment.

E-Cadherin is a 120 KDa transmembrane glycoprotein, which belongs to a large family of calcium dependent adhesion molecules (CAMs) (8, 9). By mostly homotypic interactions (10), E-Cadherin plays an important role in morphogenesis of tissues during embryogenesis and in mature epithelia E-Cadherin is involved in cell-cell adhesion (11, 12). In vitro and in vivo experiments showed that absence of membranous E-Cadherin in carcinoma cells goes along with increased invasiveness and metastatic ability (13-15). It has become clear that besides cellular adhesion, E-Cadherin is also involved in signal transduction via connections with Catenins and the actin cytoskeleton. The Catenin anchorage complex, which interacts with the cytoplasmic domain of E-Cadherin, consists of a heteromere of at least three proteins: Alpha-, Beta- and Gamma-Catenin. Alpha-Catenin, a 102 kDa protein ubiquitously expressed in cells, binds to actin and to Beta-Catenin. In turn, Beta-Catenin, a 92 kDa protein, is directly linked to E-Cadherin (16, 17). Both Beta- and Alpha-Catenin have binding sites for several onco- and tumor suppressor proteins, suggesting that these might modulate the function of the E-Cadherin-Catenin adhesion complex (18-20). In adenocarcinomas of the prostate, breast, esophagus, lung, stomach and colon, and in squamous cell carcinomas of the esophagus and of head and neck, decreased E-Cadherin and/or Alpha-Catenin expression was found to correlate with high tumor grade and poor patients' prognosis (21-29). Recently, high frequencies of decreased Beta-Catenin expression were observed in several gastro-intestinal cancers (30).
In the present report we describe the expression of E-Cadherin, (EC), Alpha- (AC) and Beta-Catenin (BC) in 65 esophageal adenocarcinomas associated with Barrett's esophagus and in 15 lymph node metastases. Expression of these proteins, as assessed by immunohistochemistry with specific monoclonal antibodies, is evaluated with respect to various pathological parameters and patient survival.

7.3 Materials and methods

7.3.1 Clinico-pathological data

Esophageal-cardia resection specimens of 65 patients with esophageal adenocarcinoma associated with Barrett's esophagus, encountered between 1985 and 1992 at the Academic Hospital-Dijkzigt Rotterdam, were used in the study. Forty nine patients were male, 16 female, with a mean age of 67 (Range 34-80

years). All patients had undergone esophageal-cardia resection with microscopically complete removal of the carcinoma. Hematoxylin-eosin stained sections were used for tumor staging and grading. According to pTNM criteria, carcinomas were categorized into malignancies without (Stage 0, I, IIa) and with loco-regional or distant lymph node metastases (Stage IIb, III). Carcinomas clearly showing glandular structures, with well developed intercellular junctions throughout the tumor, were classified as well/moderately differentiated carcinomas. Carcinomas were classified as poorly/undifferentiated when showing focally or diffusely solid growth without glands and with high cellular pleomorphism. Survival times were calculated from date of operation to death resulting from recurrence of disease, or to last clinical control date.

7.3.2 Tissues

Of each resection specimen one archival formalin fixed paraffin embedded tissue block, representing the deepest levels of tumor invasion into the esophageal wall, was selected for immunohistochemical study. In 15 cases lymph node metastases were included for immunohistochemical evaluation. Adjacent squamous cell epithelium or non-dysplastic columnar epithelium served as internal positive control for immunohistochemistry.

7.3.3 Monoclonal antibodies

For E-Cadherin staining we used the culture supernatant of a hybridoma clone, 5H9, raised against a 80 kDa tryptic fragment of E-Cadherin from human A-431 carcinoma cells (Organon Technika Boxtel The Netherlands) (31). Anti- Alpha-Catenin IgG1 monoclonal antibody (Affinity, Exeter UK) has been raised against

Figure 1. Staining results of immunohistochemistry by the avidin-biotin-peroxidase method, Di-aminobenzidin tetrachloride was used as a chromogen (black). AB: Normal expression as shown by homogeneous membrane staining of the epithelial cells (arrows) of Alpha-Catenin at the squamo-columnar junction (A), and E-Cadherin in non-dysplastic Barrett's esophagus (B). C: Homogeneous staining of all carcinoma cell-membranes (arrows) indicating normal E-Cadherin expression in a well differentiated pT3 esophageal adenocarcinoma. D-F: Reduced expression with positive (arrows) and negative (arrowheads) cells of: E-Cadherin in a poorly differentiated pT3 adenocarcinoma (D), and of Alpha- (E) and Beta- Catenin (F) in a moderately differentiated esophageal adenocarcinoma. G: Normal expression, homogeneous membrane staining (arrows), of Alpha-Catenin expression in a well differentiated adenocarcinoma. H: No membrane staining (arrowheads), but positive nuclear staining (arrows) of Beta-Catenin in a moderately differentiated adenocarcinoma. Magnification A: 90x, B-H: 180x.

Figure 1 (A,B)

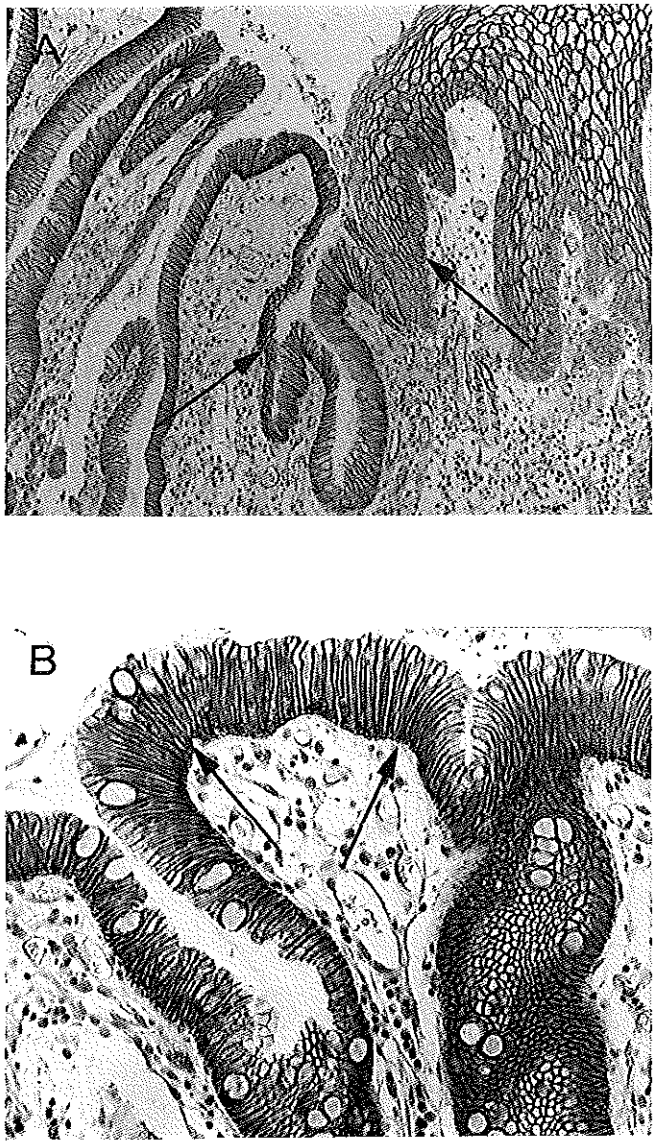
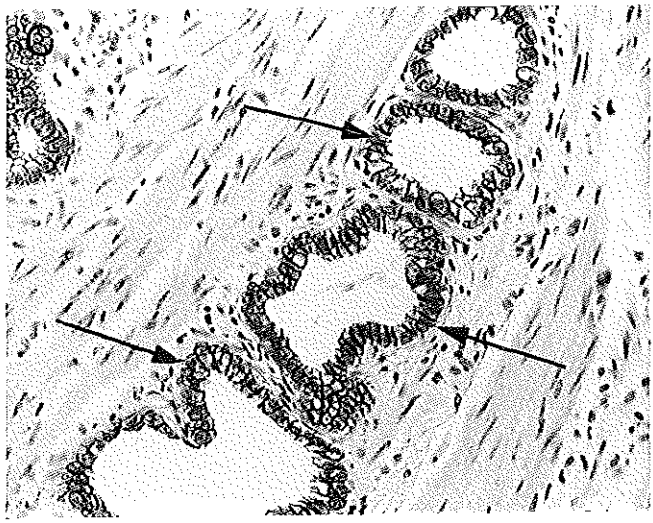


Figure 1
(C,D)



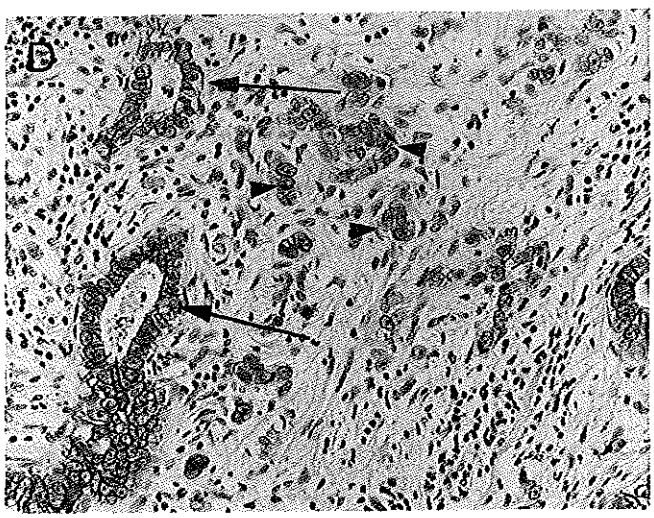


Figure 1 (E,F)

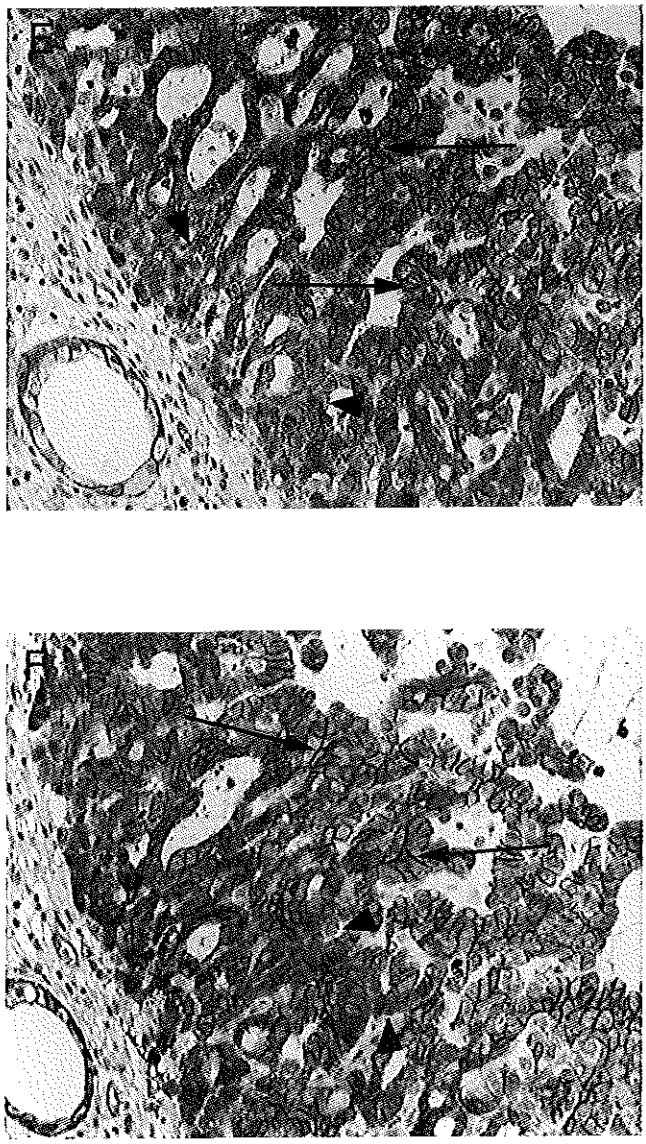
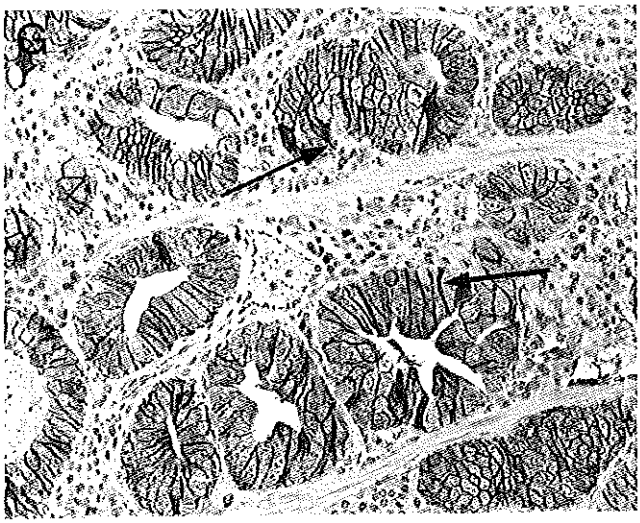
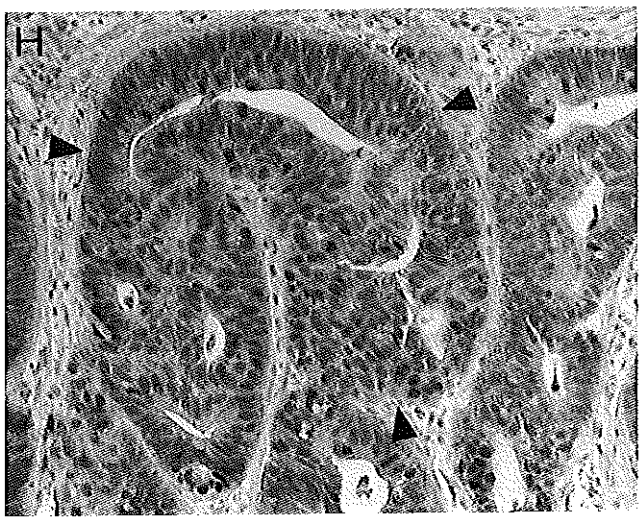


Figure 1 (G,H)





a 19.4 kDa C-terminal protein fragment corresponding to amino acids 729-906 of mouse Alpha-Catenin. This antibody was diluted 1:100 in PBS. A monoclonal IgG1 Anti-Beta Catenin (Affinity), raised against 23 kDa protein fragment corresponding with amino acid residues 571-781 of mouse Beta-Catenin, was diluted 1:200 in PBS.

7.3.4 Immunohistochemistry

Five μm paraffin sections were mounted on 3-amino-propyl-tri-ethoxy-silane (Sigma, St. Louis, USA) coated slides and stored overnight at 60°C. Sections were deparaffinized, endogenous peroxidase was blocked in 3% H_2O_2 in methanol (20 min) and rinsed in PBS. As antigen retrieval procedure for E-Cadherin, tissues were digested in 1% protease (Sigma) with 1% Ca^{++} in PBS solution (10-20 min at 37° C). Prior to Alpha- and Beta-Catenin staining the sections were heated in a microwave oven (in 0.01 M Citrate buffer Ph 6.0) for two cycles of five min. Primary antibodies were incubated overnight (at 4°C). As detection system we used streptavidin-biotin-peroxidase (Dako, Glostrup Denmark) with diaminobenzidin tetrachloride (Fluka, Basel Switzerland) as chromogen. Tissues were counterstained with hematoxylin.

7.3.5 Evaluation of E-Cadherin (EC), Alpha- (AC) and Beta-Catenin (BC) expression.

Immunohistochemical staining of EC, AC and BC in the subsequent tissue sections was scored semi-quantitatively by two independent investigators in two areas per carcinoma and per lymph node metastasis(10x objective).

7.3.6 Statistical analysis

Correlations between E-Cadherin, Alpha-, Beta- Catenin, grade and stage were calculated in cross-tables. Significance was tested by the Fisher's exact test. Survival probabilities were calculated by the Kaplan-Meier method and probabilities were compared by logrank test. Multi-variate analysis to determine independent prognostic value of the various parameters was performed by Cox regression. P=0.05 (two sided) was considered the limit of significance.

7.4 Results

7.4.1 Immunohistochemical staining patterns in control tissues and in

adenocarcinomas.

Homogeneous membranous immunostaining for E-Cadherin (EC), Alpha- (AC) and Beta-Catenin (BC) was found in all cases of normal squamous epithelium, normal cardia mucosa and non dysplastic Barrett's mucosa (figure 1A,B). These tissues adjacent to the adenocarcinomas served as internal control for the staining procedures. In squamous epithelium immunostaining was present homogeneously

Table 1: Cross tabulation of immunohistochemical staining results of E-Cadherin, Alpha- and Beta-Catenin with respect to stage and grade in 65 esophageal adenocarcinomas.

	Pts	E-Cadherin		Alpha-Catenin		Beta-Catenin	
		normal	reduced	normal	reduced	normal	reduced
Total	65	17	48	28	37	18	47
Stage[a]:							
0,I,IIa (%)	33	15(88)[b]	18(38)[b]	21(75)[c]	12(32)[c]	12(67)[d]	21(45)[d]
IIb,III (%)	32	2(12)	30(63)	7(25)	25(68)	6(33)	26(55)
Grade:							
Well/moder (%)	33	17(100)[e]	16(33)[e]	20(71)[f]	13(35)[f]	14(78)[g]	19(40)[g]
Poor/undiff (%)	32	0 (0)	32(67)	8(29)	24(65)	4(22)	28(60)
E-Cadherin:							
normal (%)	17			17(61)[h]	0(0)[h]	11(61)[i]	6(13)[i]
reduced (%)	48			11(39)	37(100)	7(39)	41(85)
Alpha-Catenin:							
normal (%)	28					16(89)[j]	12(25)[j]
reduced (%)	37					2(11)	35(75)

[a] Stages: 0=pTisN0M0 I=pT1N0M0; IIa=pT2/3N0M0; IIb=pT1/2N1M0; III=pT3/4N1-3M0 *[b-j]*
Column percentages. [b]$p<0.001$ [c]$p=0.001$ [d]$p=0.17$ [e]$p<0.001$ [f,g]$p<0.01$ [h-j]$p<0.001$

on the intermediate cells and on the basal layer (but not at the basal membrane side). In non dysplastic Barrett's and cardia epithelia intercellular membrane staining was seen between all columnar cells.

Membrane staining for all three proteins was also found in the adenocarcinomas and lymph node metastases. In the adenocarcinomas and lymph node metastases three main staining patterns were recognized: Homogeneous positive staining with more than 90% positive cells (figure 1C,G). Heterogeneously staining carcinomas with mixed negative and positive cells or tumor areas (figure 1D,E,F), but with at least 30% negatively staining cells. And a group of carcinomas with negative staining with no or less than 10% positive cells (figure 1H). For further analysis, carcinomas displaying a homogeneous positive pattern

were regarded as staining normally, whereas the latter two patterns, heterogeneous and negative were taken together and classified as reduced staining. Occasionally, in carcinomas with reduced AC or BC staining, weak to intense cytoplasmic staining for both proteins and for BC also nuclear staining was observed (figure 1H).

Table 2: Esophageal adenocarcinoma specific five years survival probabilities, subsequently stratified for stage, grade, and immunoreactive E-Cadherin, Alpha-and Beta-Catenin expression.

	patients	5-yrs[1]	SD[2]	p-value[3]
Overall	65	32%	0.07	
Stage[4]:				
0,I,IIa	33	64%	0.10	
IIb,III	32	7%	0.10	0.0001
Grade of differentiation:				
Well/moderately	33	55%	0.10	
Poor/undifferentiated	32	10%	0.10	0.0001
E-Cadherin expression:				
E-Cadherin normal	17	74%	0.12	
E-Cadherin aberrant	43	14%	0.7	0.001
Alpha-Catenin expression:				
Alpha-Catenin normal	28	61%	0.10	
Alpha-Catenin aberrant	37	11%	0.06	0.001
Beta-Catenin expression:				
Beta-Catenin normal	18	64%	0.14	
Beta-Catenin aberrant	42	21%	0.07	0.01

[1]Kaplan-Meier's five year survival probability. [2]Standard deviation. [3]p-value calculated by Logrank test.
[4]Stages: 0=TisN0M0; I=pT1N0M0; IIa=pT2/3N0M0; IIb=pT1/2N1M0; III=pT3/4N1-3M0.

7.4.2 E-Cadherin (EC), Alpha- (AC) and Beta-Catenin (BC) expression in esophageal adenocarcinoma.

Of the 65 esophageal adenocarcinomas 33 (51%) were classified as well/moderately differentiated and 32 (49%) as poorly/undifferentiated. Thirty-three (51%) of the carcinomas had no metastases (stage 0,I,IIa), but 32 (49%) had loco-regional or distant lymph node metastases (stage IIb, III ; table 1). Reduced EC, AC and BC immunostaining was found in 48 (74%), 37 (60%) and 47 (72%) of the cases, respectively (table 1).

AC immunostaining correlated significantly with BC staining. EC immunostaining correlated significantly with both AC and BC (p-values <0.01, Fisher's exact test; table 1). Staining of all three proteins was reduced in 35(54%) cases. Forty-three (66%) of the cases showed reduced staining of at least two, 54 (83%) of at least one of the proteins.

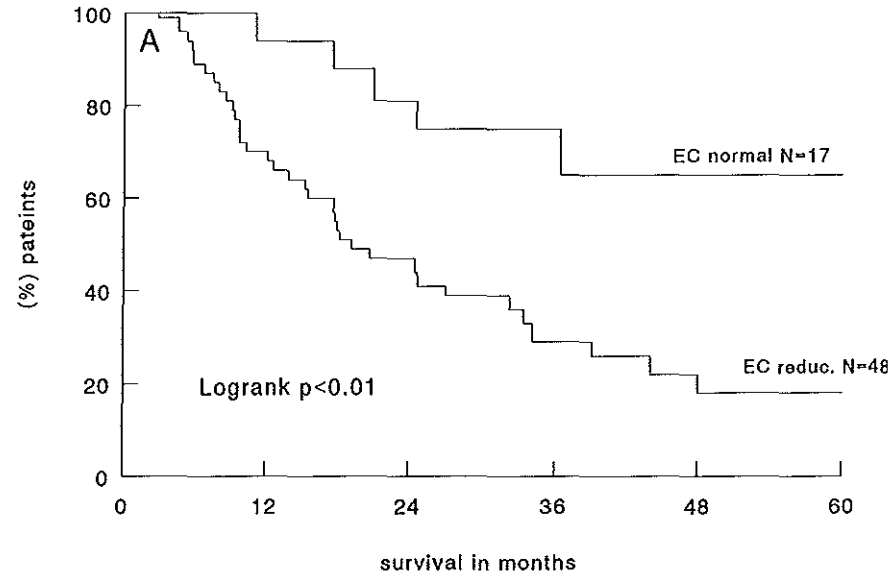
Reduced immunostaining in poorly and undifferentiated carcinomas of EC, AC and BC was found in 67, 65, and 60% versus 33, 35, 40% in the well and moderately differentiated cases, resp. Staining of EC, AC and BC correlated significantly with tumor grade (p-values <0.01, Fisher's exact test; table 1).

In carcinomas with metastases reduced EC, AC and BC staining was seen in 63, 68, and 55% versus 38, 32 and 45% in carcinomas without metastases, respectively. Immunostaining of only EC and AC correlated significantly stage of disease (p-values <0.01, Fisher's exact test; table 1).

Median follow-up period of patients was 23 months (Range 3-114 months). Overall five year survival was 32%. Significant differences in survival were found when patients were grouped according to stage (stage 0, I, IIa versus IIb, III), grade (well/moderate versus poor/undifferentiated), and for EC, AC and BC (normal versus reduced staining), (p-values <0.05, logrank; table 2; figure 2). Upon multi-variate analysis, in addition to stage, no significant prognostic value remained for grade ($p=0.15$). Adjusted for stage, BC was still an important prognostic factor (reduced versus normal: relative death rate=2.67; $p=0.03$). The same applied to EC (relative death rate=2.72, $p=0.04$), but not to AC ($p=0.09$). Figures 2C and 2D show the effect of normal and reduced BC staining within the groups of patients without metastases(stage 0, I, IIa; figure 2C), and with metastases (stage IIb, III; figure 2D). Due to strong correlation between EC, AC and BC expression, data did not allow to define which of these parameters might be superior to the other in predicting survival.

Figure 2. A-D: Kaplan Meier survival curves. X-axis: Survival in months; Y-axis: % of patients. The interrupted line in A and B shows five years survival of the whole group; in C and D of stage 0,I,IIa and stage IIb,III, respectively. A,B: Significant differences of survival probabilities for patients with esophageal adenocarcinomas are shown, as subsequently stratified for reduced versus normal expression of E-Cadherin(EC) and Beta-Catenin (BC) (Logrank test $p<0.05$). C: Further stratification within the group of patients without metastases (stage 0, I, IIa), following reduced and normal expression of Beta-Catenin, illustrates a significant difference in survival between patients without metastases and normal BC expression, and without metastases but reduced BC expression (logrank test p,0.05). D: Further stratification following BC expression within the group of patients with metastases, shows no significant difference between the two groups. Notice that only 6 out of 32 patients with metastases have reduced BC expression.

Figure 2
(A,B)


A
100
80
60
40
20
0
(%) pateints
EC normal N=17
EC reduc. N=48
Logrank p<0.01
0
12
24
36
48
60
survival in months

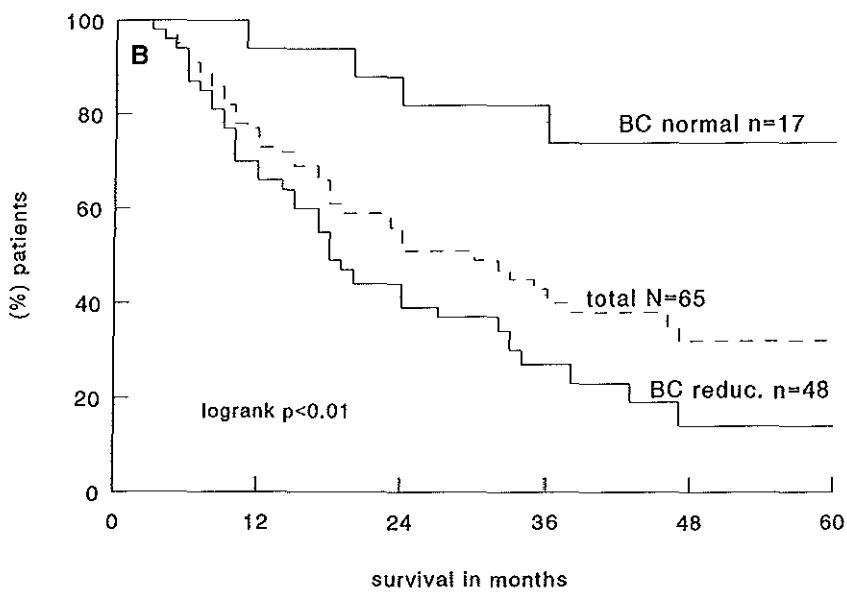
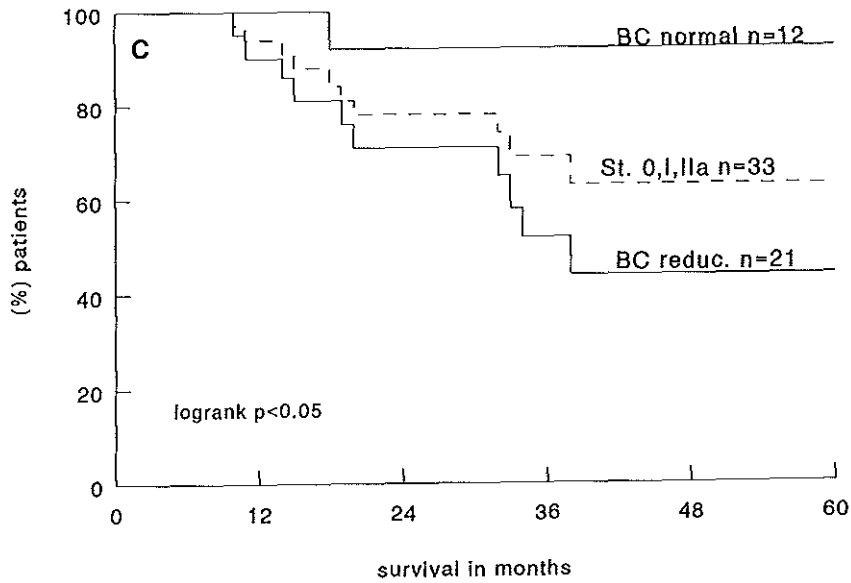
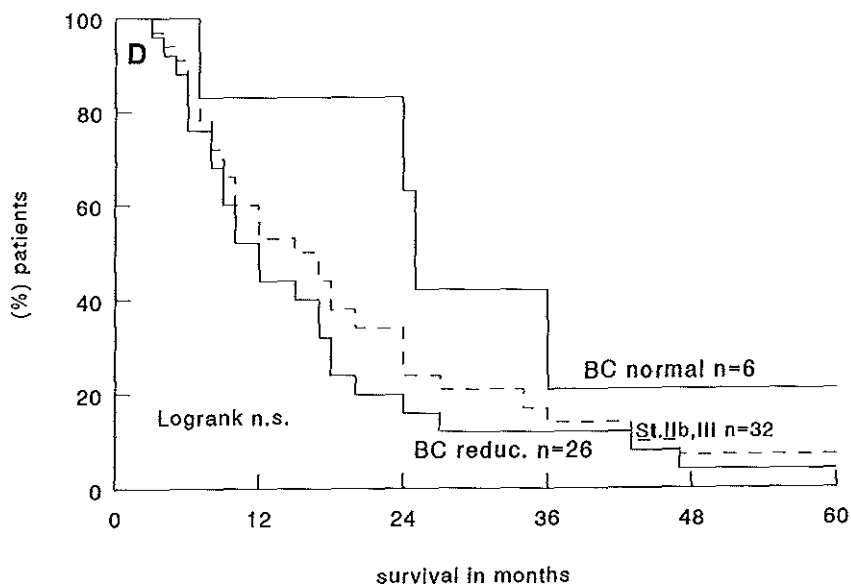

B
100
80
60
40
20
0
(%) patients
BC normal n=17
total N=65
BC reduc. n=48
logrank p<0.01
0
12
24
36
48
60
survival in months

Figure 2
(C,D)


C
BC normal n=12
St. 0,I,IIa n=33
BC reduc. n=21
logrank p<0.05
(%) patients
100
80
60
40
20
0
0
12
24
36
48
60
survival in months


D
BC normal n=6
St.IIb,III n=32
BC reduc. n=26
Logrank n.s.
(%) patients
100
80
60
40
20
0
0
12
24
36
48
60
survival in months

7.4.3 E-Cadherin (EC), Alpha- (AC) and Beta-Catenin (BC) expression in lymph node metastasis.

In the 15 investigated lymph node metastases, heterogeneous or homogeneous positive membrane staining for EC and BC was found in 14 (93%) and for AC in 13 (86%) cases. All these lymph node metastases corresponded with heterogeneously or normally expressed EC, AC and BC in the primary cancers. All but one of the negatively staining (less than 10% positive cells) lymph node metastases arose from heterogeneously stained primary carcinomas. One metastasis, which was negative for AC corresponded with a normally AC expressing carcinoma.

7.5 Discussion

The E-Cadherin-Catenin complex is part of a larger system, including in addition to Alpha- and Beta-, also Gamma-Catenin (plakoglobin), which is linked to the actin cytoskeleton (16, 17, 32). As E-Cadherin (EC) is responsible for tight cellular connections, expression of EC between epithelial cells is concentrated at tight junctions, adherens junctions and desmosomal regions (33). The Catenins, which connect EC with the actin cytoskeleton, are essential for EC function since deletion of the intracellular Catenin binding domain of EC results in loss of adhesive function, even when the extracellular portion of EC is intact (16, 17, 34-35). In carcinomas several mechanisms may impair the function of the E-Cadherin-Catenin complex, which might result in increased invasiveness and metastatic ability of malignant cells. Gene mutations or deletions and decreased expression, and transcriptional and translational abnormalities have been found for EC and Alpha-Catenin (AC) in several malignancies (36-38). Alternatively, down-regulation of the protein complex appears to occur through phosphorylation or competitive binding of Beta-Catenin (BC) to tumor suppressor proteins, such as APC, oncoproteins for instance C-erbB-2, and growth factors such as EGF-R (18-20, 39-42). Consequently, overexpression of such onco proteins and growth factors or mutated APC in carcinomas may influence tumor behavior through modulation or down regulation of the E-Cadherin-Catenin complex (43-45). In esophageal adenocarcinomas overexpression of EGF-R and C-erbB-2 have been frequently found. In several studies C-erbB-2 has been correlated with poor patients' prognosis (43-45). Frequent 5q (APC) allelic loss has been documented in esophageal adenocarcinomas (46-48). However, in these malignancies APC

gene mutations appears to be rare (49).
In various gastro-intestinal cancers a high percentage of tumors with reduced EC, AC and BC expression have been observed. This phenomenon generally appears to be correlated with poor differentiation grade or increased tumor stage (24-28). In squamous cell carcinoma of the esophagus and colon carcinoma, reduced EC, AC and BC expression has been found in 70%-80% of cases (25, 26, 28, 30). In gastric cancers reduced EC, AC and BC expression has been observed in 55% (33/60), 70% (42/60) and 47% (9/19) of the cases, respectively (25, 27, 30). E-Cadherin gene mutations were discovered in 50% of diffuse type gastric carcinomas(36). In scirrhous gastric adenocarcinomas, however, the frequency (55%) of reduced AC expression was much higher than that of reduced EC expression (18%)(50).
Recently Bongiorno et al., showed reduced expression of E-Cadherin in 86% (37/43) of esophageal adenocarcinomas, correlating with stage of disease. However, positive EC staining was observed in 100% (12/12) of the lymph node metastases(24). In esophageal adenocarcinomas, to our knowledge, no literature data is available concerning AC and BC expression in esophageal adenocarcinomas. In the present study we observed reduced BC expression as frequently as reduced EC and slightly more frequently than reduced AC expression in esophageal adenocarcinomas. EC and AC, but not BC were significantly correlated with stage of disease (Table 1). Nevertheless, BC status correlated significantly with survival, independent of stage of disease.
At the level, immunohistochemical findings were compared with clinico-pathological data. Reduced EC, AC and BC expression was found in between 60 and 75% of cases. Moreover, expression of the three proteins was strongly correlated. Reduced expression of EC and AC correlated with unfavorable clinico-pathological parameters, such as poor differentiation grade, advanced stage and poor survival. Interestingly, reduced BC expression did not correlate with stage of disease but strongly correlated with poor differentiation grade and shorter survival. In our study almost all (90%) lymph node metastases expressed all three proteins. The slightly higher number of cases with altered EC staining observed by Bongiorno et al., as compared to the present results, may be explained by methodological differences and the higher number of advanced stage carcinomas in their study (24). The unexpected observation of homogeneous positive expression of the proteins in lymph node metastases, even from carcinomas with reduced expression, may be explained by transient down regulation of the protein complex in cancer cells followed by (re)expression of

the complex in metastasized cells, re-establishing tissue architecture, which requires cell adhesion. Future investigations on the regulation of the expression of these proteins may elucidate this enigma.

In conclusion EC, AC and BC expression in esophageal adenocarcinomas are significantly correlated. Reduced expression of EC, AC and BC is correlated with less favorable clinico-pathological parameters. Moreover, EC and BC expression are prognostic parameters, independent of stage, which might be used to identify patients which run a higher risk for developing disease recurrence.

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Chapter 8

CD44st and CD44v6 Splice Variant Expression in Barrett's Esophagus and Barrett's Adenocarcinoma.

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8.1 Abstract

CD44 is a family of glycoproteins involved in cell-cell and cell-matrix interactions. The standard CD44 form (CD44st) of only 90 kDa is present on most hematopoietic and various epithelial cell types. Larger CD44 forms between 140-230 kDa exist as alternatively spliced exons are inserted in the extracellular domain of the CD44 protein. Expression of CD44 with insertion of the variable exon 6 product (CD44v6) in several malignancies is associated with increased metastatic potential. To analyze the role of CD44 in adenocarcinoma of the esophagus, we studied expression of CD44st and CD44v6 in 35 cases of Barrett's esophagus, in 70 resected esophageal (Barrett's) adenocarcinomas and in 22 lymph node metastases by immunohistochemistry. Expression of CD44st and CD44v6 in Barrett's esophagus was compared with grade of dysplasia and Ki-67 defined proliferation rate. In the adenocarcinomas expression of CD44st and CD44v6 was evaluated with respect to clinico-pathological parameters. In Barrett's esophagus, CD44st expression increased along with the rate of proliferation and dysplasia ($p < 0.01$). In contrast, increased CD44v6 expression was seen in an early stage of malignant transformation. In esophageal adenocarcinomas, increased CD44st and CD44v6 expression was observed in 50% (35/70) and 83% (58/70), respectively. No significant correlations were found between expression of CD44st and CD44v6 and grade or stage of the carcinomas. A trend towards improved five years survival was found for the group without increased CD44v6 (24% versus 55%; $p = 0.06$). In the lymph node metastases CD44st and CD44v6 were positive in 45% (10/22) and 64% (14/22) cases. In conclusion, in Barrett's esophagus CD44st expression increases along with dysplasia and the proliferation rate. Increased CD44v6 occurs early during malignant transformation. Furthermore, esophageal adenocarcinoma patients with increased CD44v6 expression tend to have poorer five years survival.

8.2 Introduction

Barrett's esophagus is a disorder predisposing for esophageal adenocarcinoma. Progression of Barrett's esophagus to adenocarcinoma occurs through gradually increasing dysplasia, along with increased proliferation and accumulation of genetic changes (1-9). Only a fraction of Barrett's esophagus will progress into esophageal adenocarcinoma (10-13). Once this invasive malignancy occurs,

survival of patients is poor, even after seemingly radical surgical intervention (14, 15). Therefore, it is important to find efficient screening parameters, which can identify Barrett's esophagus patients at high risk for malignant transformation. An additional problem is the rapidly increasing incidence of esophageal adenocarcinomas. Since the vast majority of Barrett's esophagus patients have little or no symptoms and therefore is not surveyed, most of the malignant cases are detected when dysphagia and weight loss occur and disease is advanced. Despite surgical intervention, overall five years survival of patients with esophageal adenocarcinoma is poor. The presence of metastases is one of the most important factors determining the prognosis of the patient (14, 15). Little biological data is available concerning progression and metastatic behavior of esophageal adenocarcinomas. Understanding these mechanisms and identifying factors involved in these processes are of major concern and could improve treatment protocols.

The CD44 protein is a multi functional cell surface molecule, involved in processes as lymphocyte homing, lymphocyte activation and extracellular matrix and intercellular adhesion (16). As a result of alternative splicing of 10 exons (v1-v10) more than 20 isoforms have been described. The standard hemopoietic variant (CD44st), sized 90kD, lacks all 10 variable variants and is expressed on a variety of tissues such as epithelium, fibroblasts and cells of hematopoietic origin (17). Gunthert et. al. (18), detected the association between the expression of certain CD44 splice variants and metastatic behavior of cells. Transfection experiments proved that naturally non-metastasizing cells, when transfected with the CD44-v6 splice variant, were transformed into metastatic cells (19). Although the role of CD44 splice variants is not completely understood, the presence of certain splice variants were shown to correlate with metastatic behavior of several cancers, including gastrointestinal malignancies (20-24). In most malignancies the CD44-v6 splice variant is overexpressed and then associated with unfavorable clinico-pathological features. Alternatively, tissue specific overexpression of splice variants has been documented. For instance, upregulation has been found in cervical carcinoma of CD44v7-8, in diffuse type gastric carcinomas of CD44v5, in gastric and renal carcinoma of CD44v9-10 (20, 22, 25, 26). In colon carcinomas expression of CD44v8-10 has been associated with hematogenous metastases, while in general alternatively spliced variants correlate with poor prognosis (27, 28). Interestingly, in colon adenomas CD44v6 expression is correlated with dysplastic features (29-31).

In the present study we evaluated, by immunohistochemistry, CD44st and

CD44v6 expression in archival material of 35 cases of Barrett's esophagus, 70 of Barrett's adenocarcinomas and 22 lymph node metastases of the esophageal adenocarcinomas. In Barrett's esophagus CD44 immunostaining results were compared with dysplasia and proliferation rate as reflected in Ki-67 expression. In adenocarcinomas staining results were evaluated with respect to histopathological parameters and patient survival.

8.3 Material and Methods

8.3.1 Clinico-pathological data

Barrett's oesophagus was defined as columnar epithelial lining of the oesophagus at least three cm proximal to the gastroesophageal junction. Only cases with specialized columnar type of metaplasia, characterized by the presence of intestinal columnar cells and goblet cells, were included. Dysplasia was graded into three categories following criteria revised by Reid et al, i.e.; negative, indefinite or low grade dysplasia, and high grade dysplasia (32). Thirty five cases of Barrett's esophagus, encountered between 1987 and 1992 at the Academic Hospital-Dijkzigt Rotterdam, were included for immunohistochemical study, 26 (74%) male and 9 (26%) female. Mean age was 62 (range 28 to 81). Thirty two cases of Barrett's esophagus were biopsy specimens of patients without esophageal adenocarcinoma; three Barrett's esophagus were esophageal resection specimens with high grade dysplasia but without invasive carcinoma.

Seventy cases of resected esophageal adenocarcinomas associated with Barrett's oesophagus, encountered between 1985 and 1992, were included in this study. In addition in 22 cases lymph node metastases were investigated as well. Fifty three of these patients were male, 17 female, mean age was 67 (Range 34-80 years). All patients had undergone cardia-esophageal resection with microscopically complete removal of the carcinoma. Hematoxylin-eosin stained sections were used for tumor staging and grading. According to pTNM criteria, carcinomas were categorized in malignancies without (Stage I, IIa) or with loco-regional or distant lymph node metastasis (Stage IIb, III). Carcinomas which clearly showed glandular structures, with well developed intercellular junctions throughout the tumor, were classified as well/moderately differentiated carcinomas. Carcinomas were classified as poorly/undifferentiated when focally or completely characterized by lack of differentiation and high nuclear pleomorphism. Survival times were calculated from the date of operation to death resulting from

recurrence of disease, or to last clinical control date.

8.3.2 Tissues

Of each resection specimen the formalin fixed paraffin embedded tissue block representing deepest level of tumor invasion in the esophageal wall, and in 22 cases also one paraffin block with lymph node metastases was selected for immunohistochemical investigations. Of the Barrett's biopsy specimens, per case only those biopsies representing the highest degree of dysplasia were used for immunohistochemical evaluation. Adjacent squamous cell epithelium or non dysplastic columnar (cardia) epithelium served as internal positive control.

8.3.3 Monoclonal antibodies

Monoclonal IgG1 antibodies, Mab VFF-18 (diluted 1:100 in PBS), which specifically recognizes the epitope encoded by exon v6 of the variant portion, and Mab SFF-2 (diluted 1:100 in PBS), which reacts with an epitope of the non-variable portion of the human CD44 molecule were used for CD44v6 and CD44st staining respectively (Bender Med Systems, Vienna Austria). The Mib-1 IgG monoclonal antibody (diluted 1:50 in PBS) which specifically recognizes the Ki-67 antigen expressed in cycling cells, was used to determine proliferation in Barrett's esophagus (Dako, Glostrup Denmark).

8.3.4 Immunohistochemistry

Five μm paraffin sections were mounted to 3-amino-propyl-triethoxy-silane (Sigma, St. Louis, USA) coated slides and stored overnight at 60°C. Sections were deparaffinized, endogenous peroxidase was blocked in 3% H_2O_2 in methanol for 20 minutes and rinsed in phosphate buffered saline (PBS). For enhancement of Ki-67, CD44st and CD44V6 staining, the sections were heated in a microwave oven (submerged in 0.01 M Citrate buffer pH 6.0) for two cycles of five minutes. Appropriately diluted monoclonal antibodies were applied to sections and left for overnight incubation at 4°C. As a detection system we used streptavidin-biotin-peroxidase (Dako, Glostrup Denmark) with diaminobenzidin tetrachloride (Fluka, Basel Swiss) as chromogen. Tissues were counterstained with hematoxylin.

8.3.5 Evaluation of proliferation (Ki-67 antigen staining), CD44st, and CD44v6 expression in Barrett's esophagus

In Barrett's esophagus, Ki-67 staining and staining of CD44st and CD44v6 were

scored by two independent investigators. Three increasing levels of expression were defined in Barrett's esophagus. Compartment 1 (C1) was defined as the glandular or crypt portion of the mucosa; compartment 2 (C2) included staining in the neck area of the villus; compartment 3 (C3) also included the luminal side of the villus (fig. 1B).

8.3.6 Evaluation of CD44st and CD44v6 expression in esophageal adenocarcinomas and lymph node metastases

Immunohistochemical staining of CD44st and CD44v6 in subsequent tissue sections was scored semi-quantitatively, in 10% intervals (0-10%, 10-20%, etc) by two independent investigators in three previously selected areas per carcinoma (10x objective). Percentages as scored by the two investigators were averaged per tumor area. In order to compare immunohistochemical results with clinicopathological data, eventually one average score per carcinoma was calculated. Since CD44st and CD44v6 are normally expressed in the crypt zone of non dysplastic intestinal type (metaplastic) epithelium, carcinomas were considered as having increased CD44st or CD44v6 expression when on average more than 20% of cells was positive.

8.3.7 Statistical analysis

In Barrett's esophagus differences in expression of CD44st, CD44v6 and Ki-67 expression were calculated by Wilcoxon signed rank test. Correlations between these parameters and dysplasia in Barrett's esophagus, and the correlation between the CD44st and CD44v6 in adenocarcinomas were evaluated by calculating Spearman's rank correlation coefficient.

Figure 1. A (α, β, γ): Immunohistochemical staining (IHC) showing nuclear staining of Ki-67 (α), and membranous staining of CD44st (β) and CD44v6 (γ) in normal stratified squamous epithelium of the esophagus (arrows). Staining of the three epitopes is found in the same area of the epithelium. Notice that staining of CD44v6 is more intense than of CD44st. B-D: subsequent sections of a Barrett's esophagus with low grade dysplasia, stained for Ki-67, CD44st and CD44v6 respectively. B: The defined areas for scoring IHC results are shown: Compartments C1: gland and crypt zone; C2: C1+ neck zone and C3: C2+ luminal side. Ki-67 positive are shown in C2 (arrow heads). C, D: CD44st and CD44v6 positive cells are found in C3 (arrows). Notice that virtually all epithelial cells are stained for CD44v6. E, F: Subsequent sections of a moderately differentiated esophageal adenocarcinoma stained for CD44st and CD44v6, respectively. E: Most carcinoma cells lack CD44st staining (arrows), whereas stromal cells and leukocytes show positive membrane staining (arrow heads). F: CD44v6 staining is present on almost all carcinoma cells (arrows), whereas stromal cells are negative (arrow heads).

Figure 1
(A)

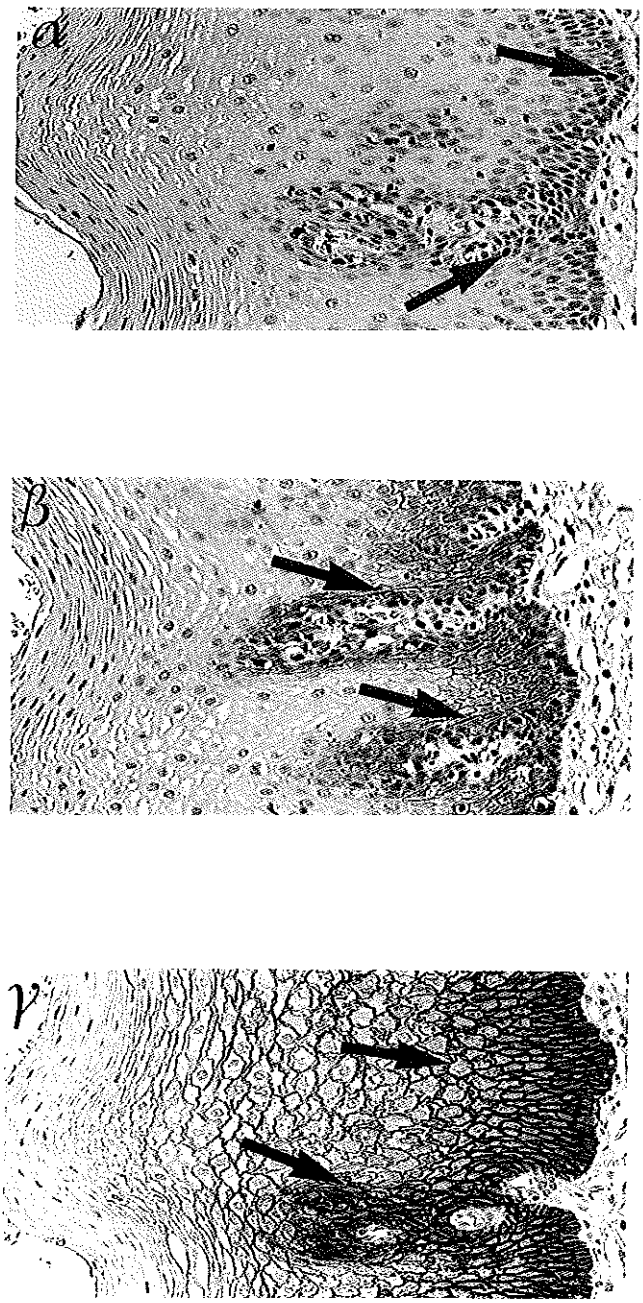


Figure 1
(B,C,D)

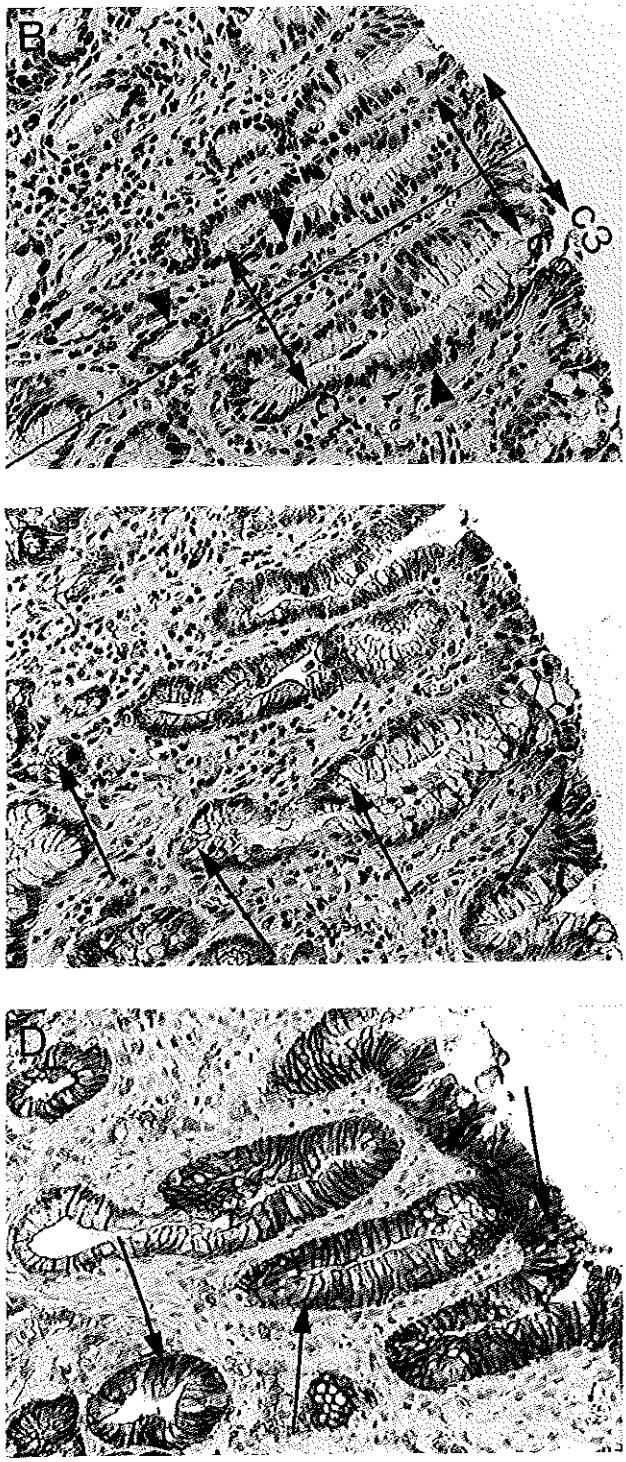
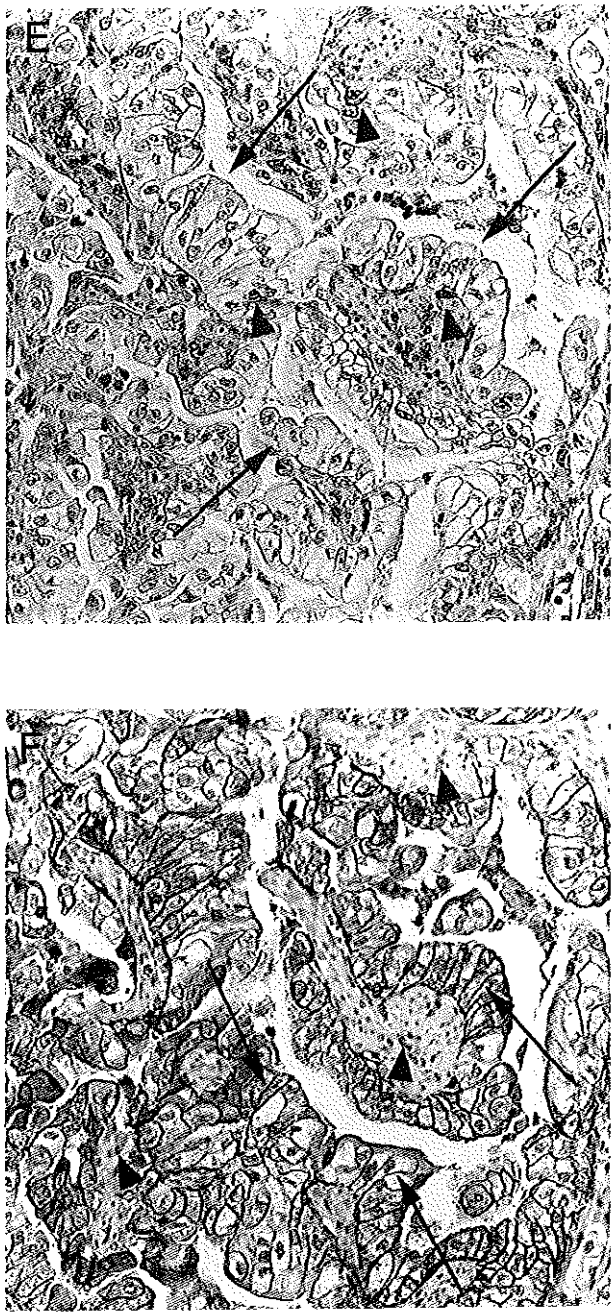


Figure 1
(E,F)



Cross tabulations were performed to assess correlations between CD44st and CD44-V6 expression, and grade and stage of the carcinomas. Significance was tested by the Fisher's exact test (two tailed). Survival probabilities were calculated and compared by the Kaplan-Meier method and logrank test. Multivariate analysis to determine independent prognostic value of the various parameters was performed by Cox regression. For all tests, $p=0.05$ (two sided) was considered the limit of significance.

8.4 Results

8.4.1 CD44st and CD44v6 in control tissues, Barrett's esophagus and esophageal adenocarcinomas

Homogeneous membrane immunostaining for CD44st and CD44v6 was found in all cases of normal squamous epithelium and in the crypts and glands of normal cardia mucosa and non dysplastic Barrett's epithelium (figure 1A, B). These tissues adjacent to the adenocarcinomas served as internal controls for the staining procedures. In squamous epithelium membrane immunostaining was present homogeneously on the intermediate cells and on the basal layer, but not on the basal membrane side of the basal cells (figure 1A). In columnar cardia epithelium, intercellular membrane staining was only seen in the gland and crypt zone. In both squamous epithelium and columnar epithelium CD44v6 staining was more often found and more intense than CD44st (figure 1A). Membrane staining for CD44st, but not for CD44v6, was seen for a variety of other different cell types, including lymphocytes and stromal cells.

8.4.2 Dysplasia, Ki-67 antigen (proliferation), CD44st and CD44v6 in Barrett's esophagus

Thirty five cases of Barrett's esophagus were analyzed for dysplasia, Ki-67 antigen expression, and CD44st and CD44v6 immunoreactivity (figures 1B-D). Fourteen cases were non dysplastic, 16 showed indefinite or low grade dysplasia and five cases were highly dysplastic (figure 2).

In Barrett's esophagus without dysplasia proliferation was observed in C1 in 13/14 and in C2 in 1/14. In all non dysplastic cases CD44st was seen in C1 only. In contrast, CD44v6 was seen in C1, C2 and C3 in 8/14, 5/14 and 1/14 Barrett's esophagus without dysplasia.

In cases indefinite for dysplasia or with low grade dysplasia, Ki-67 antigen was

observed in C1, C2 and C3 in 6/16, 8/16 and 2/16; CD44st in 9/16, 6/16 and 1/16; and CD44v6 in 3/16, 4/16 and 9/16 cases, respectively (figures 1B-D, 2). In Barrett's esophagus with high grade dysplasia Ki-67 antigen staining was positive in C2 and C3 in 2/5 and 3/5; CD44st in 1/5 and 4/5 cases, respectively. In all five cases of high grade dysplasia CD44v6 staining was seen in C3.
Both Ki-67 and CD44st staining correlated significantly with grade of dysplasia (table 1; Spearman rank corr coef. 0.7, $p<0.001$). Overall, CD44v6 staining, compared to CD44st and Ki-67 expression, was significantly more frequently observed in C2 and C3 (figure 2; table 1, Wilcoxon signed rank test $p<0.01$). CD44v6 expression did not correlate with dysplasia.

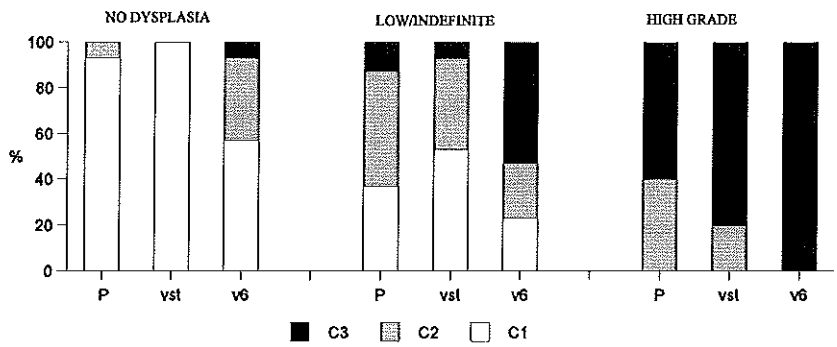


Figure 2: Y-axis: Percentages of patients with Barrett's esophagus without dysplasia, with indefinite/low grade dysplasia and with high grade dysplasia, respectively. X-axis: P: proliferative activity assessed by Ki-67 antigen immunohistochemistry (IHC). st: CD44st expression. v6: CD44v6 expression. C1: immunohistochemical stainging in gland and crypt zone of villi; C2: in C1 and neck zone; C3: in C2 and luminal side of villi. Notice that Ki-67 and CD44st immunostaining in C2 and C3 occurs as dysplasia increases. CD44v6 immunostaing in C2 and C3 is seen in all cases of high grade dysplasia but as well in 77% of cases with indefinite/low grade dysplasia and 43% of cases without dysplasia.

8.4.3 CD44st and CD44v6 in esophageal adenocarcinomas

Of the 70 esophageal adenocarcinomas 37 (53%) were classified as well/moderately differentiated and 33 (47%) as poorly/undifferentiated. Fifty percent of the carcinomas had no (stage 0, I, IIa), the other 50% had loco-regional or distant lymph node metastases (stage IIb, III ; table 2).
In the adenocarcinomas, a median score of 21% (S.D. 26) for CD44st positive cells was calculated. For CD44v6 expression a median score of 58% (S.D. 27) was calculated. There was no correlation between CD44st and CD44v6

Table 1: Cross tabulation of immunohistochemical staining results with monoclonal antibodies against the CD44st and CD44v6 epitopes in 70 esophageal adenocarcinomas.

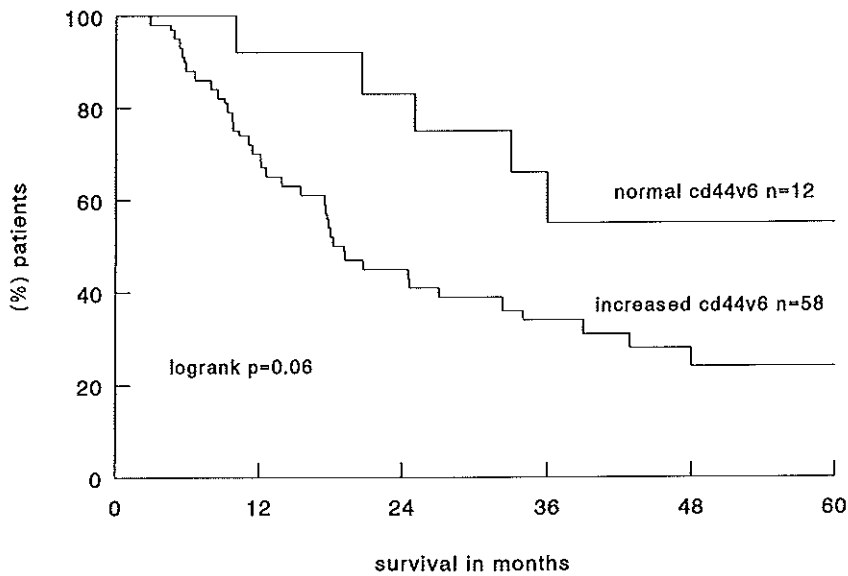
	Pts	CD44st normal	CD44st increased	p[1]	CD44v6 normal	CD44v6 increased	p[1]	5-yrs	p[2]
Total (%)	70	35(50)	35(50)		12(17)	58(83)			
Stage[3]:									
0,I,IIa (%)[4]	35	15(43)	20(57)		8(67)	27(47)		45%[5]	
IIb,III	35	20(57)	15(43)	0.17	4(33)	31(53)	0.17	0%	<0.001
Grade[6]:									
well/moder	37	21(60)	16(46)		11(92)	26(45)		35%	
poor/undiff	33	14(40)	19(54)	n.s.	1(8)	32(55)	<0.01	12%	<0.01
CD44st									
normal	35				9(75)	26(45)		37%	
increased	35				3(25)	32(55)	n.s.	25%	n.s.
CD44v6									
normal	12							55%	
increased	58							24%	0.06

1) p-value by two tailed Fisher exact test. 2) p-value by logrank test. 3) Stage: 0=pTisN0M0 I=pT1N0M0 IIa=pT2-3N0M0; IIb=pT12N1M0 III=pT3-4N1-3M0. 4) (%) column percentages. 5) Five years survival probability calculated by Kaplan-Meier method. 6)Grade: Well/Moderately; poorly-undifferentiated.

Immunostaining. Increased CD44st and CD44v6 expression (>20% positive cells) was seen in 49% (34/70) and 83% (58/70), respectively (figure 1, table 2). Overall survival correlated significantly with stage and grade of disease. Overall five years survival of patients without metastases at time of resection was 45% compared to 6% for patients with metastases at time of resection (logrank test $p<0.001$). For patients with well and moderately differentiated adenocarcinomas five year survival was 35% compared to 12% for those with poorly or undifferentiated adenocarcinomas (logrank test $p<0.01$). Survival of patients with normal or increased expression of CD44st did not differ. A trend was found for the group with increased CD44v6 to shorter survival (55% versus 24%; logrank test $p=0.06$; figure 3).

Upon multi-variate analysis stage was the only prognostic factor, which, independently of grade, CD44st and CD44v6 expression, predicted survival (Cox regression analysis $p<0.01$).

Figure 3: Kaplan Meier survival curves showing five year survival of patients with esophageal adenocarcinomas with normal (n=12) versus increased (n=58) CD44v6 expression. A trend is found towards poorer survival for patients with increased CD44v6 expression (logrank test p=0.06)



8.4.4 CD44st and CD44v6 in lymph node metastases

Twenty two lymph node metastases were investigated for CD44st and CD44v6 expression. Increased CD44st expression was found in 45% (10/22) and CD44v6 in 64% (14/22) of cases. In the lymph node metastases no significant correlation was found between CD44v6 and CD44st expression. Expression of CD44st in the lymph node metastases correlated significantly with CD44st expression in the primary adenocarcinoma; concordant staining was found in 85% of cases ($p<0.02$). Increased CD44v6 expression in primary adenocarcinomas and lymph node metastases was concordant in 73% (16/22) of cases ($p=0.1$). In five cases CD44v6 was expressed in the primary adenocarcinoma but not in the lymph node metastasis, whereas in one case CD44v6 expression was seen in the lymph node metastasis but not in the corresponding adenocarcinoma.

8.5 Discussion

Development of esophageal adenocarcinomas in Barrett's esophagus occurs through increasing dysplasia, concurrent with increased proliferation and accumulation of genetic abnormalities (1-9). Overall, patients with esophageal adenocarcinomas have poor prognosis. The presence of metastases is the most important factor predicting patient survival (14, 15).

Expression of CD44st and certain CD44 splice variants have proven to be predictive for behavior of several malignancies. The CD44v6 splice variant expression has not only been associated with unfavorable clinicopathological characteristics in several carcinomas (20-24), but has also been observed in premalignant lesions such as in colon adenomas (29-31).

In this study we present data on CD44st and CD44v6 expression in 35 cases of Barrett's esophagus with different degrees of dysplasia, in 70 esophageal adenocarcinomas associated with Barrett's esophagus, and also in 22 lymph node metastases. Although in control epithelia a consistently higher number of cells stained positive for CD44v6 than for CD44st, in these epithelia CD44st and CD44v6 staining was clearly correlated.

In Barrett's esophagus we compared CD44st and CD44v6 expression with grade of dysplasia and proliferation rate. CD44st expression increased along with increased dysplasia and proliferative activity, but CD44v6 did not. In literature a similar correlation between CD44 and proliferation was noted for colonic epithelium and adenomas (33). Also in gastric intestinal metaplasia increased CD44 expression was observed (20). CD44v6 expression in Barrett's esophagus did neither correlate with dysplasia nor with proliferation nor with CD44st expression. Excessive CD44v6 expression was seen in all cases of Barrett's esophagus with high grade dysplasia but was also observed in many cases with low grade and some cases without dysplasia. This indicates that increased CD44v6 expression is an early event during neoplastic transformation in Barrett's esophagus. In contrast, in colon adenomas increased CD44v6 expression has almost exclusively been observed in areas with high grade dysplasia (29-31).

In the esophageal adenocarcinomas, increased CD44st was observed in a lower number of cases than CD44v6 expression (50% versus 83% respectively). Gunthert et al. (34), observed that not only epitopes of the variable portion of the CD44 molecule, but also the standard part of the CD44 molecule may be differentially expressed. As we used a monoclonal antibody which recognizes only one epitope of the CD44st domain, this may explain why a number of

carcinomas with increased CD44v6 immunostaining did not stain for CD44st. Alternatively, differences in epitope affinity of the anti CD44st and CD44v6 antibodies, resulting in different staining intensities, may have confounded the staining results.

The proportion of cases with increased CD44st and CD44v6 found in this study for esophageal adenocarcinomas is comparable to that found for other gastro-intestinal carcinomas (20, 24). Interestingly, univariate analysis showed a trend of increased CD44v6 expression to be correlated with poorer survival ($p=0.06$). Also breast and colon carcinomas as well as in non-Hodgkin lymphomas CD44v6 expression is significantly correlated with survival (35).

Sixtyfour per cent (14/22) of the lymph node metastases showed increased CD44v6 staining. Interestingly in 5/16 cases CD44v6 expression was not increased in the lymph node metastases. Since most carcinomas showed heterogeneous CD44v6 expression, it is likely that metastatic competence is not limited to the CD44v6 positive cells. Alternatively, progression of metastasized carcinoma cells may lead to loss of CD44v6 expression.

Expression of CD44v6 has been associated with increased malignant behavior, in spite of the fact that in many epithelia these variants are normally expressed (29, 36). To differentiate between regulated and abnormal CD44v6 expression in Barrett's esophagus, investigation of the expression of splice variants expression at mRNA level would be necessary(20). Unfortunately, the paraffin embedded material used in this study did not allow these analyses. Moreover, for clinical practice RNA analyses-assays are too laborious, demanding special conditions for tissue preservation in order to obtain liable results. Assessment of progression specific expression of epitopes of the CD44 molecule which are specific tumor progression markers may be more applicable for clinical use. Recently, intron 9 of CD44 has been found to be expressed in 80% of colon adenocarcinomas and to be expressed in several cell-lines of gastro intestinal origin (37). As expression of an intron sequence is an abnormal event and presumably the result of gene derangement, immunohistochemical assessment of these epitopes may be more tumor specific. However, expression of CD44st and CD44 splice variants is complicated and yet not fully understood. This caveat calls for careful interpretation immunohistochemical staining.

In conclusion, we have found in Barrett's esophagus expression of CD44v6 splice variant early during development of neoplastic transformation, reaching a high incidence in esophageal adenocarcinomas. At present immunohistochemical staining of CD44 epitopes do not have diagnostic value for evaluation of

8.6 References

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Chapter 9

Concluding Remarks

9.1 Model

In this study several biological markers were investigated in premalignant Barrett's esophagus and esophageal adenocarcinoma. The aim was to determine the prognostic value of these markers in premalignant and malignant stages. This was done by correlating several biological parameters in different stages of disease, ranging from non dysplastic intestinal type of metaplasia through low grade and high grade dysplasia to adenocarcinoma and lymph node metastases. Based on the presented results and the literature data, we propose a model with a step wise evolution, in which the significance of these markers during the process of malignant transformation is evaluated (Figure 1). In this chapter I will refer to the paragraphs in chapter 2 in which the original literature has been discussed. In the model, secretory factors, such as O-acetylated sialomucins and sucrase-isomaltase (Chapter 2.2) and growth factors such as TGF-Alpha and EGF-R (Chapter 2.4) are frequently found in high levels in non dysplastic as well as in dysplastic intestinal type of Barrett's mucosa. Therefore, these factors do not seem to identify the group of Barrett's esophagus patients which has an

Figure 1. Molecular events as they may occur along the sequence of metaplasia to low and high grade dysplasia, and subsequently to adenocarcinoma and metastasis

Intestinal type metaplasia

⇩ O-acetylated sialomucins, Sucrase-isomaltase, EGF-R, TGF-alpha

Low grade dysplasia

⇩ Glutathione, P53, aneuploidy, Y-Chromosome loss C-erbB2, APC, MCC

High grade dysplasia

⇩ H-Ras

Adenocarcinoma

⇩ E-Cadherin, Alpha- and Beta-Catenin, CD44-v6, C-erbB2

Metastasis

increased risk for developing an adenocarcinoma.

Parameters which do seem to be associated with neoplastic progression are decreased glutathione and glutathione-S transferase enzyme activity (Chapter 2.2) increased proliferative activity (Chapter 2.3), aneuploidy (Chapter 2.8) aberrant p53 (Chapter 2.6) and Y-chromosome loss (Chapter 2.7). Typically, these markers are observed at low frequency in non dysplastic Barrett's esophagus, they are increasingly observed along with increasing dysplasia and a high frequency is reached in high grade dysplasia and in the adenocarcinomas. In a prospective follow-up study the majority of Barrett's esophagus patients with aneuploidy or increased tetraploidy in their initial biopsy specimens progressed towards high grade dysplasia or carcinoma, whereas patients without these abnormalities did not (Chapter 2.8). In chapters 5, 6 and 8, the correlation of increased proliferation rate, p53 protein accumulation, aneuploidy and Y-chromosome loss with dysplasia was studied. In chapter 5, we report increased levels of proliferation in intestinal type of metaplasia with and without dysplasia. However, detailed analysis of proliferation in Barrett's esophagus revealed that as the epithelium becomes dysplastic the zone of proliferating cells tends to expand to the luminal surface of the mucosa. A similar expression pattern was observed for CD44st (chapter 8).

The evolution from low grade to high grade dysplasia is characterized by increasing genetic instability. Proliferation rate and aneuploidy increase (Chapter 2.3, 2.8, 5, 8). In addition to p53 aberrations other tumor suppressor genes and oncogenes such as APC, and H-ras showed abnormalities (Chapter 2.5, 2.6). It is not known in which order these genetic aberrations occur and neither whether the order is at all of importance. Nevertheless, there is evidence that p53 (17p) aberrations precede aneuploidy and APC (5q) gene loss (Chapter 2.7).

For dysplastic cells to become invasive cancer cells a crucial step is the moment they acquire the ability to break through the basement membrane and invade surrounding tissues. In frankly invasive adenocarcinomas the accumulation of numerous genetic aberrations have been seen (Chapter 2). However, little data is available about genetic events specifically involved in transition from high grade dysplasia into adenocarcinoma. In a small series H-ras expression was observed in high grade dysplasia and adenocarcinomas but not in low grade dysplasia, nor in non dysplastic epithelium (Chapter 2.5). This suggests an important role of H-ras in the transition from high grade dysplasia to adenocarcinoma.

Perhaps the most fascinating step in carcinogenesis is the formation of metastases. Formation of metastases is a complex process in which many factors are involved. In this process, epithelial cells are liberated from their original

compartment, break through the basement membrane, invade lymph and blood vessels, adhere to a new environment and proliferate. It has been observed that esophageal adenocarcinomas with limited invasion of the esophageal wall already may have metastasized (Chapter 1.3). It seems that as soon as cells invade the submucosa, metastatic ability is rapidly acquired. In a few studies aneuploidy was found to correlate with the presence of metastases and poor survival (Chapter 2.8). Another study showed a correlation between C-erbB2 expression and poor survival (Chapter 2.5). Recently, cell surface molecules and their role in tumor cell behavior gained interest. Cell surface molecules, such as those involved in the E-Cadherin-Catenin complex and CD44 with its splice variants, play an important role in tumor invasiveness and metastatic ability (Chapter 2.9, 2.10). A high frequency of decreased expression of E-Cadherin was found in esophageal adenocarcinomas (Chapter 2.9). In our studies decreased expression of Alpha- and Beta-Catenin was found to occur roughly in the same frequency as E-Cadherin expression (Chapter 7). Interestingly, in a subset of carcinomas without metastases and sometimes with limited invasion of the esophageal wall, decreased expression of these proteins correlated with poor survival (Chapter 7). Another interesting cell surface molecule studied in this thesis is CD44. It has become apparent that the expression of the CD44v6 splice variant is associated with increased tumor invasiveness and metastatic ability (Chapter 2.10). We detected increased CD44v6 splice variant expression in a high percentage of esophageal adenocarcinomas (Chapter 8). A trend was found between increased CD44v6 expression and poor five year survival. Surprisingly, a high level of CD44v6 expression was found in Barrett's esophagus as well (Chapter 8).

9.2 In conclusion

In conclusion, in the progression of Barrett's esophagus via dysplasia to carcinoma and the development of metastases formation numerous parameters are involved. In this study p53 protein accumulation, aneuploidy, Y-chromosome loss, high expression of the CD44st molecule and increased proliferation rate were found to go along with increasing dysplasia. These markers may identify Barrett's esophagus at risk for neoplastic progression. Nevertheless, if these markers were to be applied for clinical decision making, their relevance should be evaluated in long term follow up studies of patients with Barrett's esophagus. For most of these parameters long term follow up studies are lacking.

The most promising marker investigated is p53. During neoplastic progression mutation of the p53 gene seems to be a key event which is followed by several other genetic alterations. In addition to histological grading of dysplasia, detection of p53 aberrations might become an important diagnostic tool.
A remarkable observation in this study was loss of the Y-chromosome, which occurs as dysplasia increases. The meaning of Y-chromosome loss is unclear. At present no specific genes involved in tumorigenesis have been located on the Y-chromosome. Therefore, Y-loss is considered a random effect, reflecting genetic instability and as such is a parameter to detect neoplastic progression in male Barrett's esophagus patients.
The transformation of high grade dysplasia into adenocarcinoma is poorly understood. Possibly the same factors involved in tumor invasiveness and metastatic behavior of esophageal adenocarcinomas are involved in this transitional step. Future investigation of biological markers involved in tumor behavior in high grade dysplasia and early carcinomas are of major importance.
With respect to tumor behavior and metastasis decreased expression of the cell surface molecules E-Cadherin, Alpha- and Beta-Catenin and increased expression of CD44v6 in esophageal adenocarcinomas were found to correlate with poor survival. Although these results may seem useful for clinical applications, the underlying mechanisms involved in up and down regulation of these factors are poorly understood and should be explored in more detail. For instance, there is a complicated interaction between the E-Cadherin-Catenin complex and several intracellular factors such as the tumor suppressor APC and the C-erbB2 oncogene. Concerning CD44 splice variants, little is known about their interactions and regulation of expression. Therefore, the use of the expression of cell surface molecules for predicting survival at this moment is premature.

Summary

Barrett's esophagus is a condition in which, due to chronic gastro-esophageal reflux, the normal squamous epithelium is transformed into columnar epithelium. Compared to the general population, patients with Barrett's esophagus have an increased risk of 30 to 120 fold for developing esophageal adenocarcinomas. Malignant transformation of Barrett's esophagus is a gradual process which is reflected in histological changes, classified as low and high grade dysplasia. Unfortunately, most patients with Barrett's esophagus have no or few symptoms (gastro-esophageal reflux), and esophageal adenocarcinoma is often detected in advanced stage of disease. During the past decades there has been an alarming rise in the incidence of esophageal adenocarcinomas. In Western countries the incidence of esophageal adenocarcinomas reaches almost 1 per 100.000 per year. The prevalence is highest in white middle aged males of higher socio-economic classes. Esophageal adenocarcinomas are frequently metastasizing tumors associated with an average five year survival of less than 20%. However, in case treated in an early stage of the disease, survival improves significantly.

In this thesis studies concerning neoplastic progression of Barrett's esophagus and metastases formation in esophageal adenocarcinomas are reported. In our studies we analyzed several biological parameters in non dysplastic and dysplastic Barrett's esophagus, in esophageal adenocarcinomas and metastases. In a pilot study aneuploidy, p53 protein accumulation and numerical chromosomal aberrations such as chromosome 17- and Y-loss in adenocarcinoma and Barrett's esophagus were detected. P53 protein accumulation was evaluated in over a hundred biopsy specimens of patients with Barrett's esophagus with varying degrees of dysplasia and in 24 esophageal adenocarcinomas. In approximately 80% of the adenocarcinomas p53 protein had accumulated. In Barrett's esophagus a significant correlation was found between p53 protein accumulation and increasing dysplasia. In a subsequent study aneuploidy and the status of X and Y-chromosome was investigated in a series of 50 biopsy specimens of patients with Barrett's esophagus and in 23 adenocarcinomas. Aneuploidy and Y-chromosome loss were found to correlate with dysplasia. The frequencies of both parameters reached 70-100% in high grade dysplasia and adenocarcinomas. Other parameters investigated in Barrett's esophagus have been the expression of the cell surface molecules CD44st and CD44v6 splice variant and proliferation rate. Expression of these parameters has been investigated in different compartments of the Barrett's esophagus mucosa. As dysplasia increased proliferation and

CD44st expression were seen more often at the luminal side of the crypts. CD44v6 splice variant expression, a cell surface molecule associated with aggressive tumor behavior, was highly expressed in both non dysplastic and dysplastic epithelium. The metastatic behavior of esophageal adenocarcinoma has been studied by evaluation of cell surface markers with respect to clinicopathological parameters. These markers are associated with increased invasiveness and metastatic ability of carcinomas. Decreased expression of the adhesion molecules, E-Cadherin, Alpha- and Beta-Catenin were found in 60 to 70% of carcinomas. Decreased expression of all three proteins correlated with poor differentiation grades. Independent of stage, E-Cadherin and Beta-Catenin correlated with poor survival. Increased expression of the cell surface molecules CD44st and its CD44v6 splice variant was found in 50 and 83% respectively. There was a trend between CD44v6 expression and five year survival.

In this thesis global knowledge has been acquired concerning carcinogenesis, tumor behavior and metastases formation. However, many questions remain to be answered concerning the complex biological mechanisms which are involved in expression of the various parameters. On the other hand, long term follow up studies of Barrett's esophagus patients are necessary to proof the clinical value of the several parameters. As knowledge of the biological mechanisms involved in carcinogenesis increases, this knowledge will be increasingly applied for choosing the best treatment modality for the individual patient.

Samenvatting

Barrett oesofagus is een aandoening, waarbij het normale niet verhoornend meerlagig plaveisel epitheel van de oesofagus, door langdurige gastro-oesofageale reflux, is veranderd in cilindrisch epitheel. Patiënten met een Barrett oesofagus hebben vergeleken met de normale bevolking een 30 tot 120 maal verhoogd risico voor het krijgen van een oesofagus adenocarcinoom. Maligne transformatie van Barrett epitheel is een geleidelijk proces, dat wordt gekenmerkt door histologische veranderingen. Binnen deze histologische veranderingen wordt onderscheid gemaakt tussen niet dysplastisch, licht en sterk dysplastisch epitheel. Het merendeel van de patiënten met een Barrett oesofagus heeft geen (reflux) klachten, waardoor veel oesofagus adenocarcinomen in een laat stadium worden ontdekt. Gedurende de afgelopen decennia is de incidentie van oesofagus adenoarcinomen alarmerend toegenomen. In Westerse landen is deze incidentie bijna 1 per 100.000. De belangrijkste risico groep voor het krijgen van een oesofagus adenocarcinoom wordt gevormd door blanke mannen van middelbare leeftijd uit een hoog socio-economische milieu. Oesofagus carcinomen zijn geassocieerd met vroege metastasering en een vijf jaars overleving van minder dan 20%. De overleving verbetert significant indien de ziekte in een vroeg stadium wordt ontdekt.

In dit proefschrift is de maligne progressie van Barrett oesofagus en het progressieve gedrag van oesofagus adenocarcinomen onderzocht. Verschillende biologische factoren werden bestudeerd in niet dysplastisch en dysplastisch Barrett epitheel, in oesofagus adenocarcinomen en in lymfklier metastasen. In een 'pilot' studie werden aneuploidie, p53 proteïne accumulatie en numerieke chromosomale afwijkingen, zoals verlies van chromosomen 17 en Y ontdekt. Deze bevindingen werden in de daarop volgende studies verder geëvalueerd. Aberrante accumulatie van het p53 eiwit werd in meer dan 100 biopten met verschillende graden van dysplasie en in 24 oesofagus adenocarcinomen bekeken. Accumulatie van p53 werd in ongeveer 80% van de adenocarcinomen gezien. In Barrett oesofagus correleerde p53 accumulatie significant met toenemende dysplasie. In de studie die hierop volgde werden aneuploidie en de status van het X en Y-chromosoom bestudeerd in 50 biopten van patienten met Barrett oesofagus en in 23 oesofagus adenocarcinomen. Zowel aneuploidie en verlies van het Y chromosoom correleerden significant met toenemende dysplasie. Beide parameters hadden een frequentie van 70-100% in biopten met sterke dysplasie en in de adenocarcinomen. Andere parameters die in Barrett oesofagus werden

onderzocht zijn proliferatie, CD44st en CD44v6 expressie. Expressie van deze factoren werden in verschillende compartimenten van de villi in het Barrett epitheel gescoord. Naarmate de dysplasie toeneemt werden expressie van CD44st en proliferatie van het epitheel vaker in het luminale compartiment van de villi gezien.

Het metastaserend gedrag van de oesofagus adenocarcinomen werden onderzocht door evaluatie van de cel membraan markers, E-Cadherine, Alpha-, Beta-Catenine, CD44st en CD44v6. Expressie van deze molekulen werd met betrekking tot clinico-pathologische parameters geëvalueerd. Aberrante expressie van deze moleculen zijn geassocieerd met toegenomen invasiviteit en metastase capaciteit van tumoren. Verlaagde expressie van de adhesie moleculen E-Cadherine, Alpha- en Beta-Catenine werd in 60-70% van de adenocarcinomen waargenomen. Deze verlaagde expressie correleerde met een slechte differentiatie graad van de adenocarcinomen. Verlaagde expressie van E-Cadherine en Beta-Catenine correleerden significant met een slechtere vijf jaars overleving. Toegenomen expressie van CD44st en CD44v6 werden in respectievelijk 50 en 83% van de tumoren gezien. Een trend werd gevonden tussen verhoogde expressie van CD44v6 en vijfjaars overleving.

Ten slotte wordt in dit proefschrift een model voorgesteld waarin de prognostische waarde van verschillende markers worden geëvalueerd. Proliferatie, aneuploidy, p53 en Y-chromosoom verlies zijn potentieel factoren die in een vroege fase patienten met Barrett oesofagus kunnen identificeren met een verhoogde risico op het ontwikkelen van een oesofagus adenocarcinoom. In oesofagus adenocarcinomen zijn met name E-Cadherine en Beta-Catenine de veelbelovende prognostische factoren.

In dit proefschrift werd algemene kennis opgedaan over tumorigenese en tumor gedrag. Er zijn echter nog veel onbeantwoorde vragen met betrekking tot de complexe biologische mechanismen die ten grondslag liggen aan de verschillende observaties. Het uitvoeren van follow-up studies van patienten met Barrett oesofagus zijn essentieel om de klinische relevantie van de verschillende onderzochte factoren in Barrett oesofagus aan te tonen. De verwachting is dat, naarmate kennis betreffende tumorigenese en tumor gedrag toeneemt, het gebruik van biologische parameters om de beste behandeling voor de individuele patient te kunnen kiezen steeds belangrijker zal worden.

List of abbrevations

AC: Alpha-Catenin
APC: Adeno-polyposis coli gene
BC: Beta-Catenin
CD44st: Standard part of CD44 molecule
CD44v6: V6 splice variant of CD44 molecule
DAB: Diaminobenzidine tetrachloride
DCC: Deleted in colon carcinoma gene
DO-7: Monoclonal antibody against p53
EC: E-Cadherin
EGF: Epidermal growth factor
EGF-R: Epidermal growth factor receptor
FCM: Flow cytometry
IHC: Immunohistochemistry
ISH: In situ hybridization
kDa: kilodalton
LOH: Loss of heterogeneity
MCC: Mutated colon carcinoma gene
Mib-1: Monoclonal antibody against Ki-67 antigen
ODC: Ornithine decarboxylase
P53: Protein of 53 kDa
PBS: Phosphate buffered saline
PCNA: Proliferating cell nuclear antigen
PCR: Polymerase chain reaction
PI: Proliferation index
17p: Short arm of chromosome 17
15q: Long arm of chromosome 15
Rb: Retinoblastoma gene
RFLP: Restriction fragment length polymorphism
SSCP: Single strand conformation polymorphism
TGF-Alpha: Transforming growth factor -alpha

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Curriculum Vitae

Kausilia Krishnawatie Krishnadath werd op 7 januari 1966 geboren te Paramaribo. Na het behalen van het VWO diploma aan de St. Stanislascollege te Delft, is zij in 1984 de opleiding geneeskunde gaan volgen aan de Erasmus Universiteit te Rotterdam. Het propedeuse examen werd in 1990 behaald. Als keuze stage werd er in 1989 gedurende 5 maanden onderzoek verricht in Zuid India. Een Coassistentschap dermatologie werd in 1991 gevolgd te Paramaribo. In 1992 studeerde zij Cum Laude af als basisarts. Hierna was zij werkzaam op de afdeling pathologie aan de Erasmus Universiteit, waar zij is gestart met het onderzoek naar prognostische factoren in Barrett oesofagus en oesofagus adenocarcinomen. In 1993 werd zij AIO onder leiding van Prof. Dr F.T. Bosman en Prof. Dr H.W. Tilanus. Het onderzoek heeft geresulteerd in dit proefschrift, dat zij d.d. 26 juni 1997 zal verdedigen. Sinds 1996 is zij in opleiding tot gastroenteroloog in het Dijkzigt ziekenhuis te Rotterdam.

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