# Translational Research on Esophageal Cancer
# From Cell Line to Clinic

**Jurjen Boonstra**

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# Translational Research on Esophageal Cancer
# From Cell Line to Clinic

Translationeel onderzoek naar slokdarmkanker
Van cellijn tot kliniek

**Proefschrift**

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op gezag van de rector magnificus

Prof. Dr. H. G. Schmidt

En volgens besluit van het college voor promoties

De openbare verdediging zal plaatsvinden op 2 november 2011 om 9:30

**Jurjen Johannes Boonstra**
geboren te Holten

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## PREFACE

In the Netherlands more than 1600 patients are newly diagnosed each year with esophageal cancer (www.kankerregistratie.nl). Cancers of the esophagus comprise two major histological entities, squamous cell carcinoma and adenocarcinoma. Most patients with esophageal cancer have advanced disease at the time diagnosis, because symptoms (such as dysphagia) arise late in the course of the disease. At the time of diagnosis, two of three patients will have tumors that are considered inoperable because of patient co-morbidities or tumor extension. Over the past two decades, treatment has evolved from single (surgery alone) to multimodality therapy (surgery in combination with chemotherapy or chemoradiotherapy) and become the standard of care for curative treatment. Despite improvements in both diagnostic and therapeutic techniques over the years, esophageal cancer continues to have a poor prognosis, with 5-year survival rates between 10 and 13%.

Although the incidence of esophageal adenocarcinoma (EAC) has steeply increased over recent years, little is known about the molecular mechanisms underlying the genesis of these tumors. It has been thought that symptomatic gastroesophageal reflux leads ultimately to pre-malignant metaplastic epithelium, also called Barrett's esophagus that predispose for EAC. Understanding of the genetic alterations that underlie these tumors could contribute to the identification of potential targets for therapy. In this context, cell lines derived from adenocarcinoma of the esophagus, could be powerful tools in the search for genetic alterations that are potentially involved in the carcinogenesis of EAC and to investigate novel therapeutic strategies.

This thesis focuses on cell lines as pre-clinical model for adenocarcinoma of the esophagus and on the role of preoperative chemotherapy in treatment of patients with esophageal cancer. A database from the department of surgery, containing clinical and pathological data on all patients operated on esophageal carcinoma, was used for studying different clinical aspects of esophageal cancer treatment. Most laboratory experiments described in this thesis were done at the laboratory for molecular diagnostics, department of pathology, Erasmus University Medical Center, Rotterdam. Furthermore, the author of this thesis was able to perform two months of research at the pathology department of the Memorial Sloan Kettering Cancer Center (New York), to verify the authenticity of two EAC cell lines.

J. J. Boonstra,
Berkel en Rodenrijs, May 2011

## CONTENTS

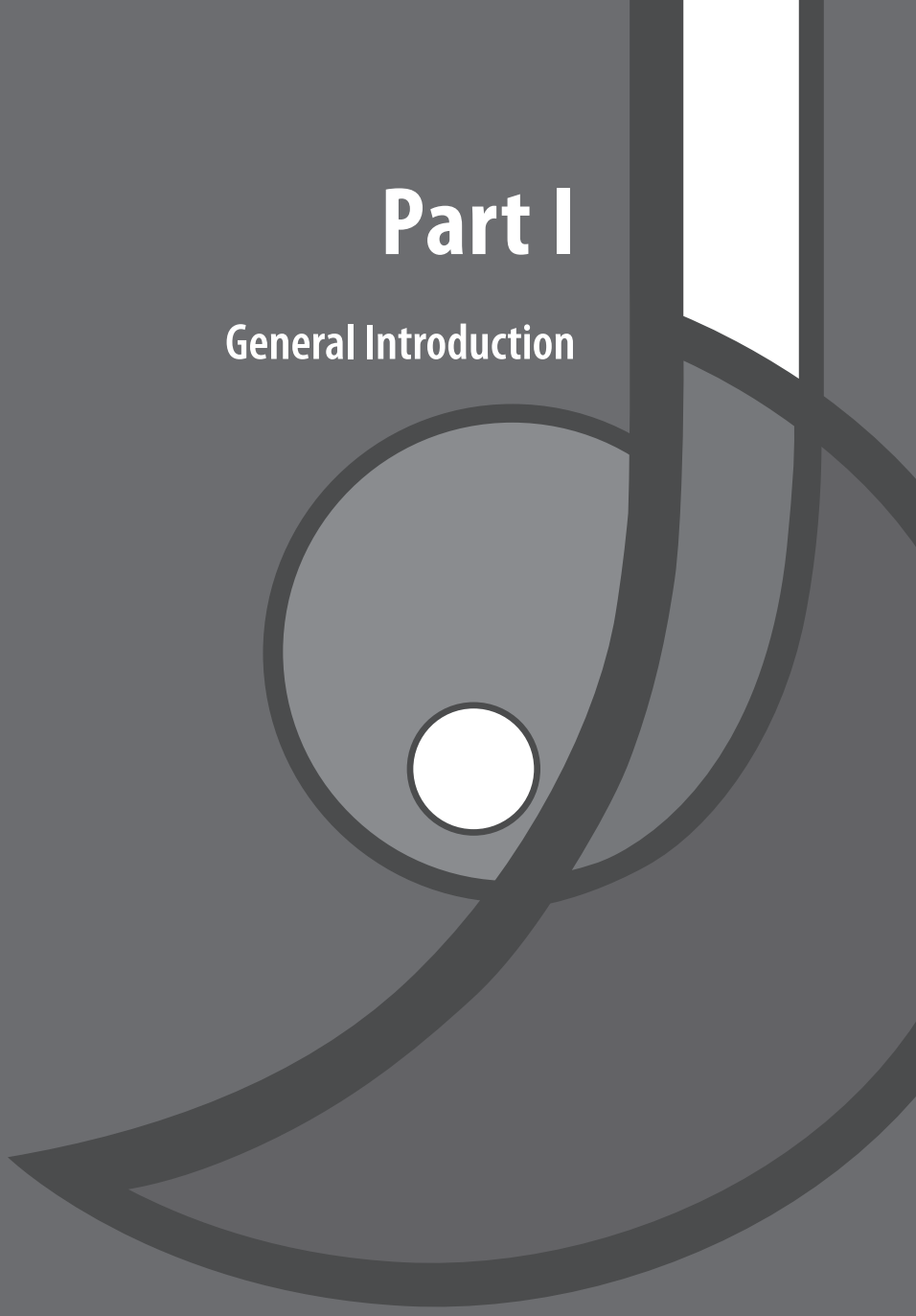
"All interest in disease and death is only another expression of interest in life"

*Thomas Mann (1875-1955)*

To Hester, Isabel and Esmee
To my parents

# Part I

## General Introduction



## GENERAL INTRODUCTION

### Epidemiology

Worldwide esophageal cancer is a significant and an increasing health problem. In 2005, there were 497,700 new cases, and the prevalence is expected to increase by approximately 140% by 2025 (1). Esophageal squamous cell carcinoma (ESCC) accounts for most of the cases of esophageal cancer worldwide (2); however, in a number of Western countries, including the Netherlands, there has been a modest decline in the incidence of ESCC and a steep rise in the frequency of esophageal adenocarcinoma (EAC) (Figure 1).

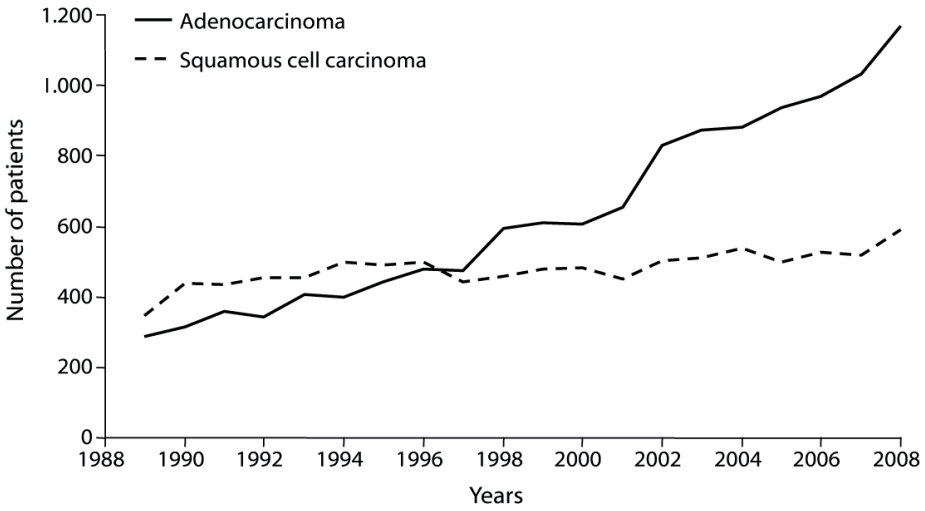



**Figure 1.** Incidence rates of esophageal cancer according to histological subtype

### Etiology

Chronic inflammation plays a key role in the initial development of both ESCC and EAC. In ESCC, the chronic inflammation is thought to be precipitated by toxins, including cigarettes and alcohol. Carcinogens produced by tobacco, particularly nitrosamines, are known to produce the precise types of guanine nucleotide conversion found in crucial genes involved in HNSCC development, such as *TP53* (3). The mechanisms by which alcohol consumption exerts its carcinogenic effect have not been defined fully. One of the mechanisms by which alcohol exert their carcinogenic effect is by acetaldehyde, the main metabolite of ethanol, which can damage DNA and trap glutathione, an important peptide in detoxification of carcinogens (4). In EAC, the inflammation is thought to be triggered by gastro-esophageal reflux, abdominal obesity, and cigarette smoking (5). The refluxate contains numerous substances in addition gastric acid, including bile salts, pancreatic enzymes, and ingested foods and their metabolites, which can cause acute and chronic inflammation of the oesophageal epithelium (6).

Abdominal obesity, in addition to promoting gastroesophageal reflux, is increasingly being recognized as causing a state of low-level systemic inflammation, characterized by increased plasma levels of pro-inflammatory cytokines and receptors (7). In addition, cigarette smoking can cause inflammation both systemically and in the oesophageal epithelium in response to swallowed smoking products (8). In turn, a chronic state of systemic and localized inflammation promotes DNA damage, DNA replication stress and genomic instability (9), which increase the risk of developing clones containing small- and large-scale genomic alterations, eventually leading to EAC (Figure 2).

**Molecular pathogenesis**

Both EAC and ESCC are thought to develop through a series of acquired mutations or epigenetic changes. These genetic changes allow normal squamous epithelial cells to progress towards cancer cells by acquiring eight essential physiologic capabilities: proliferation without exogenous stimulation, resistance to growth inhibitory signals, avoidance of apoptosis, unlimited replication, angiogenesis, invasion and metastasis, reprogramming of energy metabolism and evading immune destruction (10). Many of the genetic changes, which lead to these physiologic capabilities, will confer a growth advantage on the mutated cells, and lead to clonal expansion at the expense of other cells. Figure 2 shows the changes that have been shown to occur in the pathogenesis of EAC and ESCC.

In ESCC this multistep process is morphologically characterized by esophagitis followed by basal cell hyperplasia, squamous dysplasia, carcinoma in situ and advanced carcinoma (11). EACs are thought to arise from Barrett's epithelium, which is characterized by columnar metaplasia with intestinal differentiation that has replaced the normal squamous cell lining of the esophagus. Progression is thought to follow the metaplasia, dysplasia, adenocarcinoma sequence (12).

The majority of ESCC are located in the upper and middle esophagus (from the level of the manubrium sterni to the trachea bifurcation). Most EACs arise in the distal esophagus (which starts at the level of the trachea bifurcation and extends to the gastro-esophageal junction). The gastro-esophageal junction is characterized by the "Z" line, which forms the transition zone from squamous cell epithelium to cylindrical epithelium from the stomach. Some clinicians and scientists distinguish carcinomas that arise in the distal esophagus from carcinomas that arise in the proximal stomach, the so called "gastric cardia" (13). However, this classification seems artificial, because epidemiological, histomorphological and molecular genetic data suggest that this is one clinical entity (14, 15). In addition, the therapeutic approach for both types of tumors is identical. Therefore, the term esophageal adenocarcinoma (EAC) in this thesis comprises both carcinomas of the distal esophagus and gastric cardia.

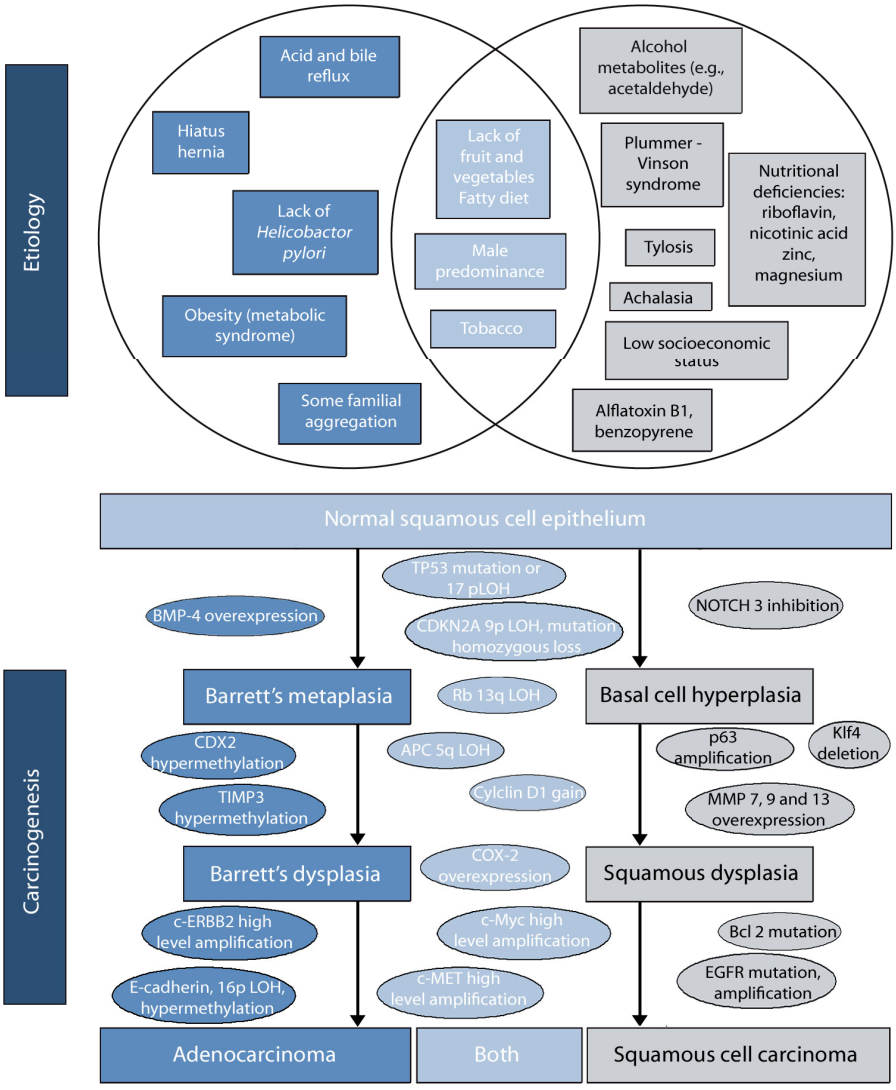



**Figure 2.** The common and distinct known etiology and carcinogenesis of esophageal squamous cell carcinoma (ESCC) and adenocarcinoma (EAC). Summary of the genetic alterations involved in the progression of normal squamous cell epithelium towards EAC and ESCC. The areas in which EAC and ESCC overlap are indicated in light blue (partly adapted from Bird-Lieberman et al (22)).

### Clinical manifestations and diagnosis

Most patients with esophageal cancer have advanced disease at the time diagnosis, because symptoms, such as dysphagia, odynophagia and weight loss develop late in the course of the disease when the tumor is large and obstruction the esophageal lumen. The diagnosis is established by pathological assessment of biopsies obtained by gastro-duodenoscopy. Then, assessing the operability of the patient (is the patient fit for surgery) and tumor staging need

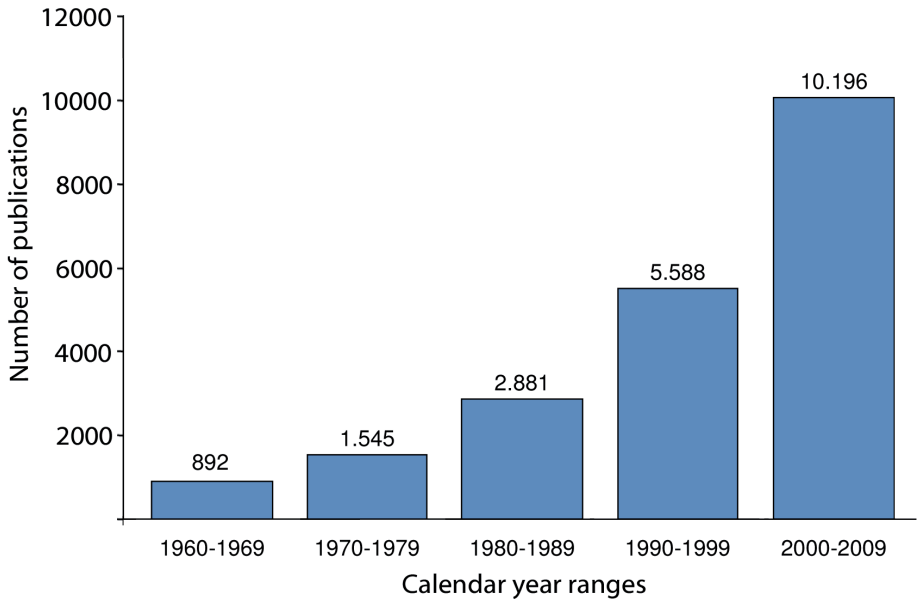



**Figure 3.** Number of publications listed by the National Library of Medicine library for the search term "esophageal cancer", from 1960 to 2009, by decade. Not the doubling of publication over each decade.

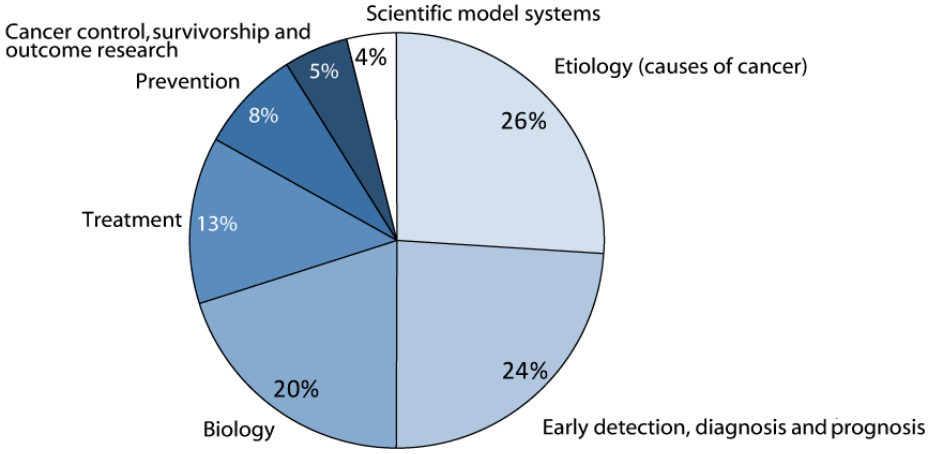



**Figure 4.** National Institute of Health esophageal cancer research portfolio. Indicated are the percentage of total dollars by scientific area in the year 2009 (http://fundedresearch.cancer.gov).

to be done. Staging of esophageal cancer is based on the tumor-node-metastasis (TNM) classification (16). The depth of tumor invasion (T-stage) can accurately assessed by endoscopic ultrasonography (EUS) (17). Lymph node involvement (N-stage) can be determined by computed tomography (CT) of chest and abdomen, CT-scan combined with positron emission tomography (PET) and EUS (with or without fine-needle aspiration). However these staging modalities have a limited sensitivity and specificity for detection of lymph nodes metastases (17). The presence of distant metastases (M-stage) can accurately assessed by CT-scan of chest and abdomen, CT-scan combined with PET and external ultrasound of the neck. At the time of diagnosis, two of three patients will have tumors that are considered inoperable because of patient co-morbidities or tumor extension (18).

**Treatment**

Treatment of patients with cancer of the esophagus is complex and needs a multidisciplinary approach (19). Only a minority of patients present with an early cancer of the esophagus. These patients, with high grade dysplasia and intramucosal carcinomas, require endoscopic therapies (such as endoscopic mucosal resection, radiofrequency ablation, or cryotherapy) in experienced centers (as reviewed by (20)). However, the fast majority of patients present with advanced esophageal carcinoma. Surgical resection remains an essential cornerstone in the treatment of localized disease especially in patients with adenocarcinoma. Recently, the preliminary results of the phase III multicenter CROSS trial, involving 364 patients in the Netherlands with resectable EAC and ESCC have been presented (21). The median survival of patients randomized for preoperative chemoradiotherapy was 49 months, compared to 26 months 26 months for those who were allocated to surgery alone. In the Netherlands, these results have changed the standard of care for patients with advanced esophageal carcinoma from surgery alone into neoadjuvant chemoradiotherapy followed by surgical resection.

**Research**

In the past decades the number of publications has increased tremendously.(Figure 3) The number of publications recorded by the NCBI in 2009 on esophageal cancer (using MESH term esophageal neoplasms) was 1387 (0,2% of the total number of publications recorded). In the United States, the National Cancer Institute invested in 2009 about 28.8 million in esophageal cancer research (http://www.cancer.gov/aboutnci/servingpeople/cancer-statistics/snapshots), which comprises 0.6% of the total investment in cancer research. As illustrated by figure 4, most research is focused on biology, etiology and diagnosis and prevention (accounting for 75%) of esophageal cancer.

## OUTLINE OF THE THESIS

This thesis is divided into two major parts: Human esophageal adenocarcinoma cell lines and Clinical aspects of esophageal cancer treatment.

**Human esophageal adenocarcinoma cell lines**

Model research on esophageal adenocarcinoma (EAC) relies entirely on a relatively small set of established tumor cell lines, because appropriate animal models and familial cases for EAC are lacking. **Chapter 1** reviews the history of the EAC cell lines and their utility in translational research and biomedical discovery.

To study the histological characteristics of EAC cell line TE-7, tumor cells were injected subcutaneously into nude mice. To our surprise the xenograft of EAC cell line TE-7 showed the histological characteristics of a squamous cell carcinoma and not of an adenocarcinoma. This prompted us, in **chapter 2**, to investigate the authenticity of all 15 TE cell lines by short tandem repeat (STR) profiling, mutation analyses, xenografting and array-CGH. In **chapter 3**, all available EAC cell lines are verified. In collaboration with the establishers of the cell lines, the original EAC tissues for 13 of the 14 cell lines have been traced in pathology archives and made available for study. The availability of the primary tissues make it possible to authenticate these EAC cell lines by comparing the genotype of the cell line with the genotypes of patient's normal and tumor tissue.

So far, little is known about the cellular and molecular mechanisms that underlying the origin of adenocarcinoma of the esophagus. Cell lines and xenografts derived from human EAC consist of pure populations of tumor cells without admixture of normal stromal or inflammatory cells, which makes detection of molecular genetic changes much easier. In **chapter 4,** these verified EAC cell lines (and xenografts) are used to screen for homozygous deletions. These somatically acquired homozygous deletions are one of several mutational mechanisms through which the proteins encoded by tumor-suppressor cancer genes are inactivated. Mapping of these deletions in the cancer genome could potentially lead to the identification of new potential tumor suppressor genes that play a role in the carcinogenesis of adenocarcinoma of the esophagus.

**Clinical aspects of esophageal cancer treatment**

Treatment of patients who present with cancer of the distal esophagus and positive celiac lymph nodes is controversial. According to the TNM classification (5$^{th}$ edition) established by the UICC and the AJCC, lymph nodes around the celiac axis are considered non-regional nodes and as such classified as M1a disease. But are M1a patients truly metastatic (and thus not approachable for cure) or do they have extensive locoregional disease and thus are potentially curable? In **Chapter 5**, we evaluated our treatment strategy of neoadjuvant chemotherapy followed by surgery with curative intent in this subset of patients.

**Chapter 6** describes the long-term results of a large randomized controlled trial in patients with squamous cell carcinoma of the esophagus who were assigned to chemotherapy followed by surgery or surgery alone. Even after twenty years, these results contribute to the ongoing debate about the optimal (preoperative) therapy for patients with ESCC.
Single nucleotide polymorphisms (SNPs) in the germline are the most common type of host genetic variations. Gene-related functional SNPs can potentially lead to differences in protein expression and/or function. In this way, SNPs in proto-oncogenes and tumor suppressor genes can potentially alter the risk for metastatic or aggressive tumor, resulting in differences in clinical outcome. In **Chapter 7,** we determine whether clinical outcome after surgical resection of EAC or ESCC is associated with functional polymorphisms in different proto-oncogenes and tumor suppressor genes.

Finally, **chapter 8** is a summary of the preceding chapters and the major conclusions are drawn. In **chapter 9,** the results of the thesis are discussed and directions for future research are provided.

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# Part II

## Human esophageal adenocarcinoma cell lines

# Chapter 1

## Translational research on esophageal adenocarcinoma: from cell line to clinic

Jurjen J. Boonstra[1,2*], Hugo W. Tilanus and Winand N. M. Dinjens[1]

*Submitted*

## ABSTRACT

Human esophageal adenocarcinoma (EAC) cell lines have made a substantial contribution to elucidating mechanisms of carcinogenesis and drug discovery. Model research on EAC relies almost entirely on a relatively small set of established tumor cell lines, because appropriate animal models are lacking. Nowadays, more than 20% of all fundamental translational research studies regarding EAC are partially or entirely based on these cell lines. The ready availability of these cell lines to investigators worldwide have resulted in more than 250 publications, including many examples of important biomedical discoveries. The high genomic similarities (but certainly not completely identical) between the EAC cell lines and their original tumors provide rational for their use. Recently, in a collaborative effort all available EAC cell lines have been verified resulting in the establishment of a reliable panel of ten EAC cell lines. It could be expected that the value of these cell lines increases as unlimited source of tumor material, because new biomedical techniques require more tumor cells and the supply of viable tumor cells is diminishing due to neoadjuvant chemo(radio)therapy of patients with EAC. Here, we review the history of the EAC cell lines and there utility in translational research and biomedical discovery.

## INTRODUCTION

Esophageal cancer is a significant and increasing health problem. In 2008, there were 450.000 new cases worldwide, which rank this tumor type among the world's most frequent malignancies (1). Although esophageal squamous cell carcinoma (ESCC) cases have steadily declined, the incidence of esophageal adenocarcinoma (EAC) has increased more rapidly than for any other cancer type. This has led to increased interest in fundamental research on molecular mechanisms underlying the origin of EAC. The current scale of biomedical cancer research on EAC requires an extensive source of tumor materials. It would appear that resection specimens of EAC could satisfy such needs. However, the extensive use of neo-adjuvant chemo(radio)therapy, severely limits the availability of viable tumor cells. This has increased the importance of preoperative biopsies, only these biopsies contain a relatively small portion of tumor cells which can be used for research (as for example SNP-arrays or genome-wide sequencing). Larger biopsies are optional, but increase the risk of gastrointestinal bleeding and perforation. Taking more biopsies (for research purposes) is impaired by increasingly stringent requirements for protection of patients' privacy rights by institutional review boards and by government legislation. These developments generate an increasing and ongoing demand for *in vivo* and *in vitro* models of EAC. Thus fare, only a small set of EAC cell lines is available for experimental cancer research and appropriate animal models are lacking. Recently, we verified the authenticity of all available EAC cell lines and confirmed the authenticity of ten EAC cell lines (2). In this review, we focus on the utility of these cell lines that have been derived from human EAC for the study of this tumor type.

## METHODS

Two searches were performed of the National Library of Medicine database (http://www.nlm.nih.gov/pubs/). The first search was done to determine the total number of publications (published in English) on esophageal adenocarcinoma in the period between 1 January 2000 and 31 December 2009. Therefore, we used the following medical search heading terms (MESH-terms): "esophageal neoplasms" and "adenocarcinoma". This search yielded 3229 publications. Eighty-three articles were excluded, because the subject was not EAC. Then, we subdivided the publications (N=3146) into three categories namely 1) review articles (reviews, comments, editorials, letters and news); 2) clinical research articles (clinical trials, clinical database research and epidemiological studies); 3) fundamental and fundamental translational articles (DNA, RNA, protein, *in vitro* and animal studies). Within this last category, we searched for studies in which EAC cell lines were used.

A second search was performed to identify the majority of publications that used EAC cell lines. The following MESH terms were used "esophageal neoplasms" and "adenocarcinoma"

and "tumor cells, cultured". On May 1, 2011, this search yielded 251 publications. Because we were involved in the establishment of the majority of EAC cell lines, we used our knowledge of literature to select relevant articles that illustrate the major purpose of this review, the utility of cell lines for the study of EAC. It should be noticed that this article is not a comprehensive review of all the literature. References and topics were selected to serve as representative examples.

**The use of EAC cell lines over the past decade**

The increase of interest in research on EAC is illustrated by Figure 1. Over the past decade, the total number of studies has increased with 34%. Interestingly, the distribution of the three study types (reviews, clinical research or fundamental research) does not change. Also the use of EAC cell lines in publications has increased (Figure 2). In almost 20% of all fundamental research studies on adenocarcinoma of the esophagus, EAC cell lines have been used (Figure 3). These figures reflect the extent of EAC cell line use in research on this tumor type.

**History of esophageal adenocarcinoma (EAC) cell lines**

Human EAC cells were first successfully cultured in Japan, in 1984 ((3, 4); Figure 4). The first cell line derived from a primary EAC was TE-7. Thereafter, only 16 EAC cell lines have been established namely: SK-GT-4, SK-GT-5 and BE-3 (5); SEG-1, BIC-1 and FLO-1 (6); JROECLI9 (OE19), JROECL33 (OE33), JROECL47 and JROECL50 (7); OACM5.1 and OACP4CE (8); KYAE-1 (9); JH-EsoAd1 (10); ESO26 and ESO51 (2).

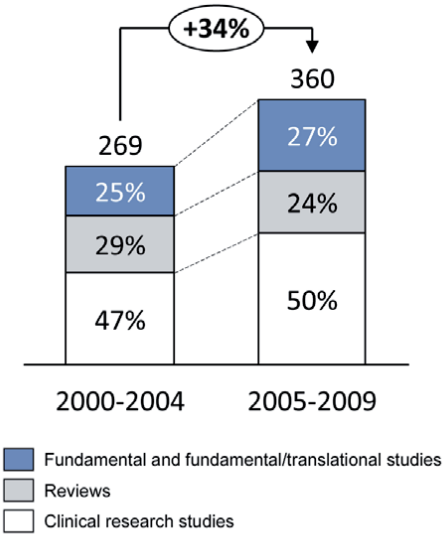


**Figure 1.** Depicted are the mean number of publications/per year on esophageal adenocarcinoma in two time periods. In the last five-years there is an increase of publications of 35%. The distribution of type of publications is nearly unchanged.

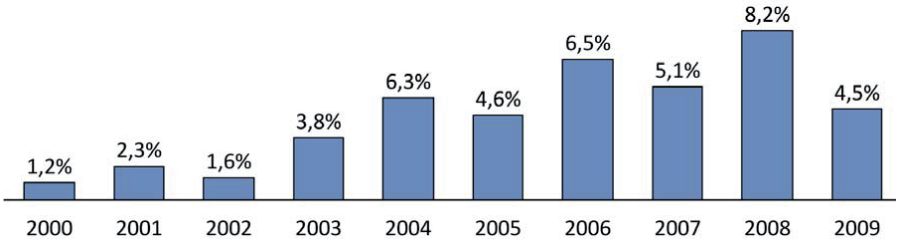


**Figure 2.** The percentage of publications on esophageal adenocarcinoma (EAC), wherein EAC cell lines have been used. Note the steadily incline of the use of cell lines among the years.

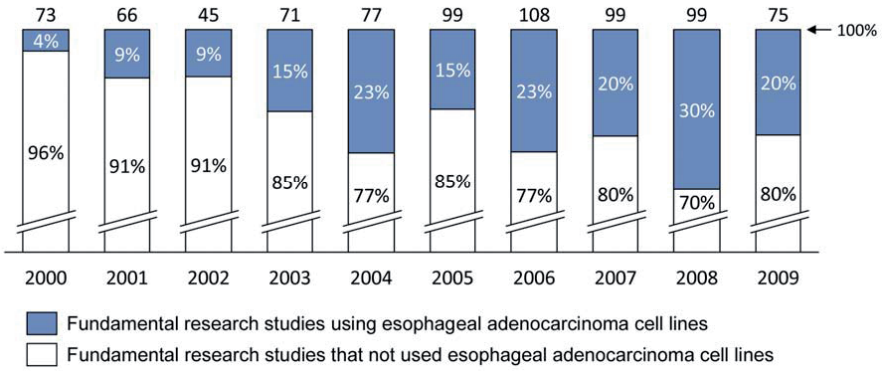


**Figure 3.** Depicted (with the numbers above the bars) are all the fundamental/translational research studies per year. Fundamental/translational research studies using cell lines are blue. Fundamental/translational research studies that did not use cell lines are white. Note that over de past six years, in almost 20% of all fundamental research studies on adenocarcinoma of the esophagus, EAC cell lines have been used.

There are several reasons for the limited number of available human EAC cell lines. At first, the establishment of cell lines is mainly based on trial and error, with relatively low success rates for EAC. The known number of attempts to establish cell lines is most likely far below the total number of attempts since most failed efforts will not have appeared in literature. Why cell lines from EAC are so hard to establish is poorly understood. It depends on many factors like the number of viable tumor cells and the ratio between these tumor cells and fibroblasts in the sample. Furthermore, a variety of nutritional and environmental conditions must be met for cells to thrive in culture which are often enigmatic to the establishers resulting in failure of *in vitro* growth of tumor cells. The major cause of failure appears to be the lack of tumor cell attachment to the plastic flasks and fibroblast overgrowth, as reported by several investigators (5, 7, 8). Secondly, before the era of multimodality treatment of EAC, the availibiliy of resection specimens with large amouts of viable tumor cells was abundant. The need for an infinite source of material (one of the major advantages of cell lines) was limited, which resulted in a lack of drive among researchers to establish EAC cell lines. At third, several

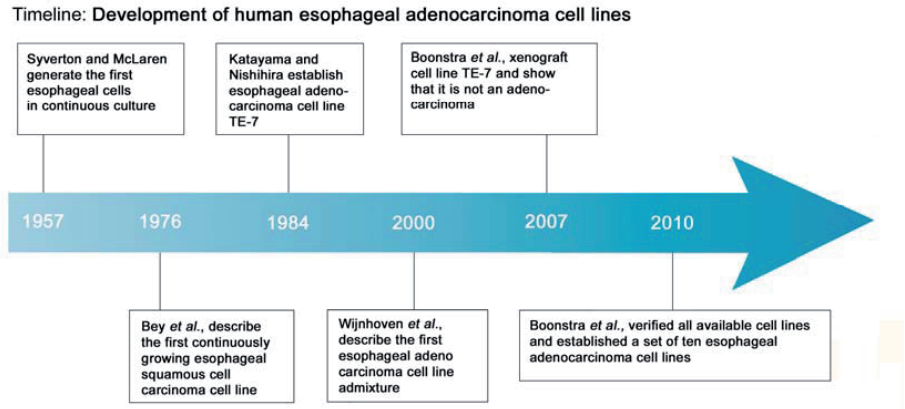


**Figure 4.** Timeline esophageal adenocarcinoma cell line development.

cases of cell line contamination among EAC cell lines has been identified, which reduced the number of reliable EAC cell lines. Approximately three years after the first publication of the first European series of cell lines, we discovered that cell lines JROECL47 and JROECL50 were admixtures of the human colon carcinoma cell line HCT 116 (11). In 2007, we revealed that EAC cell line TE-7 had the phenotype of a squamous cell carcinoma after xenografting and shared the same genotype as four other esophageal squamous cell lines of the Japanes TE series (12). In a very recent report, we showed that three of the most commonly used EAC cell lines (SEG-1, BIC-1 and SK-GT-5) were misidentified and were actually derived from other forms of human cancers. Ten cell lines, FLO-1, KYAE-1, SK-GT-4, OE19, OE33, JH-EsoAd1, OACP4C, OACM5.1, ESO26, and ESO51 were proven to derive from their original tissues. All these EAC cell lines, together with their genotyping information, have been deposited in publicly available cell line repositories to promote and facilitate future solid research on EAC. In the remaining part of this review, only studies and data of the verified cell lines will be discussed.

**Cell lines as experimental systems to study cancer biology and for translational research**

EAC cell lines offer certain advantages and disadvantages when compared to tumor materials. An excellent discussion on these issues is presented in a recent review (13). Cell lines are populations of pure tumor cells without admixed stromal or inflammatory cells, which greatly aiding tumor cell characterization. Furthermore, cell lines are capable of infinite replication, providing a limitless source of materials and permitting their dispersion to laboratories worldwide. This allows scientists to compare their results from identical materials.

The relevance of human EAC cell lines depends on how representative these cell lines are as model for all adenocarcinomas of the esophagus, and on how closely they resemble the tumors from which they were derived. Most EAC cell lines were derived from poorly differen-

tiated adenocarcinoma (except cell line JH-EsoAD1 that is derived from a moderately differentiated adenocarcinoma), which illustrates that these cell lines represent only a subtype of all EAC. In general it has been thought that cell lines arise from subpopulations of the original tumor that have inherent properties that allow them to grow as immortal cultures. As a result of selection of the most robust fast growing subpopulation, cell lines may also be relatively undifferentiated. Thus, from an already poorly differentiated EAC the most undifferentiated subpopulation grows out to a cell culture.

The EAC cell lines retain properties of the cells of origin; however, they show also differences compared to the *in vivo* tumors. Previously, we have demonstrated that the EAC cell lines retain the *TP53* mutations that were detected in the primary tumors (2). Furthermore, a study that genetically characterized four EAC cell lines (OE19; OE33; OACP4CE and OACM5.1) by 24-color FISH and array-CGH, showed multiple chromosomal regions of gains and losses. Among these aberrations, losses on 3p, 4, 5q, 9p, 13q, 18q, and 21 were detected in at least 3 out of the 5 samples. Likewise, gains were detected on 1q, 8q, 17q, 19q, 20, and X (14). These findings are consistent with data obtained by array-CGH and SNP-arrays in studies using primary tumor tissue (15). However, also differences between primary tumors and the corresponding cell lines have been observed. As for example, expression of surface marker HLA-DR has been observed in the primary tumor of which cell line OE33 has been derived, however, the cell line does not express this marker (7). These results emphasise the need for more investigations regarding the question how representative these EAC cell lines are for their primary tumor. Moreover this kind of research has become feasible, since the patient's derived tumor materials have been traced.

**The role of EAC cell lines in elucidating mechanisms of carcinogenesis**

Chronic inflammation seems to have a central role in the development of EAC and its precursor lesions (16). A great part of the experimental evidence that supports the hypothesis that chronic gastro-esophageal reflux causes genetic and epigenetic changes that lead to the onset of Barrett's metaplasia and its progression to carcinoma relies on EAC cell line research. Experiments using EAC cell line FLO-1 and a non-cancer derived SV40 immortalized squamous epithelial cell line HET1A (established by Harris et al. (17)) showed that specific components of the refluxate (especially sodium deoxycholate) can induce double strand DNA breaks (18). In addition, deoxycholic acid exposure is genotoxic to EAC cells (OE33) through induction of reactive oxygen species (19). *In vitro* experiments using EAC cell lines have significantly contributed to the identification of signalling pathways that are being activated by exposure to acid and bile. One of the pathways is the arachidonic acid pathway. The key enzyme in this pathway is cyclooxygenase-2 (COX-2), which catalyzes the conversion of arachidonic acids into prostaglandins. Cell line SK-GT-4 served as *in vitro* model in the first study that investigated the effect of bile acids on expression of COX-2 in human EAC (20). It was shown that dihydroxy bile acids activate transcription of COX-2 in SK-GT-4 cells. Soon thereafter,

studies using large collections of endoscopic biopsies of patients with Barrett's esophagus, Barrett's dysplasia, and EAC revealed enhanced COX-2 gene or protein expression in a large proportion of EAC (21, 22). Also the nuclear factor-kappa-B (NF-kB) pathway is thought to play a pivotal role in mucosal inflammatory response to (duodeno) gastro-esophageal reflux. NF-kB comprises a ubiquitous transcription factor that regulates host inflammatory and immune responses by affecting the expression of a wide range of genes coding for cytokines, enzymes, apoptosis, proliferation and adhesion molecules (23). The first experimental evidence for activation of the NF-kB pathway came from a surgical model of reflux esophagitis (24). The link between NF-kB pathway activation in EAC comes from studies using EAC cell lines (as reviewed by Abdel-Latif et al (25)). *In vitro* studies using cell lines OE33 and SK-GT-4 demonstrated that acid and bile have the capacity to induce NF-kB expression (26).

**The role of EAC cell lines in drug testing**

EAC cell lines have been used for many years as drugs discovery tools. As reviewed by Sharma et al. (27), the rapidly expanding use of cancer cell lines to predict the clinical efficacy of new agents is already affecting the course of drug development and is now becoming an important tool for the biotechnology and pharmaceutical industries, in which efforts that are focused on molecularly targeted cancer therapies are accelerating. Genome-wide profiling of copy number alterations of EAC samples and cell lines have identified regions that harbour potential targets for therapy, such as amplifications on chromosomes 7q21-22 and 17q21 harboring the c-MET and ERBB2 genes, respectively (14, 15). Overexpression and amplification of c-ERBB2 in EAC was described first in resection specimens (28, 29). Several years later, c-ERBB2 gene amplification and overexpression was reported in EAC cell lines OE19 and OE33. *In vitro* testing of a monoclonal antibody against the c-ERBB2 receptor showed marked growth inhibition in both EAC cell lines (30). These results led to the design of a phase I-II clinical trial among patients with EAC (31, 32). The c-MET oncogene is also frequently overexpressed and amplified in EAC samples and cell lines (33, 34). *In vitro* experiments showed that the c-MET inhibitor PHA665752 induced apoptosis and reduced cell motility and invasion in cell line FLO-1 (35). These results led to the initiation of a phase II trial to evaluate the therapeutic benefit of c-Met inhibition in EAC and gastric adenocarcinoma (NCT00725712; http://clinicaltrials.gov/).

EAC cell lines do also play a role in studying mechanisms of chemo- and/or radioresistance, which remains a significant clinical problem, with only ~25% of patients achieving a complete pathological response after neoadjuvant chemoradiation therapy (36). By continuously exposing drug and/or radiosensitive EAC cells to treatment *in vitro* over a period of time, it is often possible to eliminate the majority of cells while selecting for the expansion of relatively rare drug resistant clones. Recently, a radio resistant sub line of cell line OE33 has been established (37). In addition, a recent patent claimed the establishment of 5-fluorouracil resistant sub lines of cell lines OE19 and OE33 (http://www.freepatentsonline.com/; United States

Patent Application 20110014303). Further investigations are needed to compare the various properties of the parental cells and the selected chemo- or radio resistant cells, to identify specific molecular mechanisms of drug resistance.

**Xenografts from EAC cell lines**

The major limitations of tumor cell lines are their lack of stromal, vascular, and inflammatory components. These deficiencies can be partially overcome by xenograft transplantation of EAC cell line suspensions into immunodeficient mice. Ectopic xenografts could be generated from all EAC cell lines ((2); xenografts from cell line FLO-1 were recently obtained by us in mouse strain NOD/SCID/IL2-Rγ(null) (NSG)). The most widely used translational application of these EAC cell line xenografts has been the in vivo testing of different chemotherapeutic agents such as trastuzumab or temozolomide (38, 39). Furthermore xenografts have also been used to identify biomarkers for response to chemotherapy. In a recent study, EAC cell line xenografts (derived form cell line OE19) were used to identify plasma protein biomarkers in response to treatment with epirubicin, cisplatin or 5-fluorouracil. Three of seven plasma protein markers identified in the mouse xenograft model were also detected in patient samples, which reflect the usefulness of this mouse model in modeling EAC (40).

Recently, an orthotopic mouse model has been generated by transplanting esophageal tumor fragments (derived from a previous subcutaneously injection of OE19 cells into the flanks of a female NMRI nude mice), into the abdominal esophagus of the mice (38). Up to 71% of the animals showed metastases. Orthotopic transplantation models are thought to represent a more clinically relevant tumor model with respect to tumor site and metastasis; however its limitations include technical skill, time and cost. Also the therapeutic efficacy is more difficult to assess in contrast to the relative ease of subcutaneous tumor measurements.

## CONSIDERATIONS

There remains considerable skepticism in the scientific community about the validity resulting from the use of cell lines. The major concerns are genetic instability during long time culture, selective growth of subpopulations and cross contamination or misidentification. Almost all EAC arise in association with chromosomal instability that leads to gains, losses or loss of heterozygosity (LOH) of large regions of chromosomes (41, 42). Chromosomal instability might be caused by *TP53* mutations (43). The presence of *TP53* mutations in all original tumors of which the EAC cell lines were derived, indicate that genomic instability is already present at time the cell lines were established. This inherent genomic instability and the considerably shorter population-doubling times of cell lines may result in an increased frequency of mutational changes during *in vitro* growth. However, it has been demonstrated that the mutation rate during prolonged cell culture is limited (44, 45). Cancer cell lines con-

stantly generate variants with phenotypic and/or genotypic differences from the predominant population caused by exposure to different conditions (such as media, sera, trypsin, carbon dioxide levels, humidity and temperature). Similar to tumor cells *in vivo*, cells *in vitro* adapt their phenotype, by epigenetic (potentially reversible) or genetic mechanisms (irreversible), to the conditions to which they are exposed. Variants that are better adapted to the new conditions are likely to be selected. Despite our efforts to authenticate all available EAC cell lines, cross-contamination or misidentification of EAC cell lines is a continuous threat. The best prevention to overcome, at least partially, most of these concerns is to limit the number of passages and to always come back to cells frozen during the early passages. However, most of the cancer cell lines used have been passaged hundreds of times. So this precaution may be applied in the future but does not apply to the cell lines as they are commonly used.

## CONCLUSIONS

Human EAC cell lines, while not ideal model systems, offer a robust model to study pathogenesis and that can serve as a filter to fast track the most promising compounds and enables high-throughput science over short periods (such as c-MET and c-ERBB2 inhibitors). It could be expected that the value of these cell lines increases as unlimited source of tumor material, because new biomedical techniques require more tumor cells and the supply of viable tumor cells is diminishing due to neoadjuvant treatment of patients with EAC.

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# Chapter 2

## Mistaken identity of widely used esophageal adenocarcinoma cell line TE-7

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## ABSTRACT

Cancer of the esophagus is the 7th leading cause of cancer death worldwide. Esophageal carcinoma cell lines are useful models to study the biological and genetic alterations in these tumors. An important prerequisite of cell line research is the authenticity of the used cell lines, because the mistaken identity of a cell line may lead to invalid conclusions. Estimates indicate that up to 36% of the cell lines are of a different origin or species than supposed.
The TE-series, established in late 70s and begin 80s by Nishihira et al in Japan, is one of the first esophageal cancer cell line series that was utilized throughout the world. Fourteen TE cell lines were derived from human esophageal squamous cell carcinomas and one, TE-7, was derived from a primary esophageal adenocarcinoma (EAC). In numerous studies this TE-7 cell line was used as a model for EAC since it is one of the few EAC cell lines existing. We investigated the authenticity of the EAC cell line TE-7 by xenografting, short tandem repeat profiling, mutation analyses and array-CGH and demonstrated that cell line TE-7 shared the same genotype as the esophageal squamous cell carcinoma cell lines TE-2, TE-3, TE-12 and TE-13. In addition, for more than a decade independent TE-7 cultures from Japan, USA, UK, France and The Netherlands had the same genotype. Examination of the TE-7 cell line xenograft revealed the histology of a squamous cell carcinoma. We conclude that the TE-7 cell line, used in several laboratories throughout the world, is not an adenocarcinoma - but a squamous cell carcinoma cell line. Furthermore, the cell lines TE-2, TE-3, TE-7, TE-12 and TE-13 are one identical squamous cell carcinoma cell line from unknown origin.

## INTRODUCTION

Cancer of the esophagus is the 7th leading cause of cancer death worldwide (1). Although esophageal squamous cell carcinoma (ESCC) cases have steadily declined, the incidence of esophageal adenocarcinoma (EAC) has increased more rapidly than for any other cancer type and parallels rises in obesity and reflux disease (2). Despite the common occurrence of ESCC and EAC, little is known about the molecular mechanisms underlying the genesis of these tumors. Tumor-type specific cell lines are useful models to study cell biological and molecular characteristics of the comparable tumors. Therefore, established tumor cell lines are of great importance, especially from tumor types from which only few lines exist. An important prerequisite of cell line research is the authenticity of the used cell lines, because the mistaken identity of a cell line may lead to invalid conclusions (3). Estimates indicate that up to 36% of the cell lines are of a different origin or species to that being claimed (4-6). One of the major causes is cross-contamination between cell lines (see for example: http://www.sanger.ac.uk/genetics/CGP/Genotyping/synlinestable.shtml)(3, 7). Cross-contamination of cell lines can be the result of poor culture technique when a cell line culture is contaminated with another cell line. Alternatively, cross-contamination of cell lines can be due to clerical error by mislabeling of cell cultures or frozen stocks.

The TE-series, established by Nishihira et al in Japan, is one of the first esophageal cancer cell line series that was utilized throughout the world (8, 9). Of the 15 TE cell lines generated 14 were derived from human ESCC and one, TE-7, from a primary EAC (10). This last cell line is one of the few EAC cell lines generated and therefore frequently used as an *in vitro* model of EAC throughout the world. The high incidence of cross-contamination prompted us to investigate the authenticity of the TE-series and especially the TE-7 cell line by xenografting, short tandem repeat (STR) profiling, mutation analyses and array-comparative genomic hybridisation (array-CGH).

## MATERIALS AND METHODS

### Cell cultures, DNA isolation and xenografts

Cell lines TE-1 – TE-15, except TE-7, were obtained from the Japanese Cell Resource Centre for Biomedical Research (Sendai). Cell line TE-7 was obtained from the originator Dr T. Nishihira. Cells were cultured under standard conditions in RPMI 1640 supplemented with 10% Fetal Calf Serum. To study the histological characteristics of the TE-7 cell line, 5 x 106 trypsinized tumor cells were injected subcutaneously in female NMRI nude mice. Xenografts were removed and routinely processed for histological and immunohistochemical examination. The animal experiments were licensed and done in accordance with approved protocols by the Erasmus MC, University Medical Center Rotterdam, The Netherlands. DNA was isolated from

cultured cells and xenografts using the PureGene Genomic DNA isolation Kit (Qiagen, Venlo, The Netherlands). DNA was diluted to a concentration of 100ng/µl to perform PCR.

**STR profiling**

The cell lines TE-1 – TE-15 were genotyped by a short tandem repeat profiling system using the loci of the Powerplex 16 System (Promega Madison WI, USA). The Powerplex 16 system comprises fifteen STR loci, including Penta E, D18S51, D21S11, TH01, D3S1358, FGA, TPOX, D8S1179, vWA, Penta D, CSF1PO, D16S539, D7S820, D13S317, D5S818 and the sex chromosome marker Amelogenin. Amplification was performed using 1 ng of template DNA applying the Powerplex 16 system following the manufacturer's recommendation. Multiplex PCR reactions were carried out by using fluorescent dye-linked primers. Labeled products were detected by electrophoretic size fractionation on an ABI 3100 genetic analyzer. The data were analyzed by using Genescan and Genotyper software (Perkin Elmer, Foster City, USA) to categorize peaks according to their size in relation to an internal standard run. This analysis enabled every peak to be allocated a size corresponding to the number of repeat units present.

**Mutation analyses**

Because p53 is frequently mutated in both EAC and ESCC (14, 15) all exons and intron-exon boundaries of the p53 gene were commercially sequenced in the TE-7 cell line (Asper Biotech Ltd, Tartu, Estonia). The detected p53 mutation in TE-7 was investigated in the other 14 cell lines by in house sequencing at the Department of Pathology, Erasmus MC, University Medical Center Rotterdam. Primers used were p53 intron 3 forward 5'-CAACGTTCTGGTAAGGACAAG-'3 and intron 4 reverse 5'-GGGATACGGCCAGGCATTG-3'. PCR reactions were carried out in a volume of 15µl containing 100ng of genomic DNA, 8.3µl H2O, 5µl Mg2+free buffer, 25mM MgCl2, 0.3µl of 10mM deoxynucleotide triphosphates, 20pmol of each primer and 1U Taq polymerase (Promega, Madison WI, USA). PCR-conditions were 35 cycles of 95oC for 45 sec, 61oC for 45 sec, 72oC for 30 sec, with a 10 min extension at 72oC for 10 min following the last cycle. Amplified products were analyzed on 1.5% agarose gels. These PCR products were bi-directionally sequenced using an Applied Biosystems 3100 genetic analyser (Perkin Elmer, Foster City, USA).

Cell lines TE-3, -7 and -12 have been described to harbor a heterozygous Cyclin D1 exon 5 mutation (CCND1 NM_053056.2: c.861CAC; Pro287Ala), and cell lines TE-2 and -13 a homozygous single nucleotide polymorphism (SNP) in exon 5 of the Axin1 gene (AXIN1 BC044648.1: c.1689C>T; Asp563Asp) (12, 13). Both these DNA variants were investigated by bi-directionally DNA-sequencing of PCR products, with the above-described procedure. PCR products were generated by standard procedures with primers forward 5'-GCAGAACATGGACCCCAAGG-3' and reverse 5'- GACTGTCAGGGGAGCACCTG-3' for Cyclin D1 and forward 5'-GTGGGCACGTGGCCAAGATG-3' and reverse 5'-GCTGTGCTGTGGTGGACGTG-3' for Axin1.

**Array-CGH**

The array-based CGH procedure was performed as described previously. Slides containing triplicates of approximately 3,500 large insert BAC clones spaced at density over the full genome were produced at the Leiden University Medical Center, Leiden, The Netherlands. The particular clone set used to produce these arrays is distributed to academic institutions by the Welcome Trust Sanger Institute (http://www.ensembl.org/) at no cost and contains targets spaced at a density of about 1 Mb over the full genome, a set of subtelomeric sequences for each chromosome arm, and a few hundred probes selected for their involvement in oncogenesis. After hybridization, the slides were scanned with a ScanArray Express HT (Perkin Elmer Life Sciences, Boston, MA) to collect 16-bit TIF images through Cy3 and Cy5 filter sets. The spot intensities were measured with GenePix Pro 5.1 software (Axon Instruments, Leusden, The Netherlands). Further analyses were performed using Microsoft Excel 2000 (16, 17).

## RESULTS AND DISCUSSION

Human tumor cell lines are indispensable models to study molecular and cell biological characteristics of human tumors. For this, the correct identification of a tumor cell line is crucial, as a mistaken identity of a cell line may lead to invalid conclusions with regard to the tumor-type studied. Well-established methods to investigate the authenticity of tumor cell lines are STR profiling, SNP typing, mutation analyses and karyotyping (3-5, 18). Using different methods, we demonstrated that cell line TE-7, widely used as an EAC cell line, is a squamous cell carcinoma cell line and shares the same genotype as four ESCC cell lines of the TE-series.

Xenografting of cell line TE-7 resulted in a poorly differentiated SCC (Figure 1A). The xenografts were positive for cytokeratin 10 and 14 and involucrin and negative for CDX2 expression confirming the squamous histology (Figure 1B, C, D). The genotype of the TE-7 xenograft and *in vitro* cell line were identical demonstrating that the xenograft tumor was derived from the TE-7 cell line. STR profiling of the TE-series revealed identical genotypes for the cell lines TE-2, -3, -7, -12 and -13 (Figure 2). For all TE cell lines, the exact number of repeats at each locus is shown in table 1. Differences in the relative peak heights at heterozygous loci were seen in the STR profile of the TE cell lines, probably caused by differential amplification efficiency between heterozygous loci, or the presence of additional copies of one allele (Figure 2). This finding is typical for cancer cells reflecting their relatively high genetic instability compared with normal cells.

In addition, a homozygous p53 splice site mutation TP53 AF307851.1: c.375+1G>A in intron 4, the previously in TE-3, -7 and -12, described heterozygous Cyclin D1 exon 5 mutation and a, in TE-2 and -13 described, homozygous SNP in exon 5 of the Axin1 gene were found in TE-2, -3, -7, -12 and -13 (Figure 3).

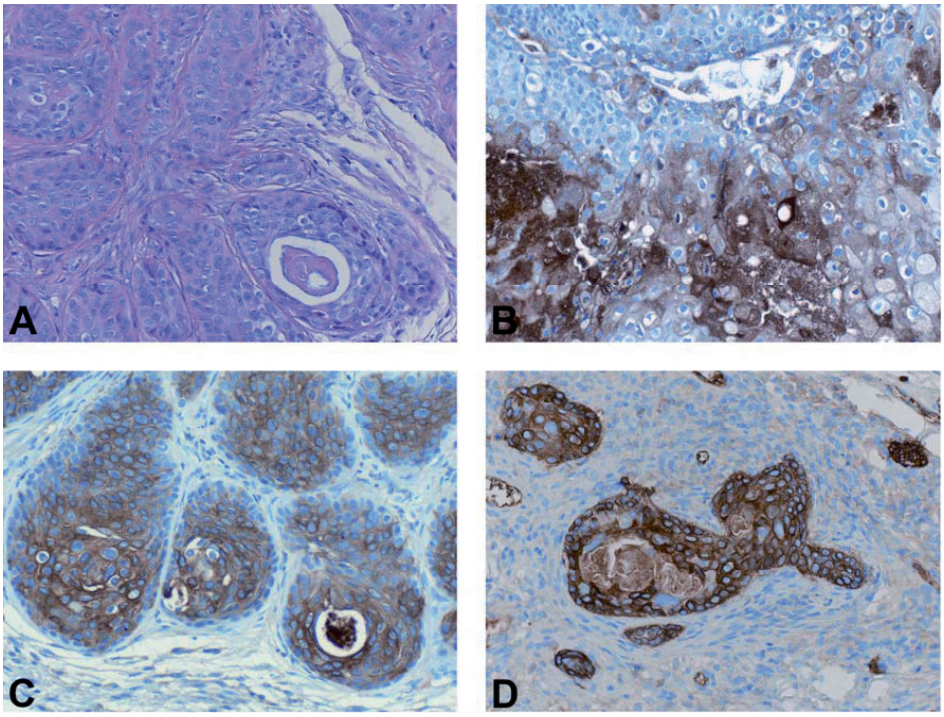


**Figure 1.** Histology of xenograft TE-7. *A*, Haematoxylin & Eosin staining showing a poorly differentiated squamous cell carcinoma, characterized by focal keratinization (Magnification 40x). *B*, *C and D*, immunostaining with involucrin, keratin 10 and 14, respectively, showing strong positive tumor cells (Magnification 40x).

Array-CGH analysis confirmed the similar genomic aberration patterns for TE-2, -3, -7, -12 and -13, however, small differences were seen, probably due to genomic instability of these cell lines (Figure 4). Recent admixture of the cell lines in our institute was excluded by Dr. Y. Morita-Fujimura (Japanese Cell Resource Centre for Biomedical Research) who confirmed these results with the TE cell lines present in the cell line bank in Japan using a STR multiplex system. Since the TE-7 cell line is widely used for decades as a model for EAC we investigated independent TE-7 DNA samples obtained from the laboratories of Dr P. Hainaut (France), Dr C. Tselepis (United Kingdom) and Dr. A. Lowe (USA). The earliest passage TE-7 DNA (1986) was obtained from the Japanese Cell Resource Centre for Biomedical Research (Dr. Y. Morita-Fujimura). All these TE-7 samples shared the same allelic pattern indicating that cross-contamination has occurred at the site of origin or in an early exchange of cell lines between laboratories. We were unable to compare the genotype of the cell lines with the patient's tissues because these were not present anymore in the pathology archive of the Tohoku University School of Medicine, Sendai, Japan.

Several literature data are in agreement with the finding that TE-7 is an ESCC cell line. Gene expression profiling revealed clustering of TE-7 with ESCC cell lines (KYSE series), and not with

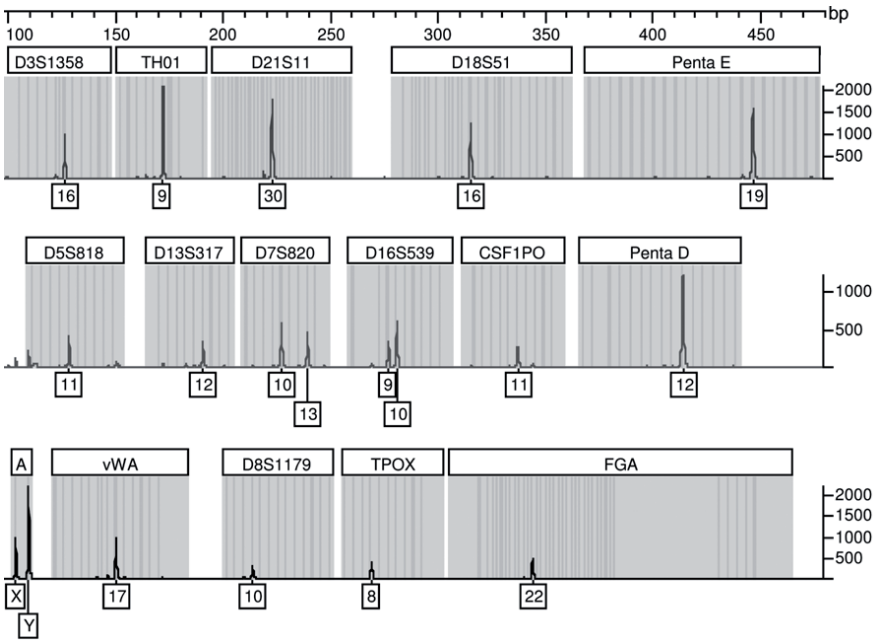


**Figure 2.** STR profile of cell line TE-7. STR loci are indicated in boxes above electropherogram; numbers of repeat units are indicated below the peaks. A, Amelogenin.

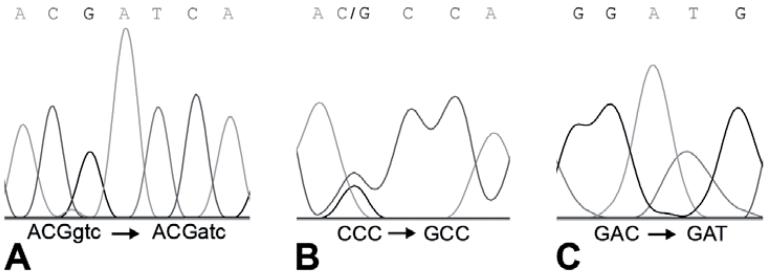


**Figure 3.** Mutation analyses of cell line TE-7. *A*, a homozygous p53 splice site mutation TP53 AF307851.1: c.375+1G>A in intron 4. *B*, heterozygous Cyclin D1 exon 5 mutation CCND1 NM_053056.2: c.861CAC (Pro287Ala). *C*, homozygous SNP in exon 5 of the Axin1 gene AXIN1 BC044648.1: c.1689C>T (Asp563Asp).

EAC cell lines (19). Another gene expression profiling study found that TE-7 cells clustered with the ESCC cell line OE-21 and not with the EAC derived cell lines OE-33 and SEG-1 (20). Furthermore, an identical Cyclin D1 mutation has been described in TE-3, -7 and 12, and an Axin1 SNP in TE-2 and -13 (12, 13). However, these observations didn't lead to questioning the authentication of these cell lines.

The impact of our findings comprises more than thirty-five reports, which have been published the last decade, using the cell lines TE-2, -3, -7, -12 and 13. Many investigators

**Table 1.** Short tandem repeat profiles of the TE-series

| | Short tandem repeat locus | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cell line** | **Penta E** | **D18S51** | **D21S11** | **TH01** | **D3S1358** | **FGA** | **TPOX** | **D8S1179** | **vWA** | **Penta D** | **CSF1PO** | **D16S539** | **D7S820** | **D13S317** | **D5S818** | **Amg** |
| TE-1 | 12, 18 | 17 | 28 | 7 | 16 | 24 | 8, 11 | 11, 13 | 18 | 10 | 10, 12 | 12 | 10, 11 | 10 | 11 | X |
| TE-2* | 19 | 16 | 30 | 9 | 16 | 22, 23 | 8 | 10, 11 | 17 | 12 | 11 | 9, 10 | 10, 13 | 12 | 11 | X, Y |
| TE-3* | 19 | 16 | 30 | 9 | 16 | 22 | 8 | 10, 11 | 17 | 12 | 11 | 9, 10 | 10, 13 | 12 | 11 | X, Y |
| TE-4 | 11, 20 | 18 | 31.2 | 6 | 15, 17 | 21 | 8, 11 | 11, 14 | 14 | 10 | 11 | 9, 10 | 11 | 11 | 10 | X |
| TE-5 | 15, 19 | 13, 16 | 30, 31.2 | 7 | 14, 15 | 23, 24 | 11 | 13, 15 | 16 | 10, 11 | 13 | 9, 10 | 11 | 10 | 9 | X |
| TE-6 | 9 | 14 | 30 | 6, 7 | 16 | 22, 24 | 12 | 14 | 17, 19 | 9 | 10, 12 | 9, 10 | 12 | 9 | 10, 13 | X |
| TE-7* | 19 | 16 | 30 | 9 | 16 | 22 | 8 | 10 | 17 | 12 | 11 | 9, 10 | 10, 13 | 12 | 11 | X, Y |
| TE-8 | 8, 17 | 14 | 30 | 6, 7 | 17 | 24, 26 | 8, 9 | 10 | 14, 18 | 9, 11 | 11, 12 | 9 | 12 | 11 | 11, 12 | X |
| TE-9 | 15, 20 | 13 | 30 | 6, 9 | 17 | 23 | 8 | 11, 13 | 17 | 10 | 11, 13 | 11, 12 | 8, 10 | 12 | 9, 13 | X |
| TE-10 | 15 | 14 | 30 | 6 | 16 | 21, 23 | 8, 11 | 15 | 14, 18 | 10 | 11 | 9, 11 | 8, 10 | 11 | 13 | X |
| TE-11 | 20 | 14, 15 | 31 | 9 | 17 | 24, 25 | 11 | 13 | 17, 18 | 10 | 9, 12 | 10, 11 | 11, 12 | 10 | 8, 14 | X |
| TE-12* | 19 | 16 | 30 | 9 | 16 | 22, 23 | 8 | 10, 11 | 17 | 12 | 11 | 9, 10 | 10, 13 | 12 | 11 | X, Y |
| TE-13* | 19 | 16 | 30 | 9 | 16 | 22 | 8 | 10, 11 | 17 | 12 | 11 | 9, 10 | 10, 13 | 12 | 11 | X, Y |
| TE-14 | 15, 21 | 15, 18 | 30 | 9 | 16 | 21 | 9, 11 | 14 | 19 | 11 | 11 | 12 | 9, 11 | 11 | 10 | X |
| TE-15 | 5, 17 | 14 | 30, 31 | 7 | 16 | 23 | 9 | 13 | 15, 18 | 9 | 12 | 9 | 11 | 12 | 9 | X |

* Note the highly similar profiles in TE-2, -3, -7, -12 and -13.

Abbreviation: Amg, Amelogenin

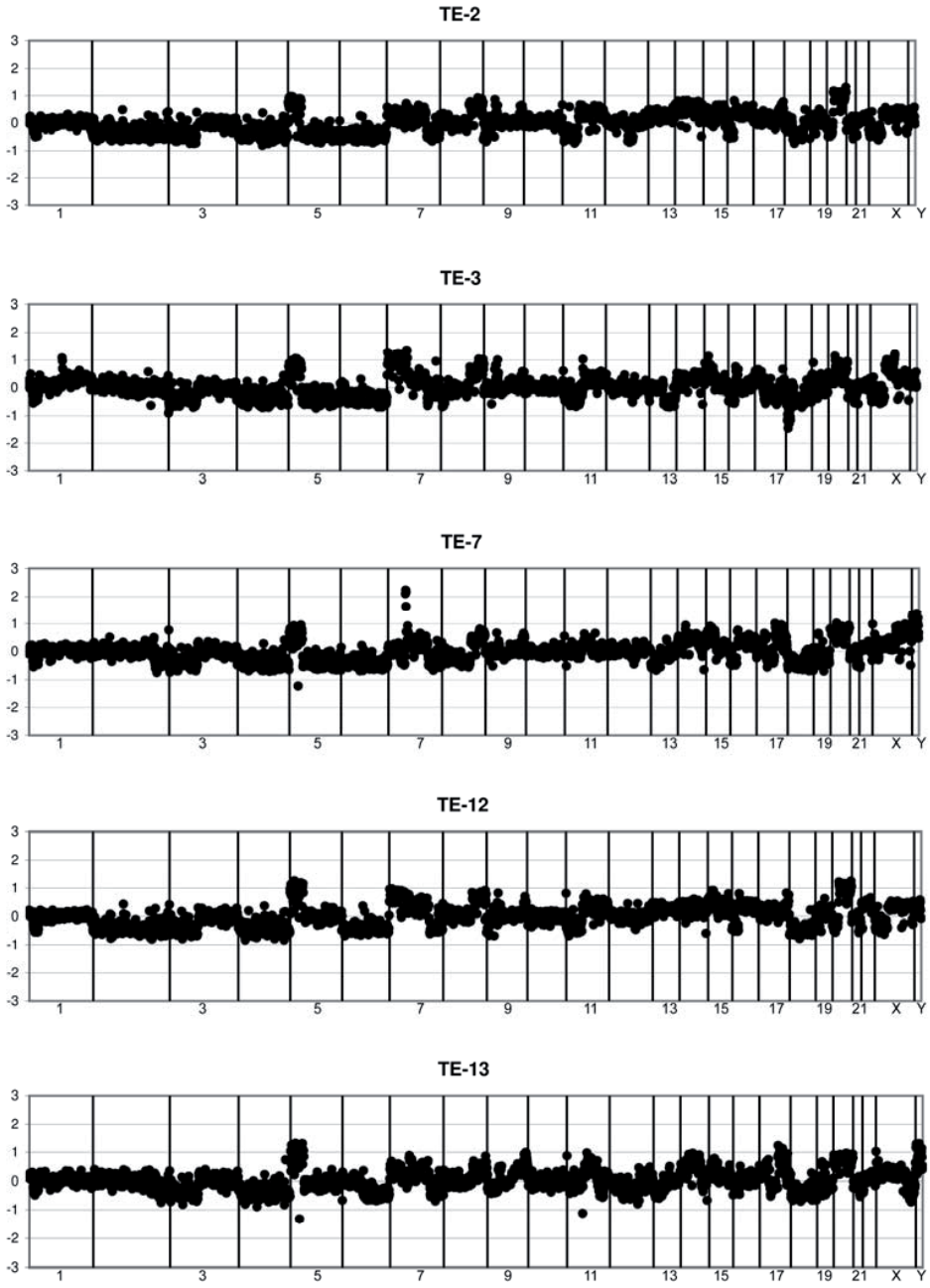


**Figure 4.** Array-CGH results of cell lines TE-2, -3, -7, -12 and -13. Note the similar genomic profiles of these cell lines.

used these cell lines to study the *in vitro* effects of chemotherapeutical agents directed too specific cellular targets as for example, the nuclear retinoic acid receptor and peroxisome proliferators-activated receptor gamma (PPARγ) (21-23). The effects of retinoic acid receptor inhibition on the growth, differentiation and apoptosis on human esophageal cancer cells were investigated in cell lines TE-1, -2, -3, -7, -8, -12 and -13. The authors reported that retinoic acid induced growth inhibition occurred only in TE-2, -3, -7, -12 and -13, all with elevated retinoic acid receptor beta (RAR-beta) expression (21). The association between retinoic acid induced growth inhibition and RAR-beta expression can now be regarded as weak, since these five responding cell lines are genotypically identical. Another investigation showed that expression of PPARγ protein was higher in an adenocarcinoma cell line (TE-7) than in a squamous cell carcinoma cell line (TE-1). The authors concluded that PPARγ inhibition in human EAC cells might due to induction of apoptosis, cell cycle arrest and reduced ornithine decarboxylase activity (22). In the light of our findings these data are misleading, since TE-7 is not an adenocarcinoma cell line.

## CONCLUSIONS

Based on the results of xenografting, STR profiling, mutation analyses and array-CGH, we conclude that the currently used TE-7 cell line is not an EAC but a squamous cell carcinoma cell line. Furthermore, cell line TE-7 shares the same genotype as TE-2, -3, -12 and -13. These five TE cell lines should be regarded as one single squamous cell carcinoma cell line of an unknown origin. Our study emphasizes that researchers should be careful in the way they establish and use cell lines, because a mistaken identity of a cell line may lead to invalid conclusions. Therefore, we suggest that future established tumor cell lines must be genotyped by SNP or STR analysis and compared with patient's tumor and normal tissue genotype. This genotyping information must be made available for the cell line users so that regular checks on the identity can be performed.

## ACKNOWLEDGEMENTS

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# Chapter 3

## Verification and unmasking of widely used human esophageal adenocarcinoma cell lines

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## ABSTRACT

For decades, hundreds of different human tumor type-specific cell lines have been used in experimental cancer research as models for their respective tumors. The veracity of experimental results for a specific tumor type relies on the correct derivation of the cell line. In a worldwide effort, we verified the authenticity of all available esophageal adenocarcinoma (EAC) cell lines. We proved that the frequently used cell lines SEG-1 and BIC-1 (in more than 100 publications) and the SK-GT-5 cell line are in fact cell lines from other tumor types. Experimental results based on these contaminated cell lines have led to ongoing clinical trials recruiting EAC patients, to more than 100 scientific publications and at least three NIH cancer research grants and 11 US patents, which emphasizes the importance of our findings. Widespread use of contaminated cell lines threatens the development of treatment strategies for EAC.

## INTRODUCTION

Cell lines derived from human cancers have been crucial to building our understanding of the molecular pathophysiology of cancer and its treatment. Of equal importance, they form an *in vitro* model system for rational drug discovery and development because they are easy to maintain and manipulate *in vitro* and in animal xenograft models. However, it has been estimated that up to one third of all cell lines have an origin other than that supposed (1). Cross contamination between cell lines and mislabeling of cultures lead to unrecognized cell line admixtures (1,2). In the past, the scientific community has recognized this problem, but decisive action has not been taken to date. Results based on experiments using contaminated cell lines might be translated to the clinic, forming the basis for clinical trials, and directly affecting the treatment of patients.

Model research on esophageal adenocarcinoma (EAC), which is the cancer type showing the steepest rise in incidence in the Western world over recent years (3), relies entirely on a relatively small set of established tumor cell lines. Appropriate animal models and familial cases for EAC are lacking (4). Cell lines are very useful to investigate molecular pathways that are involved in EAC tumorigenesis and to test experimental drugs on EAC cells *in vitro* and in vivo. Despite intensive efforts to culture EAC cells *in vitro*, only 14 permanent cell lines have been established: SEG-1, BIC-1, and FLO-1 (5), SK-GT-4, SK-GT-5 and BE-3 (6), KYAE-1 (7), OE19 and OE33 (8), JH-EsoAd1 (9), OACP4C and OACM5.1 (10), and two newly established cell lines ESO26 and ESO51 (by Grupo de Estudos de Esófago de Barrett do IPOLFG, Lisbon, Portugal). In collaboration with the primary investigators who established the cell lines, the original EAC tissues for 13 of the 14 cell lines were traced in pathology archives and made available for study (anonymously): the original tissue for cell line BE-3 (6) was not found. The availability of the primary tissues made it possible to authenticate these EAC cell lines by comparing the genotype of the cell line with the genotypes of patient's normal and tumor tissue.

## MATERIALS AND METHODS

### Cell cultures, DNA isolation, and xenografts

Except KYAE-1, all cell lines were cultured under standard conditions in RPMI-1640, supplemented with 10% Fetal Calf Serum. Cell line KYAE-1 was cultured in Ham's F12/RPMI-1640 1:1 with the addition of 10% fetal calf serum. To verify the histomorphological characteristics of each cell line, 5 x 106 tumor cells were injected subcutaneously in two female NMRI nude mice (Taconic-M&B, Ry, Denmark) or two NOD-SCID mice (in-house breeding). Xenograft tumors were removed after reaching a visible size of 0.5–1 cm in diameter and were routinely processed for histological and immunohistochemical examination (10). The mouse experiments were licensed and done in accordance with approved protocols by the Erasmus MC,

University Medical Center Rotterdam, the Netherlands. DNA was isolated from cultured cells, original tissues, and xenograft tumors using the PureGene Genomic DNA isolation Kit (Qiagen, Venlo, The Netherlands). DNA was diluted to a concentration of 100 ng/µL to perform polymerase chain reaction (PCR).

**Short Tandem Repeat (STR) profiling**

All cell lines were genotyped by STR profiling by using the Powerplex 16 System (Promega Madison WI). The Powerplex 16 system comprises fifteen STR loci, including Penta E, D18S51, D21S11, TH01, D3S1358, FGA, TPOX, D8S1179, vWA, Penta D, CSF1PO, D16S539, D7S820, D13S317, D5S818, and the sex chromosome marker Amelogenin. Amplification was performed using 1 ng of template DNA applying the Powerplex 16 system following the manufacturer's recommendation. Multiplex PCRs were carried out with fluorescent dye-linked primers. Labelled products were detected by electrophoretic size fractionation on an ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, CA). The data were analyzed by using Genemarker software (SoftGenetics LLC, State College, PA) to categorize peaks according to their size in relation to an internal standard. This analysis enabled every peak to be allocated a size corresponding to the number of repeat units present.

Inability to amplify the larger STR fragments of DNA isolated from paraffin embedded archival tissues (due to degraded DNA) forced us to use PCR primers closer to the repeat structures in order to perform single STR profiling. Using this method, eight single STR loci were investigated in the original tissue and in the corresponding cell line. The size of DNA fragments found in the cell lines could be linked to the number of repeats known from the Powerplex assay. This enabled us to correlate the length of the DNA fragments found in the original tissues to the number of repeats.

**Mutation analyses**

Because TP53 is frequently mutated in EAC, all exons and intron-exon boundaries of the TP53 gene were commercially sequenced in all the EAC cell lines (Asper Biotech Ltd, Tartu). The TP53 (Genbank accession number AF307851.1) mutations identified in the cell lines were then investigated in the original tumor tissues from which the cell lines had been derived.

## RESULTS

Ten of 13 cell lines unambiguously had the same genotype and harbored the same TP53 mutation(s) as the original tissues, proving their correct derivation (Table 1 and Appendix Table 1). The most frequently used EAC cell lines SEG-1 and BIC-1 and the SK-GT-5 cell line had genotypes different from the original tissue of which the cell line was stated. Comparison of the genotypes of SEG-1, BIC-1, and SK-GT-5 with genotypes available from databases

**Table 1.** Short tandem repeat profiles (number of repeats at each locus is indicated) of 14 established esophageal adenocarcinoma cell lines*

| | Short tandem repeat locus | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cell line** | **Penta E** | **D18S51** | **D21S11** | **TH01** | **D3S1358** | **FGA** | **TPOX** | **D8S1179** | **vWA** | **Penta D** | **CSF1PO** | **D16S539** | **D7S820** | **D13S317** | **D5S818** | **Amg** |
| SEG-1 | 5 | 13, 15 | 30 | 9.3 | 15, 18 | 21, 23 | 8 | 12 | 17 | 11, 13 | 11, 12 | 9 | 9, 12 | 13 | 9, 10 | X, Y |
| BIC-1 | 10 | 13 | 30, 30.2 | 8 | 15, 16 | 24 | 11 | 13 | 16 | 9, 15 | 13, 14 | 9, 13 | 8, 9 | 12 | 13 | X |
| FLO-1 | 5, 17 | 14, 16 | 30, 32.2 | 6 | 15 | 21 | 9, 11 | 13 | 16 | 11, 12 | 11 | 12, 13 | 8 | 11 | 12, 14 | X |
| KYAE-1 | 5, 8 | 14, 15 | 29, 31 | 6 | 15, 16 | 18 | 8 | 13, 14 | 14 | 9, 10 | 11 | 11 | 11, 12 | 11, 12 | 10, 13 | X |
| OE19 | 5, 8 | 12, 13 | 30 | 8, 9 | 15, 18 | 23, 26 | 8 | 13, 15 | 16, 17, 18 | 9 | 11, 13 | 12, 13 | 8 | 9, 11 | 11, 14 | X |
| OE33 | 12, 18 | 12 | 29, 31.2 | 7, 8 | 18 | 23 | 8, 11 | 10, 11, 12 | 17 | 9, 11 | 10, 11 | 12 | 9, 10 | 14 | 11 | X |
| OACM5.1 | 7, 14 | 16 | 28, 31 | 6, 9.3 | 16, 17 | 22 | 8 | 13, 14 | 19, 20 | 10 | 10, 13 | 10, 11 | 8 | 11, 12 | 12 | X |
| OACP4C | 20 | 12, 13 | 30 | 9 | 18 | 20 | 11 | 13 | 16 | 12 | 11 | 12 | 9, 11 | 12 | 9 | X |
| SK-GT-4 | 7 | 14 | 31.2 | 6, 9.3 | 17 | 22, 23 | 8, 10 | 13 | 17, 19 | 9 | 11, 15 | 11, 12, 13 | 7, 11 | 9, 10 | 12 | X |
| SK-GT-5 | 15, 17 | 14, 15 | 32.2 | 8, 9 | 15 | 26 | 9, 12 | 15 | 15, 18 | 9 | 10 | 11, 12 | 9, 10 | 12, 13 | 10 | X |
| BE-3 | 10, 16 | 17 | 30.2 | 9 | 16, 17 | 22 | 8, 10 | 12, 14 | 18 | 13 | 11 | 12 | 8, 11 | 11, 12 | 11, 13 | X |
| JH-EsoAd1 | 11 | 12 | 30 | 6, 7 | 16 | 24 | 8, 9 | 10 | 18, 19 | 14 | 10 | 10, 12 | 10, 12 | 11 | 11 | X |
| ESO26 | 12, 14 | 17 | 30 | 9 | 15 | 21 | 7, 9 | 13, 14 | 14, 18 | 10 | 9, 10 | 9 | 11 | 9, 11 | 12 | X, Y |
| ESO51 | 17 | 17, 20 | 30, 33.2 | 9 | 15, 16 | 21, 22 | 8 | 10, 11, 12 | 14, 15 | 9, 13 | 10 | 13 | 8 | 11 | 11 | X |

* The profile of cell line SEG-1 is identical to large cell lung carcinoma cell line H460. The profile of cell line BIC-1 is identical to colon adenocarcinoma cell line SW620. The profile of cell line SK-GT-5 is identical to gastric adenocarcinoma cell line SK-GT-2. Amg = Amelogenin.

(http://www.lgcpromochem-atcc.com/common/cultures/str.cfm) revealed that SEG-1 is lung carcinoma (large cell lung cancer) cell line H460 (ATCC_HTB-177) and BIC-1 is colorectal adenocarcinoma cell line SW620 (ATCC_CCL-227). The genotype and TP53 mutation of the SK-GT-5 cell line matched with the tissue from which the cell line SK-GT-2 was derived indicating that cell line SK-GT-5 is actually the gastric fundus carcinoma cell line SK-GT-2 (Appendix Table 2). In independent experiments, the researcher who established cell lines SEG-1 and BIC-1 (Dr. Beer) confirmed our results using the earliest passages of these cell lines. Clearly,

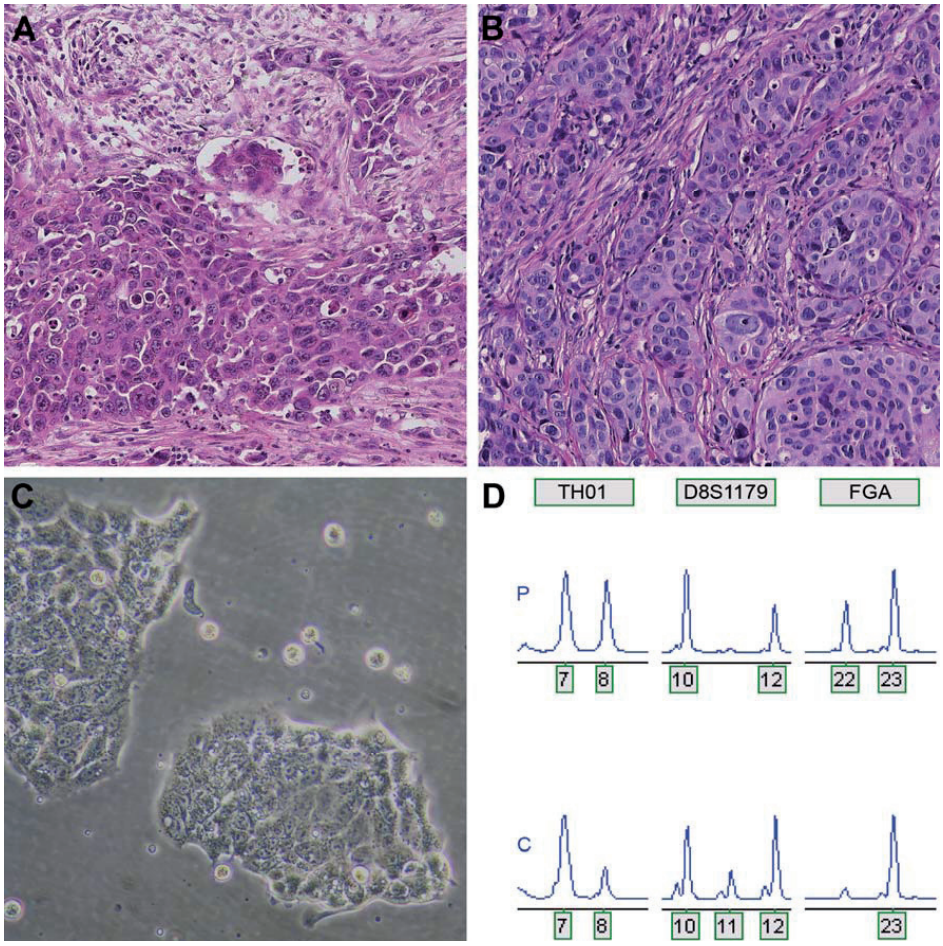


**Figure 1.** Authentication of human esophageal adenocarcinoma cell line OE33. *A and B*, Hematoxylin & eosin stained sections of the original tissue and xenograft of OE33, showing a poorly differentiated adenocarcinoma. *C*, *In vitro* growth pattern of cell line OE33. *D*, STR profile of the primary normal tissue (P) and cell line OE33 (C), indicating correct derivation of the cell line. Short term repeat loci are indicated in boxes above electropherogram; the number of repeat units are indicated below the peaks. Note: loss of heterozygosity in the cell line at loci TH01 and FGA. The additional allele (11 repeat units) of D8S1179 observed in cell line OE33 is a known phenomenon is probably due to somatic mutation or localized chromosomal rearrangements at this heterozygous locus.

contamination occurred early during establishment of the cell lines, and all of the cultures that were distributed subsequently to different laboratories were contaminated. We obtained the earliest available passage of cell line SK-GT-5 (1993) from Dr. Schrump (on referral by Dr. Altorki). This sample matched tissue from which cell line SK-GT-2 was derived, indicating that cross-contamination had occurred at the site of origin or during the early interinstitutional exchange of cell line SK-GT-5.

## DISCUSSION

After hundreds, perhaps thousands, culture passages of the 10 verified EAC cell lines, it could be questioned how representative these cell lines still are as models for their original EAC tumors. In the 10 verified EAC cell lines, 11 TP53 mutations were identified and all were found to be present in the respective original tumor tissues (Appendix Table 3), indicating consistency of the TP53 mutations during decades of *in vitro* propagation of the cell lines. Furthermore, xenografts obtained from nine of 10 verified EAC cell lines all showed a phenotype identical to the original EAC tumors, demonstrating that the cellular features and architecture of the tumor are preserved even after long-term *in vitro* culture (Figure 1).

The impact of our findings is illustrated by two clinical trials that are currently recruiting EAC patients based on experimental results using the contaminated cell lines SEG-1 and BIC-1. The first trial (http://clinicaltrials.gov/ct2/show/NCT00619242) investigates the effect of sorafenib (BAY 43-9006), a potent competitive small molecule multikinase inhibitor of the Raf/MAPK/ERK pathway, on Barrett-related EAC. Several studies suggested that exposure of cell line SEG-1 to acid increased proliferation and decreased apoptosis by activating the Raf/MAPK/ERK pathway (12-14). In addition, treatment of SEG-1 cells with Raf/MAPK/ERK inhibitors resulted in pronounced anti-proliferative effects (15-18). In the present study, we have proved that cell line SEG-1 does not represent EAC, but large cell lung carcinoma. This means that there is scant scientific evidence for activation of the Raf/MAPK/ERK pathway by acid or bile exposure in Barrett-related adenocarcinoma. The use of sorafenib in patients with Barrett-related EAC, and the recruitment of patients for this clinical trial should therefore be reconsidered.

Another potential target for therapy in EAC, which is based mainly on research with contaminated cell lines SEG-1 and BIC-1, is telomerase (19, 20). In a recent study, investigators demonstrated that treatment of SEG-1 xenografts with a specific telomerase inhibitor, GRN163L, led to loss of telomerase activity, reduction of telomere length, and inhibition of cell growth through induction of both senescence and apoptosis (21). Currently, EAC patients (among patients with other cancer types) are being recruited within a phase I clinical trial to study the effects of this telomerase inhibitor (http://clinicaltrials.gov/ct2/show/NCT00310895?term=G

RN163L&rank=2). Our findings suggest that there is little scientific evidence for treatment of EAC patients with telomerase inhibitor GRN163L.
We have identified more than 100 scientific publications in which the contaminated cell lines SEG-1, BIC-1, or SK-GT-5 were used (Appendix Tables 4 and 5). Almost half of these reports were based solely on the use of cell lines not representative for EAC and should therefore be re-evaluated because these cell lines in reality represent large cell lung carcinoma (SEG-1), colon adenocarcinoma (BIC-1), or gastric fundus adenocarcinoma (SK-GT-5). In addition, at least three NIH grants have been assigned to esophageal cancer research projects (http://crisp.cit.nih.gov/), and 11 US patents (http://www.uspto.gov/) have been granted based on the use of cell lines SEG-1 and BIC-1. Following the lead of Walter Nelson-Rees, the first person who urged scientists to stop using contaminated cell lines (22,23), this report is a call for all scientists to authenticate their cell lines. Recent advances in DNA-profiling techniques make it possible to genotype cell lines simply and cheaply. The use of verified cell lines is a shared responsibility for scientists, editorial boards of scientific journals and clinical and basic cancer research funding agencies.

## CONCLUSIONS

In summary, cell lines SEG-1, BIC-1, and SK-GT-5 are not EAC cell lines, but large cell lung cancer cell line H460, colorectal adenocarcinoma cell line SW620, and gastric fundus carcinoma cell line SK-GT-2, respectively. Cell lines FLO-1, KYAE-1, SK-GT-4, OE19, OE33, JH-EsoAd1, OACP4C, OACM5.1, ESO26, and ESO51 are derived from human EACs. All of these 10 verified EAC cell lines, together with their genotyping information, will be deposited in publicly available cell line repositories in the United States (http://www.lgcpromochem-atcc.com), Europe (www.ecacc.org.uk), and Japan (www.brc.riken.jp), to promote and facilitate future solid research on EAC.

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# Chapter 4

## Mapping of homozygous deletions in verified esophageal adenocarcinoma cell lines and xenografts

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## ABSTRACT

Human esophageal adenocarcinoma (EAC) cell lines and xenografts are powerful tools in the search for genetic alterations because these models are composed of pure human cancer cell populations without admixture of normal human cells. In particular detection of homozygous deletions (HDs) is easier using these pure populations of cancer cells. Identification of HDs could potentially lead to the subsequent identification of new tumor suppressor genes (TSGs) involved in esophageal adenocarcinogenesis.

Genome wide single nucleotide polymorphism (SNP) arrays were used to identify HDs in ten verified EAC cell lines and nine EAC xenografts. In total, 61 HDs (range 1-6 per sample) were detected and confirmed by PCR. Besides HDs observed in common fragile genomic regions (N=26), and gene deserts (N=8), 27 HDs were located in gene containing regions. HDs were noted for known TSGs, including *CDKN2A*, *SMAD4* and *CDH3*/*CDH1*. Twenty-two new chromosomal regions were detected harboring potentially new TSGs involved in EAC carcinogenesis. Two of these regions of homozygous loss, encompassing the *ITGAV* and *RUNX1* gene, were detected in multiple samples indicating a potentially role in the carcinogenesis of EAC. To exclude culturing artifacts, these last two deletions were confirmed by fluorescent in situ hybridization in the primary tumors of which the involved cell lines and xenografts were derived.

In summary, in this report we described the identification of HDs in a series of verified EAC cell lines and xenografts. The deletions documented here are a step forward identifying the key genes involved in EAC development.

## INTRODUCTION

Esophageal adenocarcinoma (EAC) is the cancer type with the steepest rise in incidence in the Western world over recent years (1). With the increasing prevalence of contributing factors (i.e., obesity and gastro-esophageal reflux disease) in developed countries, it is to be expected that the incidence will continue to rise. Despite the common occurrence of EAC, little is known about the molecular mechanisms underlying the genesis of these tumors. In the past five years, several studies have identified DNA copy number gains/amplifications amplifications on chromosomes 1q, 3q, 7p, 7q, 8q, 17q and 20q along with copy number losses (including loss of heterozygosity and homozygous deletions) on 3p, 4q, 5q, 9p, 14q, 16q, 17p and 18q in the EAC genome (2). Mapping of regions with homozygous deletions (HDs) can potentially lead to the identification of new tumor suppressor genes (3-6). However, in primary EAC tumor tissue samples HDs are difficult to detect due to the presence of contaminating non-malignant cells. A more powerful tool in detection of HDs are the use of cell lines and xenografts, that consists of pure populations of cancer cells.

To identify novel tumor suppressor genes involved in the carcinogenesis of EAC, we conducted high-density single-nucleotide polymorphism (SNP) microarrays in 10 verified EAC cell lines and 9 xenografts and focussed on areas of homozygous loss. Given the high resolution of the SNP arrays and the use of pure populations of cancer cells (without admixture of normal human tissue), we have been able to identify small homozygous deletions that have not been detected by previous studies. Furthermore, the availability of the primary tissues of which the cell lines and xenografts were derived, made it possible to verify a selection of these HDs by fluorescent in situ hybridization (FISH).

## MATERIAL AND METHODS

### Cell lines and xenografts

Verified EAC cell lines, ESO-26, ESO-51, FLO-1, JH-EsoAd1, KYAE-1, OACP4C, OACM5.1, OE19, OE33 and SK-GT-4 were cultured under standard conditions in RPMI-1640 or Ham's F12/RPMI-1640 1:1 (for KYAE-1), supplemented with 10% Fetal Calf Serum (7). Xenografts P58x, P100x, P101x, P102x, P104x, P117x, P140x, P171x, P175x were obtained after transplantation of primary tumor tissue to female nude mice, 4-6 weeks of age (8). The animal experiments were licensed and done in accordance with approved protocols by the Erasmus MC, University Medical Center Rotterdam, The Netherlands.

Paraffin embedded primary tumor tissue of all cell lines and xenografts was available. Normal frozen tissues were available for all xenografts and two EAC cell lines (OACP4C and OACM5.1); normal immortalized lymphocytes related to cell line JH-EsoAd1 were obtained from dr J. R. Eshleman (Johns Hopkins University School of Medicine, Baltimore, MD, USA). DNA was

isolated from cultured cells, original tissues and xenografts using the PureGene Genomic DNA isolation Kit (Qiagen, Venlo, The Netherlands).

**Single nucleotide polymorphim (SNP) array**

The Affymetrix 500K SNP array, used for all tumor (N=19) and corresponding normal tissue samples (N=11), was performed by Myriad Genetics Inc. (Salt Lake City, UT, USA), according to the methods described by the manufacturer (http://www.affymetrix.com/products_services/arrays/specific/500k.affx). Total genomic DNA (250 ng) was digested with a restriction enzyme (Nsp I or Sty I) and ligated to adaptors that recognize the cohesive four base pair overhangs. All fragments resulting from restriction enzyme digestion, regardless of size, are substrates for adaptor ligation. A generic primer that recognizes the adaptor sequence was used to amplify adaptor ligated DNA fragments. PCR conditions have been optimized to preferentially amplify fragments in the 200 to 1,100 bp size range. The amplified DNA was then fragmented, labeled, and hybridized to a GeneChip Human Mapping 250K Array.

**Identifying homozygous deletions by SNP-array analysis**

Copy number intensities were calculated using the public domain software package CNAG (Copy Number Analyser for GeneChip), version 2.0, and detected using the implemented hidden Markow model (9, 10). To detect only the tumor-associated copy number changes, and to exclude naturally occurring copy number variation (CNV), the DNA of the cell lines OACP4C, OACM5.1 and all xenografts was compared with corresponding normal DNA (obtained from frozen normal squamous cell epithelium of the esophagus). In addition, cell line JH-EsoAd1 was compared with the corresponding EBV- immortalized lymphocyte cell line. For the seven remaining cell lines, a database containing probe intensity and genotype data sets from the three normal tissue samples was generated and used as reference sample for copy number analysis.

Each deletion is defined by whether the log 2 ratio of probe intensity is below the baseline log 2 ratio defined by the matched normal DNA. The baseline log 2 ratio has a theoretical value of zero. Homozygous deletions were identified by CNAG2.0 with a copy number of the probes equal to zero in default settings with a smoothing window of 5-SNP probes. To validate the HDs detected by CNAG, we used NEXUS copy number software (11). The physical positions of the detected deletions were determined based on the Human hg18 assembly (NCBI Build 36.1 http://genome.ucsc.edu/).

**Confirmation of the homozygous deletion by PCR and gene-expression**

All homozygous deletions detected by SNP-array analysis were confirmed by PCR. Primers were designed within and flanking the homozygous deletions. In samples with a HD in a gene containing region, the expression of genes within this region is expected to be lower as compared with samples without the deletion. However, differences in gene expression

will only be detected when the particular genes are expressed in esophageal cancer; otherwise, if the genes are not expressed in esophageal cancer, no differences in gene-expression between the cell lines will be observed. Of all EAC cell lines mRNA was isolated using the Qiagen RNeasy kit (Qiagen, Venlo, The Netherlands). RNA quality was assessed using the Agilent Bioanalyser, requiring RNA integrity >7.0 (12). All further processing of the samples was performed according the Affymetrix GeneChip whole transcript sense target labeling assay. Affymetrix GeneChip Human Exon 1.0 ST Arrays (http://www.affymetrix.com/products_services/arrays/specific/exon.affx) were used to determine the expression level of virtually all exons present in the human genome (1.4 million probe sets covering >1 million exon clusters). Expression data were analyzed by Partek® Genomics Suite™ 6.3 (Partek Inc., St. Louis, MO, USA). A standard algorithm was used to normalize exon signals to the gene-level signal for each sample. A t-test was performed to detect differences in gene-expression levels between samples harboring a HD (containing the gene of interest), and samples without HD. P-values <0.05 were considered as statistical significant.

**Selection of candidate genes for further analysis**

Overlapping HDs in gene containing regions, present in multiple samples were selected for further analysis. A second selection criterion was a HD in a gene containing region present in a single sample and aberrant gene expression in another sample as detected by gene-expression arrays. The HDs selected for further analysis, were validated in the cell lines and/or xenografts and corresponding primary tumors, by fluorescent in situ hybridization (FISH) experiments were performed according to standard protocols with minor modifications. For dual color FISH, on cell line metaphases and paraffin embedded tumor material, centromeric or telomeric and locus specific BAC and cosmid probes were selected as described previously (13).

Mutation analysis of the candidate genes *ITGAV*, *CDH1*, *CDH3*, *CTNNA1* and *RUNX1* was done in all EAC cell lines (without HD in the particular gene region). Polymerase chain reactions (PCR) were carried out in a volume of 15µl containing genomic DNA, 8.3µl $H_2O$, 5µl $Mg^{2+}$free buffer, 25mM $MgCl_2$, 0.3µl of 10mM deoxynucleotide triphosphates, 20pmol of each primer and 1U Taq polymerase (Promega, Madison WI, USA). The primers and PCR conditions used were described previously (14-18). The PCR products were bi-directionally sequenced using an Applied Biosystems 3100 genetic analyser (Perkin Elmer, Foster City, USA). Detected mutations were validated in the primary tumors of which the cell line was derived. Protein-expression of the candidate genes was investigated by immunohistochemistry (IHC), only if suitable antibodies were available.

## RESULTS AND DISCUSSION

Human EAC cell lines and xenografts are indispensable models to study genetic alterations that potentially contribute to the carcinogenesis of this tumor type, because appropriate animal models and familial cases for EAC are lacking. In these pure populations of tumor cells identification of HDs is feasible and potentially contribute to the discovery of new tumor suppressor genes. In addition, the availability of the primary tumors of all cell lines made it possible to confirm the deletion in patient's material, and thus excluding potential *in vitro* culturing artifacts. The clinical characteristics of all tumors from which the cell lines and xenografts were derived are depicted in Table 1. Median age at diagnosis was 67 years (range, 44-89). Most patients were male (84%). The majority of tumors had an advanced stage at pathological examination. In 11 (58%) patients Barrett's mucosa was present.

In total, 61 HDs were detected by SNP-array analysis. These deletions ranged in size from 32 kb (OE33) to 4.1 Mb (OE19). Up to six deletions per sample were identified. Twenty six HDs were found related to (overlapped or bordered) six common fragile regions (Appendix Table 6). Deletions at these fragile sites have also been described by other investigators using primary EAC samples (2, 19-24). It has been thought that these deletions are a common an early event in EAC development (25). Furthermore, functional knockout mice studies indicate a

**Table 1.** Clinical characteristics of esophageal adenocarcinoma cell lines and xenografts

| Cell line or Xenograft | Gender | Age at diagnosis | Tumor location | Presence of Barrett's epithelium | Postoperative stage | Number of HDs |
|---|---|---|---|---|---|---|
| ESO26 | Male | 56 | Cardia AC | No | pT4N1M1 | 3 |
| ESO51 | Male | 74 | Distal EAC | Yes | pT3N1Mx | 2 |
| FLO-1 | Male | 68 | Distal EAC | No | pT2N1M0 | 4 |
| JH-EsoAd1 | Male | 66 | Distal EAC | Yes | pT3N0Mx | 4 |
| KYAE-1 | Male | 60 | Distal EAC | No | pT4N1M1 | 5 |
| OE19 | Male | 72 | Cardia AC | No | pT3N1Mx | 4 |
| OE33 | Female | 73 | Distal EAC | Yes | pT3N0Mx | 3 |
| OACM5.1 | Female | 47 | Distal EAC | Yes | pT3N1M1 | 4 |
| OACP4C | Male | 55 | Cardia AC | No | pT3N1M1 | 6 |
| SK-GT-4 | Male | 89 | Distal EAC | Yes | pT2N1Mx | 3 |
| P58x | Male | 44 | Distal EAC | Yes | pT1N1M0 | 3 |
| P100x | Male | 54 | Distal EAC | Yes | pT3N1M0 | 4 |
| P101x | Male | 65 | Cardia AC | No | pT3N1M0 | 5 |
| P102x | Male | 67 | Distal EAC | Yes | pT3N0M0 | 1 |
| P104x | Male | 70 | Distal EAC | No | pT3N1M0 | 2 |
| P117x | Female | 55 | Cardia AC | No | pT3N1M0 | 6 |
| P140x | Male | 67 | Distal EAC | Yes | pT3N0M0 | 1 |
| P171x | Male | 73 | Distal EAC | Yes | pT3N1M0 | 1 |
| P175x | Male | 73 | Distal EAC | Yes | pT3N1M0 | 1 |

*Abbreviations:* cardia AC, cardia adenocarcinoma; distal EAC, distal esophageal adenocarcinoma; HDs, homozygous deletions

potential tumor suppressor role of the fragile histidine triad gene (*FHIT*) located at FRA3B and the WWdomain–containing oxidoreductase gene (*WWOX*) at FRA16D in the development of forestomach adenocarcinoma (which is an equivalent of the human distal esophagus) (26, 27). Eight HDs were located in gene deserts (Appendix Table 7). The remaining HDs (N=27) were located in gene containing regions (Table 2). These results are in concordance with large surveys on HDs in human cancer cell lines, in which the majority of deletions are detected in fragile sites (regions that show increased rates of chromosome breakage in normal cells in response to agents causing replicative stress) or in gene deserts (region that not harbor known genes) (28, 29).

HDs in gene containing regions were noted for known TSGs involved in EAC carcinogenesis: *CDKN2A*/*CDKN2B*, *SMAD4* and the *DLC1* gene. To our knowledge, this is the first report that describes inactivation of *CDH3*/*CDH1* by homozygous deletion in EAC. Both genes play important roles in cell-cell adhesion, although the gene encoding P-cadherin (*CDH3*) is poorly

**Table 2.** Homozygous deletions in gene containing regions

| Cytoband | Start | Stop | Size (bp) | Sample | Representative gene within interval | Total no. genes |
|---|---|---|---|---|---|---|
| 2q32.1 | 187115467 | 187240020 | 124553 | ESO51 | *ITGAV* | 1 |
| 2q32.1 | 187160790 | 187225440 | 64650 | KYAE-1 | *ITGAV* | 1 |
| 3p12.3 | 79526088 | 80745130 | 1219042 | JH-EsoAd1 | *ROBO1* | 1 |
| 4q35.1 | 183083268 | 184639494 | 1556226 | OACP4C | *ODZ3; DCTD; WWC2; CLDN22; CDKN2AIP* | 7 |
| 4q35.2 | 188635673 | 189826114 | 1190441 | OACP4C | *ZFP42; TRIML2; TRIML1* | 3 |
| 5q11.2 | 58526447 | 58707182 | 180735 | P117x | *PDE4D* | 1 |
| 5q31.2 | 138084150 | 138148730 | 64580 | OACP4C | *CTNNA1* | 1 |
| 6p25.3 | 1533630 | 1785593 | 251963 | OACP4C | *FOXC1; GMDS* | 2 |
| 8q24.3 | 140936025 | 141407844 | 471819 | JH-EsoAd1 | *TRAPPC9* | 1 |
| 8p23.1-p22 | 12285856 | 13893340 | 1607484 | P117x | *DLC1* | 5 |
| 8q13.3 | 71445803 | 72048237 | 602434 | P117x | *NCOA2; TRAM1; LACTB2; XKR9* | 4 |
| 9p23 | 9450595 | 9627189 | 176594 | OACM5.1 | *PTPRD* | 1 |
| 9p21.3 | 21673024 | 23252297 | 1579273 | JH-EsoAd1 | *MTAP; C9orf53; CDKN2A; CDKN2B; DMRTA1* | 5 |
| 9p21.3 | 21815984 | 22231913 | 415929 | P101x | *MTAP; C9orf53; CDKN2A; CDKN2B* | 4 |
| 9p21.3 | 21845431 | 22032346 | 186915 | OACP4C | *MTAP; C9orf53; CDKN2A; CDKN2B* | 4 |
| 9p21.3 | 20656622 | 22584436 | 1927814 | SK-GT-4 | *MTAP; CDKN2A; CDKN2B* | 25 |
| 9q21.2 | 79319412 | 79349466 | 30054 | P117x | *GNA14* | 1 |
| 10q23.31 | 89543978 | 91973853 | 2429875 | P117x | *PTEN; FAS-APO* | 26 |
| 12p13.31-p13.2 | 8600421 | 10063603 | 1463182 | OACM5.1 | *A2M; CD69* | 23 |
| 12q12 | 37810640 | 39112297 | 1301657 | OE19 | *KIF21A; ABCD2; C12orf40; SLC2A13; LRRK2* | 5 |
| 12q12-q13.11 | 41286253 | 45403376 | 4117123 | OE19 | *IRAK4; NELL2* | 14 |
| 12q13.13-q13.2 | 52942936 | 53559293 | 616357 | OE19 | *ITGA5; PDE1B* | 16 |
| 16q22.1 | 67226159 | 67351131 | 124972 | P104x | *CDH3; CDH1* | 2 |
| 18q21.2 | 46859775 | 50908355 | 4048580 | P58x | *SMAD4; DCC* | 11 |
| 21q22.12 | 35120118 | 35281843 | 161725 | P100x | *RUNX1* | 1 |
| 21q22.12 | 35099379 | 35442696 | 343317 | SK-GT-4 | *RUNX1* | 1 |
| 22q11.1 | 15774370 | 15841990 | 67620 | OACM5.1 | *GAB4* | 1 |

characterised in comparison too *CDH1* (the gene that encodes E-cadherin). It has been suggested that CDH3 act as an invasion suppressor in cancer cells in which it is down-regulated during progression, but as an invasion promoter in cancer cells in which aberrant expression occurs throughout progression (30). This could be an explanation for the results of a study that showed enhanced protein expression of CDH3 in 17/24 EAC samples (31). Germline mutations in the *CDH1* gene have been associated with hereditary diffuse gastric cancer and lobular breast cancer (32). Although other mechanism for silencing of the E-cadherin gene have been described in EAC, such as promoter hypermethylation or up-regulation of E-cadherin repressors like Slug-1 (33, 34), we revealed two overlapping deletions in cell line KYAE-1 and xenograft P104x on chromosome 16q22.1, harboring the *CDH*3 and *CDH1* gene (Figure 1A). Staining for E- and P-cadherin revealed absent protein expression in KYAE-1 and P104x, and in their corresponding primary tumors (Figure 1B/C). Additional FISH analysis confirmed these findings. Phenotypically, loss of cell-cell adhesion by deletion of *CDH3/*

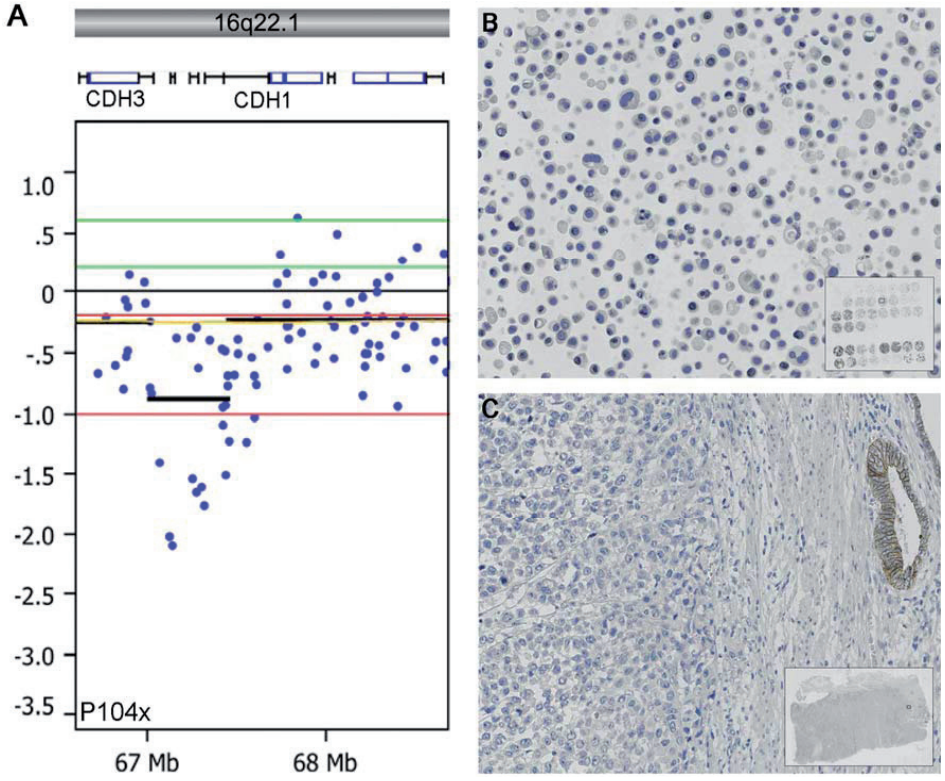


**Figure 1.** Analyses of a homozygous deletion on chromosome 16q22.1. *A*, Scatter plot of log2 copy number ratios (blue dots, left axis) for xenograft P104x showed a homozygous deletion overlapping the *CDH3* and *CDH1* gene. *B and C,* Staining for E-cadherin demonstrated absent protein expression in cell line KYAE-1 (B) and in the primary tumor of which xenograft P104x was derived (C).

*CDH1* is characterized by the round or spindle shaped appearance of the tumor cells and the capacity of the cells to disseminate (the primary tumors of cell line KYAE-1 and xenograft P104x, metastasized to lung and liver, respectively). In the cell lines without a *CDH3*/*CDH1* HD no mutations in these two genes were found, although in 4/9 cell lines LOH of the *CDH3*/*CDH1* locus was observed confirming earlier studies (35). Furthermore, these findings suggest that somatic inactivation of E- and/or P-cadherin is not restricted to gastric cancer or lobular breast cancer but does also occur in esophageal adenocarcinoma. urthermore, these results validate the sensitivity of our strategy with respect to the detection of biologically important HDs in human cancer.

Twenty-two homozygous deletions within gene-containing regions were identified, harboring potentially new TSG involved in EAC carcinogenesis. Gene-expression data of the cell lines showed that the majority of the HDs identified by SNP-array analysis resulted in reduced gene-expression; significance was reached in 8 of 15 tested genes (table 3). Homozygous deletions affecting chromosome 4q35.2 (*TRIML2*), 5q11.2 (*PDE4D*), 6p25.3 (*GMDS*), 8q24.3 (*TRAPPC9*), 9p23 (*PTPRD*), 9p21.3 (*MTAP, CDKN2A etc*), 18q21.2 (*SMAD4*) and 21q22.12 (*RUNX1*) are also described in other SNP-array studies using primary EAC samples (2, 19-24). This indicates that genes within these regions of homozygous loss could be potentially involved in EAC carcinogenesis. It can be speculated that most of the HDs within gene-containing re-

**Table 3.** Differences in gene-expression levels between cell lines harbouring a homozygous deletion (containing the gene of interest), and cell lines without homozygous deletion.

| Cytoband | Cell line (harbouring a HD) | Gene (within HD region) | P-value* |
|---|---|---|---|
| 2q32.1 | ESO51; KYAE-1 | *ITGAV* | 0.046 |
| 3p12.3 | JH-EsoAd1 | *ROBO1* | 0.29 |
| 4q35.1 | OACP4C | *WWC* | 0.07 |
| 4q35.2 | OACP4C | *TRIML2* | 0.76 |
| 5q31.2 | OACP4C; ESO26† | *CTNNA1* | <0.0001 |
| 6p25.3 | OACP4C | *GMDS* | 0.0041 |
| 8q24.3 | JH-EsoAd1 | *TRAPPC9* | 0.0016 |
| 9p23 | OACM5.1 | *PTPRD* | 0.031 |
| 9p21.3 | JH-EsoAd1; OACP4C; SK-GT-4 | *CDKN2A* | 0.0007 |
| 12p13.31-p13.2 | OACM5.1 | *CD69* | 0.068 |
| 12q12 | OE19 | *SLC2A13* | 0.19 |
| 12q12-q13.11 | OE19 | *NNT* | 0.0018 |
| 12q13.13-q13.2 | OE19 | *PDE1B* | 0.98 |
| 21q22.12 | SK-GT-4 | *RUNX1* | 0.12 |
| 22q11.1 | OACM5.1 | *GAB4* | ND‡ |

*Abbreviations:* HD, homozygous deletion; ND, not determined

* A t-test was performed to detect differences in gene-expression levels between cell lines harbouring a HD (containing the gene of interest), and cell lines without HD

† In cell line ESO26, the reduced *CTNNA1* expression is caused by a single nucleotide deletion in exon 6 (c.850_delA/c.851_delA)

‡ No expression data were available of *GAB4*, because the gene was not covered by the Human Exon ST 1 Array

gions, that are only described in the present study (and not in other reports), are "passengers", reflecting the genetic instability of these tumors and not involved in cancer development. However, it could also be possible that these deletions contribute to the carcinogenesis of this particular tumor, but do not play a major role in carcinogenesis of EAC in general (36).
Here, we focused on overlapping deletions (same region of loss in multiple samples), because it can be expected that these regions harbor genes of interest (and to select a common denominator)**.** Two chromosomal regions with homozygous deletions were selected for further investigation: chromosome 2q32.1 harboring the *ITGAV* gene (ESO51 and KYAE-1) and chromosome21q22.12 harboring the *RUNX1* gene (SK-GT-4 and P100x). A third chromosomal (chromosome 5q31.2 containing the *CTNNA1* gene) region was added because two cell lines (OACP4CE and ESO26) showed significant reduced expression of *CTNNA1* of which one (OACP4C) harboured a HD.
Two overlapping HDs on chromosome 2q32.1 were detected in cell lines ESO51 and KYAE-1 (Figure 2), affecting the promoter region and first exons of the *ITGAV* gene. This gene, encodes the integrin αV chain, which can heterodimerize with five different integrin beta chains forming transmembrane glycoprotein receptors for extracellular matrix proteins (37). Expression data showed a significantly reduced expression of *ITGAV* in both cell lines with the HD, as compared with the other cell lines. FISH analysis confirmed the presence of the HD in the primary tumor of ESO51, but not in the adjacent Barrett's epithelium. In cell line KYAE-1 the deletion could not been confirmed by FISH, due to the smaller size of the deletion (available probes in this region were too large for detection of the relatively small deletion). Mutation analysis of the cell lines without HD did not reveal mutations. Members of the integrin αV subfamily play critical roles in the homeostasis of stratified squamous epithelium. Interestingly and in contradiction to the vast majority of *in vitro* growing epithelial tumor cell lines, the αV integrin negative cell lines ESO51 and KYAE-1 both grow non-adherent to the culture flasks in floating aggregates. This has also been reported for the αV integrin negative human tongue squamous cell carcinoma cell line H357 (38). This growth in suspension of the αV integrin negative epithelial tumor cell lines is in accordance with the reported inhibition of anoikis by absence of αV integrin expression. In concordance with the concept of αV integrin inactivation in squamous cell carcinoma, it could be speculated that bi-allelic loss of αV integrin is an early event in EAC carcinogenesis, contributing to the disorganizing of the stratified epithelium of the esophagus by uncoupling integrin-mediated adhesion and signaling.
Two overlapping HDs in cell line SK-GT-4 and xenograft P100x were located on chromosome 21q22.12, containing the *RUNX1* gene (Figure 3). This gene encodes a heterodimeric transcription factor that binds to core binding factor beta (CFBbeta), and regulates downstream genes that are important in development and differentiation, as well as in cancer (39). PCR analysis confirmed the deletions in SK-GT-4 and P100x, and screening of the series cell lines and xenografts showed an additional *RUNX1* HD in EAC cell line OE33. Re-evaluation of the SNP-array data affirmed this HD; the size of the deletion was so small that it not reached

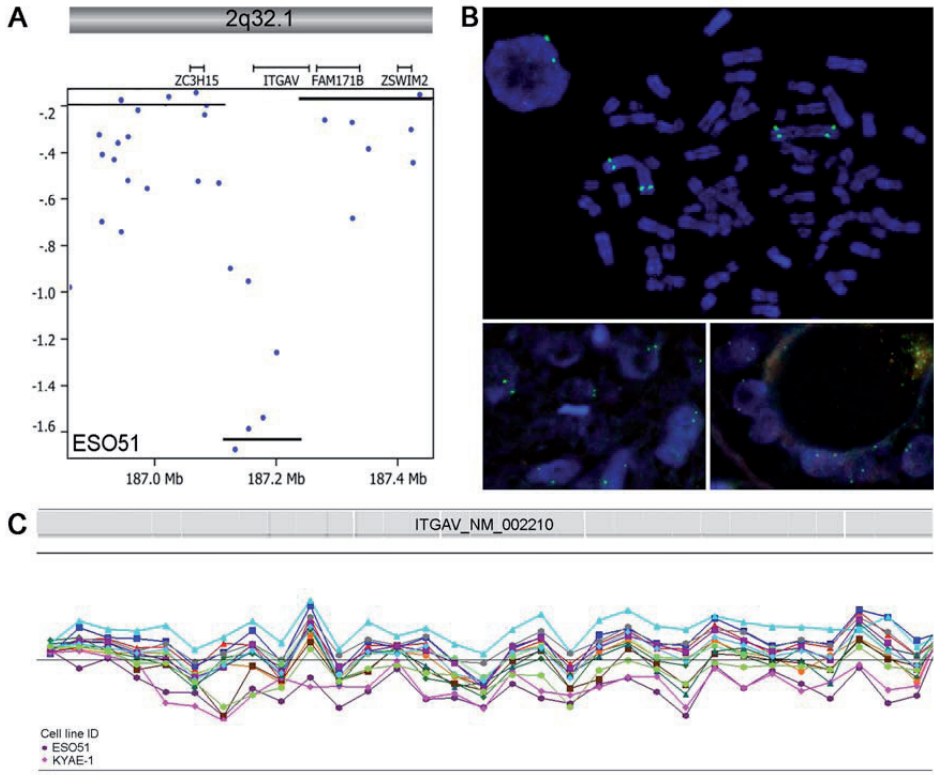


**Figure 2.** Analyses of a homozygous deletion on chromosome 2q32.1. *A*, Scatter plot of log2 copy number ratios (blue dots, left axis) for cell line ESO51 revealed a homozygous deletion containing a part of the *ITGAV* gene. *B*, FISH analysis on metaphase chromosomes and on the primary tumor of which cell line ESO51 is derived (left) demonstrated absence of the red-coloured probe (Fosmid G248P87126F7) which is located within the homozygous deletion. In the adjacent Barrett's epithelium (right) the red-coloured probe is present, which suggests that homozygous loss occurs late in EAC carcinogenesis. *C*, Expression analysis showed significantly reduced expression of *ITGAV* in cell lines ESO51 and KYAE-1.

the initial threshold for detection (the deletion encompassed <5-SNP probes). FISH analysis revealed the presence of the HD in cell line SK-GT-4 and in the primary tissue of P101x. The presence of HDs in two EAC cell lines and one xenograft, indicate the possible tumor suppressive role of *RUNX1* in EAC carcinogenesis. Sequence analysis of the remaining cell lines and xenografts did not reveal additional point mutations. It could be that *RUNX1* is silenced by promoter hypermethylation. This has been observed for the *RUNX1* family member *RUNX3*, which was found to be subject of methylation in gastric cancer carcinogenesis (40). Besides the role of *RUNX1* in human leukaemia, where functional disruption by chromosomal translocation and somatic point mutation commonly occur (41), little is known about the role of the *RUNX1* gene in epithelial cancer types. It has been demonstrated that *RUNX1* is significantly down-regulated in metastasis of different types of adenocarcinoma (42). Therefore it could

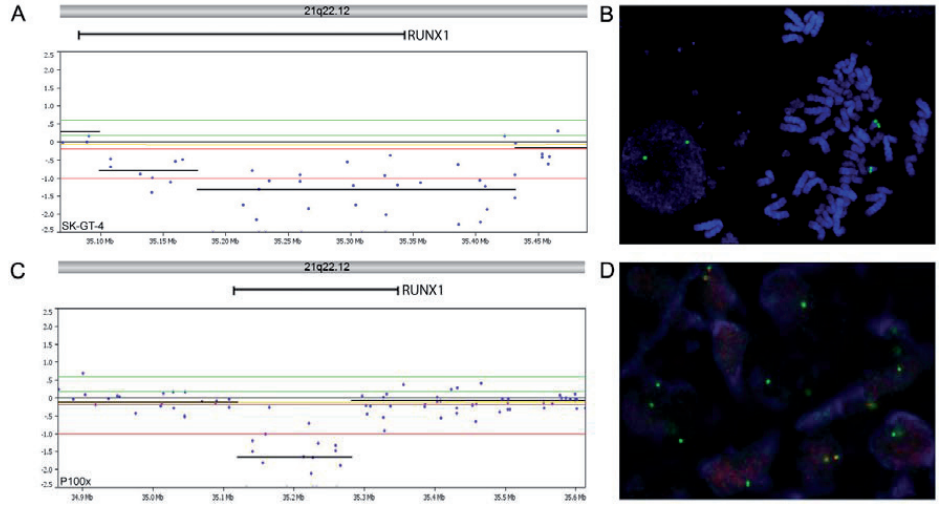


**Figure 3.** Analyses of a homozygous deletion on chromosome 21q22.12. *A and C*, Scatter plot of log2 copy number ratios (black dots, left axis) of cell line SK-GT-4 (A) and xenograft P100x (C) revealed a homozygous deletion that over spanned the *RUNX1* gene. *B and D*, FISH analysis on metaphase chromosomes of cell line SK-GT-4 (B) and on the primary tumor of which xenograft P100x is derived (D) demonstrated absence of the red-coloured probe (Fosmid G248P88378B1) which is located within the homozygous deletion.

be speculated that deletions of *RUNX1* in esophageal adenocarcinoma potentially contribute to the aggressive metastatic phenotype of these tumors.

A HD on chromosome 5q31.2 containing only the *CTNNA1* gene was detected in cell line OACP4C (Figure 4). Alpha-catenin, encoded by the *CTNNA1* gene, is known to anchor E-cadherin to the cytoskeletal actin bundle by binding the cadherin cytoplasmic domain (43). Additional gene-expression data showed a reduced expression of *CTTNA1* in cell lines OACP4C and ESO26. In the latter cell line, a single nucleotide deletion was found in exon 6 (c.850_delA/c.851_delA). However, this deletion mutation was not found in the primary tumor of cell line ESO26, suggesting that the mutation was acquired during *in vitro* culture or selection for cells harboring the *CTNNA1* gene mutation has occurred during establishment of the cell line (which represents only a minor population of the primary tumor cells). These findings are in line with reports in literature describing *CTNNA1* gene mutations predominantly in cell lines (44, 45).

In summary, this study reports on the identification of HDs in EAC cell lines and xenografts. Using these pure populations of cancer cells (verified EAC cell lines and xenografts) 22 new chromosomal regions were detected, harboring potentially new TSGs involved in EAC carcinogenesis. Here, we showed that the *ITGAV* and *RUNX1* gene are frequently affected by HDs, indicating a potential role in the carcinogenesis of EAC. Comprehensive genomic profiling such as presented will allow a more defined approach to identifying and characterizing genes involved in EAC carcinogenesis, offering the potential for improved clinical tests and treatments.

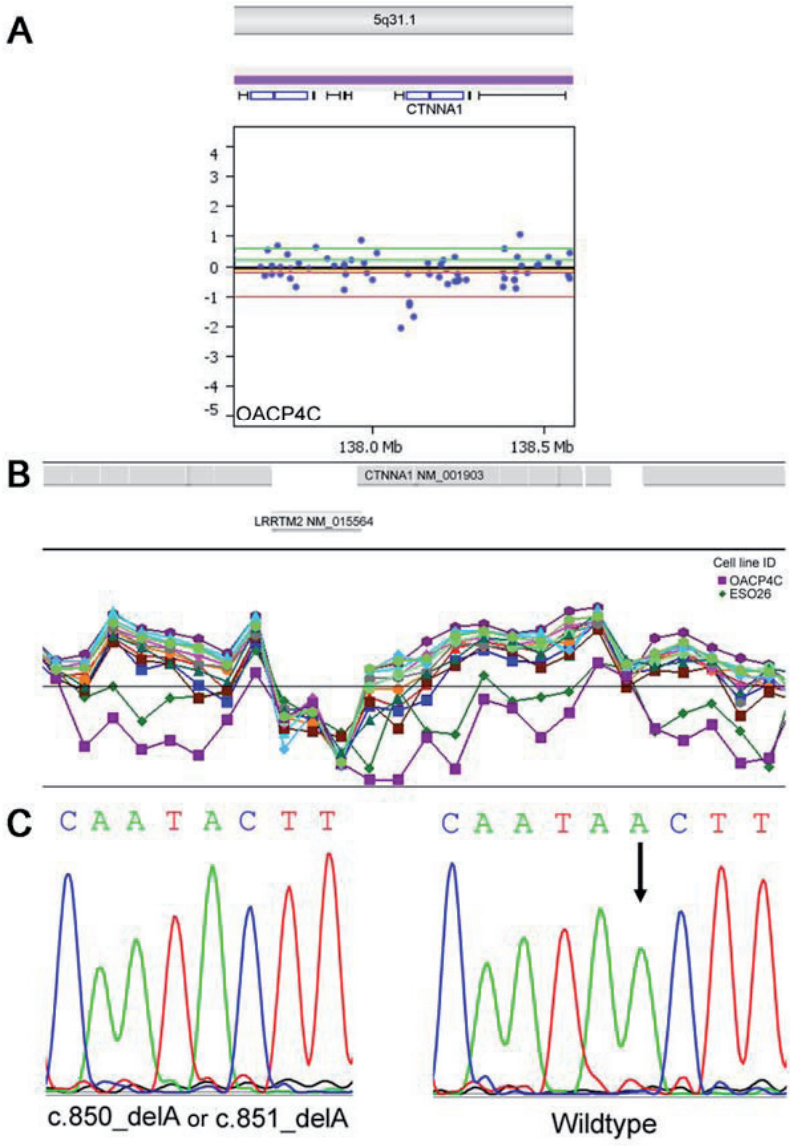


**Figure 4.** Analyses of a homozygous deletion on chromosome 5q31.2. *A*, Scatter plot of log2 copy number ratios (black dots, left axis) of cell line OACP4C showed a homozygous deletion within the *CTNNA1* gene. *B*, Expression analysis showed significantly reduced expression of *CTNNA1* in cell lines OACP4C and ESO26. *C*, Mutation analysis of cell line ESO26 showed a deletion of one base pair (c.850_delA or c.851_delA).

## ACKNOWLEDGEMENTS

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# PART II

## Clinical aspects of esophageal cancer treatment

# Chapter 5

## Chemotherapy followed by surgery in patients with carcinoma of the distal esophagus and celiac lymph node involvement

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## ABSTRACT

**Background:** Patients with carcinoma of the distal esophagus and metastatic celiac lymph nodes (M1a) have a poor prognosis and are often denied surgery. In this study, we evaluated our treatment strategy of chemotherapy followed by surgery in patients with M1a disease.

**Methods:** Thirty-eight patients who received chemotherapy for carcinoma of the distal esophagus with celiac lymph node involvement between 2000 and 2007 were identified from a prospective database. Clinical and histopathological responses to chemotherapy were analyzed and follow-up comprised review of medical charts.

**Results:** Twelve non-responding patients were not eligible for surgery. Twenty-six patients with partial responses or stable disease were operated on. The resectability rate was 96% (25/26) and tumor free resection margins (R0) were achieved in 68% (17/25). The overall survival of patients with M1a disease was 16 months. Patients who received chemotherapy alone had a median survival of 10 months; patients who underwent additional surgery had a median survival of 26 months (log-rank $p<0.001$).

**Conclusions:** The overall survival of patients with carcinoma of the distal esophagus and clinical celiac lymph node involvement is poor. Tumor free resection margins (R0) in M1a patients with clinical response to chemotherapy are likely to be achieved and contributes to prolonged survival.

## INTRODUCTION

Most patients with esophageal cancer have advanced disease at the time of diagnosis, because symptoms arise late in the course of the disease. The prognosis of patients with esophageal cancer is therefore poor (1,2). Lymphatic dissemination of a tumor in the distal esophagus occurs early and predominately to the paracardial lymph nodes, lymph nodes along the lesser curvature and to the celiac lymph nodes (CLNs) (3). According to the pTNM classification established by the UICC and the AJCC, lymph nodes around the celiac axis are considered non-regional nodes and as such classified as M1a disease (4). Controversy exists about the implications of positive CLNs. Some clinicians exclude these patients for curative treatment options, whereas others believe that these patients are still eligible for surgery (5-7). Multi-modality treatment has been suggested by some (8-12), but the optimal treatment remains unclear due to the paucity of clinical studies and lack of randomized trials. In our institution, patients with tumor-positive CLNs with a diameter of less than 1.5 cm are considered eligible for primary surgery (13). In patients with CLNs larger than 1.5 cm chemotherapy is considered with the aim of inducing tumor shrinkage followed by surgery with curative intent. The aim of this study was to evaluate the pathological and clinical outcome of this strategy in patients with carcinoma of the distal esophagus and CLN involvement.

## MATERIALS AND METHODS

The Erasmus University Medical Center is a tertiary referral center for patients with esophageal carcinoma in the Netherlands. Clinical and pathological data on all patients are collected and prospectively stored in a database. To investigate the hypothesis that a subset of patients with large celiac lymph nodes (>1.5cm) treated with chemotherapy do benefit from additional surgical resection, this database was scrutinized to identify patients with cancer of the distal esophagus who were deemed unresectable and received chemotherapy because of CLN involvement between the years 2000 and 2007. Because of the retrospective nature this study, ethical approval was not necessary.

Only patients with histological proven squamous cell carcinoma, adenocarcinoma, or undifferentiated carcinoma of the distal esophagus or gastro-esophageal junction (Siewert I/II), were included. For all patients, pretreatment staging according to TNM criteria (4), included a baseline upper gastrointestinal endoscopy, endoscopic ultrasonography (EUS), and computed tomography (CT) of the neck, chest and the upper abdomen. Lymph nodes within a 2 cm range of the celiac axis identified by means of EUS were considered malignant if one or more of the following criteria were met: hypo-echoic texture, sharp border, round shape and at least 1.0 cm in size. In addition, the largest diameter of the CLNs was measured and nodes larger than 1.0 cm detected by CT imaging were considered as malignant. Fine-needle

aspiration, PET scan or laparoscopic staging was incidentally used for assessment of the nodal status.

CLN >1 cm measured by CT scan or EUS are considered as malignant. Patients with CLNs 1.0-1.5 cm are deemed to be resectable, but CLNs >1.5cm are thought to hamper radical surgical resection and therefore this subset of patients received chemotherapy (Figure 1) (13). The chemotherapy regime consisted of paclitaxel 180 mg/m$^2$ and cisplatin 60 mg/m$^2$ in a biweekly schedule (14) or a combination of paclitaxel 100 mg/m$^2$ and carboplatin targeted at an AUC of 4 on days 1, 8, 15, 29, 36 and 43 (15). The planned number of treatment cycles was six. Toxicity was graded and reported using National Cancer Institute – Common Toxicity Criteria (NCI-CTC version 3). Tumor response, according the response evaluation criteria in solid tumors (RECIST) (16), was assessed after the third course of chemotherapy and included a CT scan of chest and abdomen. Patients with local disease progression or occurrence of distant metastasis received further palliative treatment. Patients showing response received three additional courses of chemotherapy and were then re-evaluated to determine tumor resectability. Surgery was proposed for patients that showed stable disease, partial or complete response.

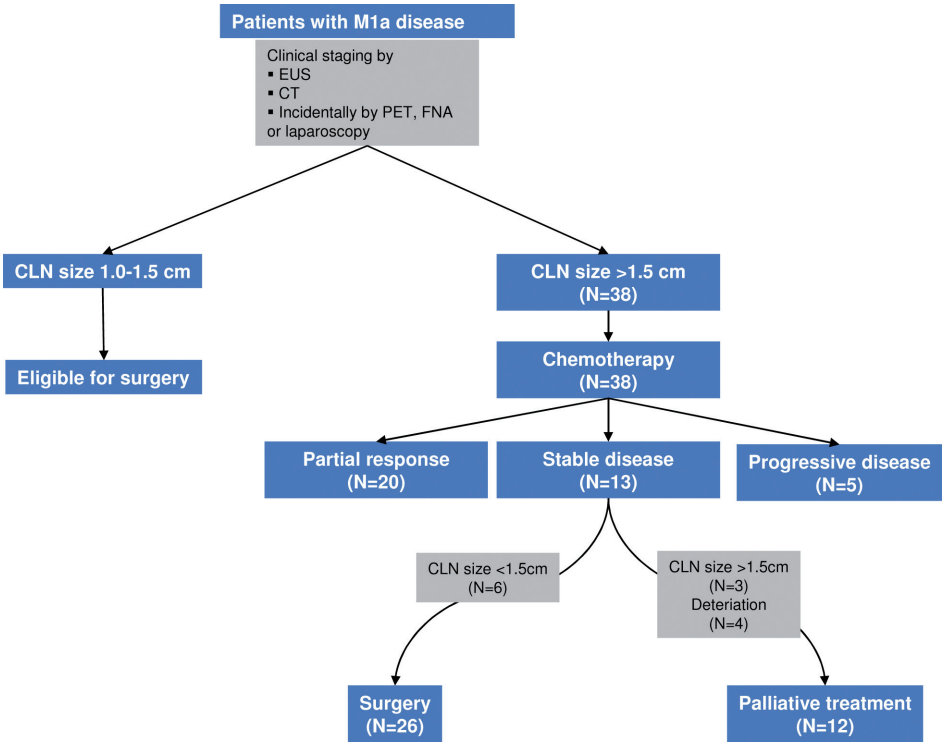



**Figure 1.** Flowchart of patients with clinical M1a disease treated with chemotherapy or chemotherapy followed surgery. CLN >1 cm are considered as malignant. Patients with CLNs 1.0-1.5 cm are deemed to be resectable, but CLNs >1.5cm are thought to hamper radical surgical resection and therefore this subset of patients received chemotherapy.

In patients without enlarged lymph nodes in the mediastinum, a transhiatal esophagectomy (THE) was preferred (17). In patients with suspicious lymph nodes in the chest, a transthoracic resection (TTE) with two-field lymphadenectomy was performed (18). The origin of the left gastric artery was routinely marked by the surgeon in the resection specimen and nodes within 2 cm of this location were marked as CLNs. The continuity of the digestive tract was restored by means of a gastric tube reconstruction with a cervical anastomosis.

Resections were considered radical if microscopically examination revealed no tumor tissue at or less than 1 mm from the circumferential, proximal, or distal margins. Pathological staging was done according to the UICC 6th edition (4). In addition, histomorphological response of solely the primary tumor to chemotherapy, was assessed and classified into four categories, grade I: more than 50% vital residual tumor cells; grade II: 10–50% vital residual tumor; grade III: less than 10% vital residual tumor cells; grade IV: complete tumor regression, no evidence of vital tumor cells (19).

Data on follow-up were collected from the prospective database and the medical charts. All patients were followed at an interval of three to four months during the first year, every six months for the second year, and annually for up to 5 year post surgery. Recurrence of disease was diagnosed on clinical grounds. However, whenever a relapse was suspected, radiologic, endoscopic, or histologic confirmation was sought. Recurrent disease was classified as local–regional (occurring in the upper abdomen or mediastinum) or distant (including cervical recurrences).

**Statistical analysis**

Statistical differences were tested using the Fisher's exact probability test for categorical variables and the t test for continuous variables. All statistical tests were two-sided. Statistical significance was set at the 5% level. Survival and follow-up were calculated from the start of chemotherapy to the date of death or the last date of follow-up (1st May 2008) at which point the data were censored. The probability of survival over time was estimated with a Kaplan-Meier curve and the log-rank test was used to determine statistical differences in survival.

## RESULTS

Between January 2000 and January 2007, 38 patients underwent chemotherapy for carcinoma of the distal esophagus with CLN involvement. These patients were not eligible for primary surgery because of large celiac lymph nodes (>1.5 cm). The baseline characteristics of these of patients are listed in Table 1. All 38 patients were staged as cT2-3N0-1M1a. In 31 patients complete EUS staging could be achieved, in five patients the celiac region could not be inspected by EUS due to tumor obstruction and two patients underwent no EUS for unknown reasons. EUS guided FNA was introduced halfway 2004 in our institute. Of the 20

**Table 1.** Patient characteristics

| Characteristics | All Patients (N=38) | CTX (N=12) | CTX + Surgery (N=26) | P-Value (CTX vs CTX + Surgery) |
|---|---|---|---|---|
| Gender | | | | *ns* |
| Female | 4 (11%) | 0 (0%) | 4 (15%) | |
| Male | 34 (89%) | 12 (100%) | 22 (85%) | |
| Age (years) | | | | *ns* |
| Median | 61 | 60 | 62 | |
| Range | 44 - 73 | 50 - 70 | 44 - 73 | |
| Histology | | | | *ns* |
| Adenocarcinoma | 31 (82%) | 10 (83%) | 21 (81%) | |
| Squamous-cell carcinoma | 5 (13%) | 2 (17%) | 3 (12%) | |
| Undifferentiated carcinoma | 2 (5%) | 0 (0%) | 2 (7%) | |
| Histological grade | | | | *ns* |
| Well differentiated | 1 (3%) | 0 (0%) | 1 (3%) | |
| Moderately differentiated | 9 (24%) | 1 (8%) | 8 (31%) | |
| Poorly differentiated | 21 (55%) | 8 (67%) | 13 (50%) | |
| Undifferentiated | 2 (5%) | 0 (0%) | 2 (8%) | |
| Unknown | 5 (13%) | 3 (25%) | 2 (8%) | |
| Tumor length (cm) | | | | *ns* |
| Median | 13 | 7 | 6 | |
| Range | 2 - 15 | 2 - 14 | 2 - 15 | |

Abbreviations: CTX, Chemotherapy; EUS, Endoscopic ultrasound; *ns*, not significant

patients staged after May 2004, nine underwent EUS-guided FNA. Cytology of the aspirate was positive for tumor cells in seven patients and was inconclusive in two patients. Diagnostic laparoscopy with histological confirmation of CLN involvement was done in two patients because of incomplete EUS staging; another patient did not receive EUS but underwent a diagnostic laparoscopy in another hospital before being referred to our institution. (Table 2)

**Chemotherapy**

Thirty-five patients were treated with a combination of paclitaxel and cisplatin; three patients received a combination of paclitaxel and carboplatin as part of a dose finding study (15). The mean and median number of treatment cycles was 5.4 and 6 (range 2-8), respectively. Twenty-five patients (66%) received the planned dose and cycles of chemotherapy. In seven patients, who received chemotherapy without additional surgery (N=12), the number of treatment cycles was reduced because of disease progression (N=4), nephrotoxicity (N=1) and clinical deterioration (N=2). Five patients, who received chemotherapy followed by additional surgery (N=26), did not complete the planned number of treatment cycles because nephrotoxicity (N=1), grade III sensory neuropathy (N=2) and clinical deterioration (N=2). One patient received eight treatment cycles. In two patients, the chemotherapy regime was switched from a cisplatin to a carboplatin regime, because of severe nephrotoxicity. Grade III or IV hematological toxicity consisted of leucopenia in five patients (13%) and neutrope-

**Table 2.** Pre-treatment staging of patients with clinical suspected celiac lymph node involvement

| Characteristics | All Patients (N=38) | CTX (N=12) | CTX + Surgery (N=26) |
|---|---|---|---|
| Staging modality | | | |
| CT | 38 (100%) | 12 (100%) | 26 (100%) |
| EUS | 36 (95%) | 12 (100%) | 24 (92%) |
| PET | 1 (3%) | 0 (0%) | 1 (4%) |
| FNA | 9 (24%) | 4 (33%) | 5 (19%) |
| Laparoscopy | 3 (8%) | 1 (8%) | 2 (7%) |
| cTNM stage | | | |
| cT stage | | | |
| T2 | 1 (3%) | 0 (0%) | 1 (4%) |
| T3 | 37 (97%) | 12 (100%) | 25 (96%) |
| cN stage | | | |
| N0 | 5 (13%) | 0 (0%) | 5 (19%) |
| N1 | 33 (87%) | 12 (100%) | 21 (81%) |

Abbreviations: CTX, Chemotherapy; CT, Computed tomography; EUS, Endoscopic ultrasound; PET, Positron emission tomography; FNA, Fine needle aspiration

nia in 24 patients (63%). One patient received anti-coagulant drug treatment because of a thrombo-embolic event. The most important non-hematological complication was a grade I-II sensory neuropathy, observed in 10 patients (26%). There was no mortality.

### Clinical response assessment

Overall clinical response evaluation showed no complete responses; 20 patients had a (53%) partial response; 13 (34%) showed stable disease and five (13%) had progressive disease. Response measurement of solely the celiac lymph nodes revealed complete disappearance in five patients, partial response (30% decreases in celiac lymph node size) in 14 patients, and in 16 patients the nodes were unchanged or progressed in size. In three patients, who underwent a diagnostic laparoscopy with complete removal of the celiac lymph node, objective regression of the lymph node could not be measured.

### Surgery

Twelve patients (32%) were not referred for additional surgery because of progressive disease (N=5), stable disease with enlarged celiac lymph nodes size >1.5 cm (N=3) or clinical deterioration (N=4). Twenty-six patients (68%) were selected for surgical resection, 20 patients had partial response and six showed stable disease (Figure 1). Except two patients, all had celiac lymph nodes <1.5 cm.

Twenty-two (84%) patients underwent THE, and three (12%) TTE. One patient (4%) had liver metastases at laparotomy and no resection was performed. Of the 25 patients who underwent surgical resection with curative intent, 17 patients (68%) had a R0 resection and eight patients (32%) had a R1 resection. The mean number of lymph nodes that were harvested

was 14 (range 1-32). The in-hospital mortality rate was 4%. The most frequent complications were of pulmonary origin (24%), followed by anastomotic leak (19%).

**Pathology**

Histomorphological analysis of the primary tumor showed a regression grade IV in four patients (16%), a regression grade III in three patients (12%), a regression grade II in one patient (4%), and 17 patients (68%) had a histopathological regression grade I (Table 3). All patients with regression grade II-IV of the primary tumor, except one who had a diagnostic laparoscopy with complete removal of the CLN, had signs of tumor regression in their CLNs. Only one patient with regression grade III of the primary tumor had still vital tumor cells present in the CLNs.

Thirteen resection specimens contained tumor positive CLNs; twelve resection specimens had tumor negative CLNs. Signs of histomorphological regression within the tumor negative CLNs were observed in six patients (who also showed regression of the primary tumor). Histomorphological regression was mainly characterized by extensive fibrosis, mucin lakes and presence of macrophages. Four patients had tumor negative CLNs without signs of regressed tumor tissue. Two patients, who underwent a diagnostic laparoscopy with complete removal of the CLN, were staged pathologically M0, but objective regression of tumor within these lymph nodes could not be measured. In four of 13 resection specimens with tumor positive CLNs, signs of tumor regression in the CLN were observed.

After chemotherapy, in five patients the celiac lymph nodes completely disappeared. Of these, two had tumor-positive CLNs without signs of regression in their resection specimens and three showed tumor-negative CLNs with histomorphological regressed tumor tissue.

**Survival and pattern of failure**

The median survival of all 38 patients was 16 months (Figure 2). At the time of analysis, 12 of 38 patients are alive. The median follow-up time of surviving patients was 55 months (range 34-97 months). None of the patients who received chemotherapy alone survived more than two years (Figure 3). Patients who underwent additional surgery had a median survival of 26 months; the 5-years survival was 19%. Patients with a R1 resection (N=8) or positive CLNs in the resection specimen (N=13) had similar survival curves as those who received chemotherapy alone (N=13) (Figure 4 and 5). The group of patients with histopathological response grade II-IV (N=8) had a significant better survival as compared to the group of patients with a histopathological response grade I (N=17) (log-rank $P$=0.025).

Most patients with M1a disease that were alive had a radical surgical resection (R0), had complete or major response to chemotherapy (Grade II-IV) and had no tumor positive CLNs in their resection specimens (Table 3). Recurrent disease after surgery was found in 11 patients; three had distant metastases and eight had both loco-regional recurrence and distant metastasis. One patient died due to surgical complications; one patient died because of unknown

reasons. Loco-regional recurrences were mediastinal lymph node metastases, retroperitoneal lymph node metastases and recurrences in the gastric tube. Distant metastases were found in liver, brain, adrenal gland and bone.

**Table 3.** Pathological characteristics of patients with clinical suspected celiac lymph node involvement treated with chemotherapy and additional surgical resection

| Study No. | Pre-treatment CLNs stage | pTNM stage | Number of involved | Resection | Regression grade | Tumor regression signs |
|---|---|---|---|---|---|---|
| | by FNA or Laparoscopy | | lymph nodes | margins | primay tumor | in CLNs |
| 1 | ND | T0N0M0 | 0 / 16 | R0 | IV | Yes |
| 2 | Laparoscopy | T3N0M0 | 0 / 7 | R0 | I | Prior laparoscopy |
| 3 | ND | T3N1M1a | 13 / 13 | R1 | I | No |
| 4 | Laparoscopy | T2N0M0 | 0 / 6 | R0 | III | Prior laparoscopy |
| 5* | ND | T3N0M0 | 0 / 8 | R0 | I | No |
| 6 | ND | T0N0M0 | 0 / 1 | R0 | IV | Yes |
| 7 | ND | T3N1M1a | 16 / 18 | R1 | I | No |
| 8* | ND | T3N1M0 | 3 / 6 | R0 | I | No |
| 9 | ND | T3N1M1a | 3 / 14 | R0 | I | No |
| 10 | ND | T0N0M1a | 3 / 11 | R0 | IV | Yes (still vital tumor cells present) |
| 11 | ND | T3N1M1a | 8 / 17 | R1 | I | No |
| 12 | ND | T1N0M0 | 0 / 18 | R0 | III | Yes |
| 13 | ND | T0N0M0 | 0 / 14 | R0 | IV | Yes |
| 14 | ND | T3N1M0 | 1 / 16 | R1 | II | Yes |
| 15* | FNA | T3N0M0 | 0 / 14 | R0 | I | No |
| 16 | FNA | T3N1M1a | 14 / 24 | R0 | I | No |
| 17 | FNA | T3N1M1a | 11 / 14 | R0 | I | Yes (still vital tumor cells present) |
| 18 | ND | T3N1M1a | 3 / 8 | R0 | I | Yes (still vital tumor cells present) |
| 19* | ND | T3N1M0 | 1 / 26 | R0 | I | No |
| 20 | FNA | T2N0M0 | 0 / 11 | R0 | III | Yes |
| 21 | ND | T3N0M1a | 1 / 9 | R0 | I | No |
| 22 | ND | T3N1M1a | 11 / 32 | R1 | I | Yes (still vital tumor cells present) |
| 23 | FNA† | T3N1M1a | 6 / 11 | R1 | I | No |
| 24 | ND | T3N1M1a | 13 / 16 | R1 | I | No |
| 25 | ND | T3N1M1a | 3 / 14 | R1 | I | No |

Abbreviations: CLN, Celiac lymph node; FNA, Fine needle aspiration ; ND, Not determined by FNA or laparoscopy

* Patients with tumor negative CLNs and without any signs of histomorphological regressed tumor tissue, indicating that these patients had possibly no celiac lymph node involvement pre-treatment
† FNA was not conclusive

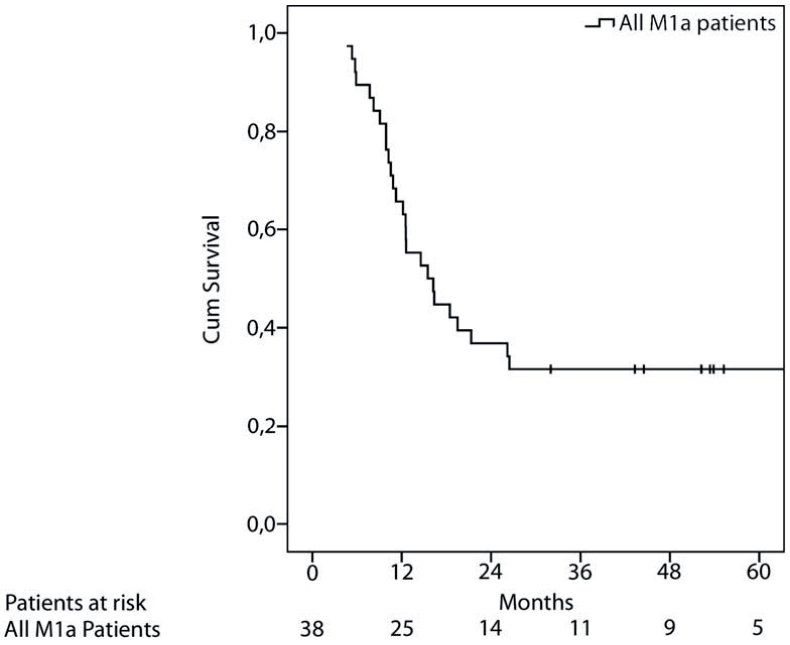



**Figure 2.** Overall survival for patients with clinical suspected celiac lymph node involvement (N=38) treated with chemotherapy or chemotherapy followed by surgery.

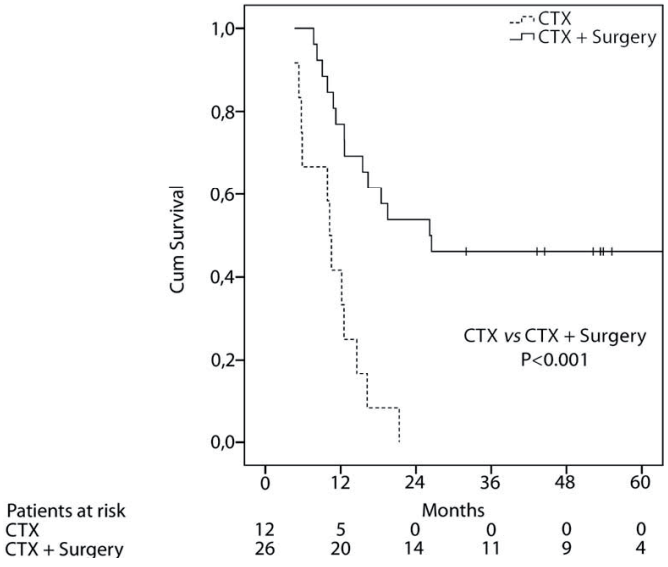
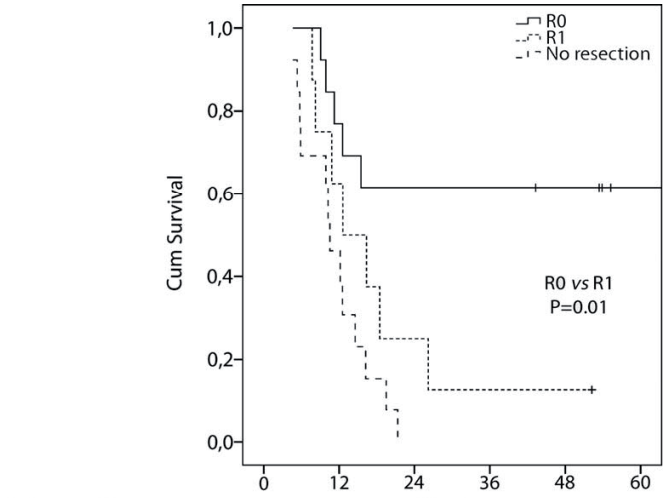
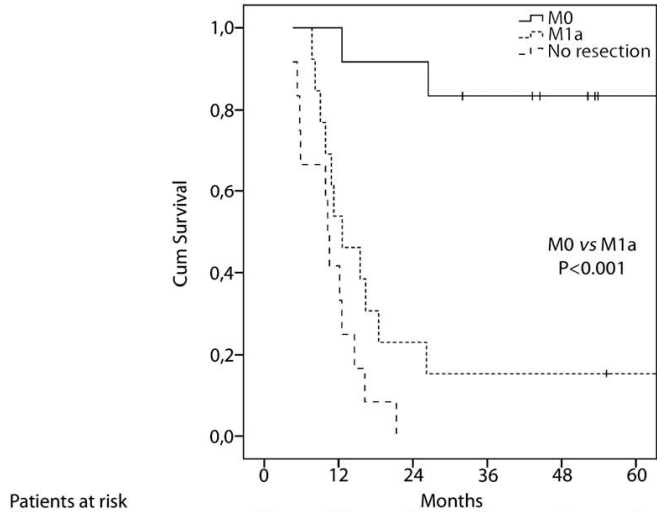



**Figure 3.** Kaplan-Meier survival curves for patients who received chemotherapy alone (N=12), and for patients who received chemotherapy and surgery (N=26).




| Patients at risk | | | | | | |
|---|---|---|---|---|---|---|
| Radical resection (R0) | 17 | 14 | 12 | 10 | 8 | 5 |
| No radical resection (R1) | 8 | 5 | 2 | 1 | 1 | 0 |
| No resection | 13 | 6 | 0 | 0 | 0 | 0 |

**Figure 4.** Kaplan-Meier survival curves of patients who underwent chemotherapy followed by radical surgical resection (N=17), patients who underwent R1 resection (N=8) and for patients who underwent no surgical resection (N=13).




| Patients at risk | | | | | | |
|---|---|---|---|---|---|---|
| M0 | 12 | 12 | 11 | 9 | 7 | 4 |
| M1a | 13 | 7 | 3 | 2 | 2 | 1 |
| No resection | 13 | 6 | 0 | 0 | 0 | 0 |

**Figure 5.** Kaplan-Meier survival curve for patients with no positive CLNs in the resection specimen (N=12; blue line), patients with positive CLNs in the resection specimen (N=13) and for patients who underwent no surgical resection (N=13).

**Table 4.** Characteristics of patients with clinical suspected celiac lymph node involvement, alive or death after chemotherapy and additional surgical resection

| Characteristics | Alive (N=12) | Death (N=13) | P value (Alive vs Death) |
|---|---|---|---|
| *Pre-treatment* | | | |
| Gender | | | *ns* |
| Women | 1 (8%) | 3 (23%) | |
| Man | 11 (92%) | 10 (77%) | |
| Age (years) | | | *ns* |
| Median | 58 | 63 | |
| Range | 44 - 69 | 48 - 73 | |
| Histology | | | *ns* |
| Adenocarcinoma | 9 (75%) | 11 (84%) | |
| Squamous-cell carcinoma | 2 (17%) | 1 (8%) | |
| Mixed or other | 1 (8%) | 1 (8%) | |
| *Pathological staging* | | | |
| Resection margins | | | 0.03 |
| R0 | 11 (92%) | 6 (46%) | |
| R1 | 1 (8%) | 7 (54%) | |
| Histomorphological regression | | | 0.011 |
| Grade I | 5 (42%) | 12 (92%) | |
| Grade II-IV | 7 (58%) | 1 (8%) | |
| Celiac lymph node status | | | 0.001 |
| M0 | 10 (83%) | 2 (15%) | |
| M1a | 2 (17%) | 11 (85%) | |
| Time of follow-up (months) | | | |
| Median | 55 | 13 | |
| Range | 32 - 96 | 8 - 26 | |

Abbraviations: *ns*, not significant

## DISCUSSION

Surgical treatment of patients with carcinoma of the distal esophagus with suspected celiac lymph node involvement is controversial. Radical resection of the primary tumor together with a radical lymph node clearance of the celiac axis is deemed impossible and the patient is denied surgery. In this study, we analyzed the results of 38 patients selected for chemotherapy followed by surgery in 26 patients, who showed clinical response to chemotherapy. The prognosis after chemotherapy followed by surgery is largely determined by tumor free resection margins, pathological response of the primary tumor and the CLN status (Table 3). Radical surgical resection (R0) in this selected group of patients was achieved in 68% (17/25). This percentage is comparable to the complete resection rates in patients with primary resectable esophageal cancer in larger series (18,20). The importance of radical surgical resection

is reflected by the group of patients who underwent a R1 resection (tumor tissue at or less than 1 mm from the circumferential, proximal, or distal margins). The median survival rate of these patients (N=8) was 11 months, which is comparable with the median survival rate of 12 months in patients who received chemotherapy alone. In contrast, the median survival in patients with a R0 has not been reached yet. Pathological response to chemotherapy is associated with long term survival; seven of eight patients (with grade II to IV pathological response) are alive after a median follow-up of 55 months. These results are comparable with another studies, in which patients who showed complete and or major pathological response to preoperative chemotherapy had 5 years survival rates between 60- 89% (21,22). Another important prognostic factor is the pathological CLN status. Patients with tumor positive CLNs in the resection specimen (N=12) had a median survival of 18 months. This is in concordance with survival rates of 14 and 23 months as described in literature, in patients with primary resectable esophageal cancer who underwent surgical resection and had microscopically positive celiac lymph nodes in the resection specimen (23,24). A recent study from our institution showed a median survival of 13 months and a 5-year survival of 8% in a cohort of 55 patients with M1a disease treated with surgery alone (23).

Twelve patients had tumor negative celiac lymph nodes, of which 4 patients had tumor negative CLNs without any signs of histomorphological regressed tumor tissue, indicating that these patients were possibly incorrectly staged as M1a. Of these patients, one patient did not underwent EUS-staging; in one patient the celiac region could not be inspected by EUS due to tumor obstruction; one patient underwent EUS-guided FNA with cytological confirmation but possibly a local-regional lymph node has been evaluated; in one patient, the involved lymph node was located adjacent to the primary tumor but within 2cm of the celiac artery, therefore, this lymph node was probably misidentified as CLN. Patients with complete response of the primary tumor or tumor negative CLNs with signs of tumor regression (N=8) had a significant better survival as compared to the rest of the patients (N=17) (log-rank $P$=0.025).

In this study, patients with clinical response to chemotherapy and CLNs <1.5 cm were deemed eligible for surgery. Obviously, there is a large discrepancy between clinical and pathological response assessment to chemotherapy. CT scan is not accurate in determining the response to chemotherapy; this is probably related to the difficulty in the differentiation between viable tumor and reactive changes, including edema and scar tissue (25). Whereas the CLN status is as important as the response of the primary tumor on clinical outcome after surgery, assessment of the celiac lymph node status after chemotherapy by EUS guided FNA or diagnostic laparoscopic could improve selection of patients that are likely benefit of additional surgical resection (26).

To our knowledge, this is the first study that reports on patients with M1a disease who received chemotherapy followed by surgery. Most studies reported on small series of patients with M1a disease who receive chemoradiotherapy eventually followed by surgery. One of the

first reports, by Christie et al (8), described the results of preoperative chemoradiotherapy in four patients with clinically M1a disease. Two patients with a complete pathological response were alive after 2 years of follow-up. One of the four patients died because of disease recurrence and another died without evidence of recurrence. In another study, 18 patients with M1a disease were treated with chemotherapy followed by definitive chemoradiotherapy (11). After completion of concurrent chemoradiotherapy, 9 patients had a clinical complete response, five showed a partial response, two had stable disease, one progressive disease, and one was not assessed. The median overall survival rate was 13.8 months, and the 2 years survival rate was only 18.6%. In a study reported by Malaisrie et al. (9), 9 patients were treated with chemoradiotherapy followed by surgery and 9 patients received chemotherapy before concurrent chemoradiotherapy and surgery; the median survival was 9 and 26 months, respectively. Frizell et al. (10) reported a 2-year survival rate of 54% in 13 patients with clinically involved celiac lymph nodes, who received a variety of treatment modalities including chemoradiotherapy alone and chemoradiotherapy followed by surgery. Eight patients developed distant disease recurrence, and one had both local and distant disease recurrence. In our opinion, the role of radiotherapy in patients with a carcinoma of the distal esophagus and CLN involvement is limited. For adequate radiotherapy a large radiation field is needed that also includes a major part of stomach that later on is needed for gastro-intestinal tract reconstruction. In addition, adequate radiation doses are limited by inclusion of the abdominal viscera (27). A multi-center randomized trial is needed to compare chemotherapy with or without radiotherapy followed by surgery in responding M1a patients.

This study has several limitations. In the majority of patients (N=28) the cytological or histological confirmation of CLN involvement before the start of treatment is lacking and therefore CLN involvement could be questioned. However, CLNs larger than 1.5 cm do have a high a priori chance that the lymph node harbor malignant cells (28). Furthermore, pathological examination of the resection specimens revealed in all, except four, tumor positive CLNs or tumor negative CLN with signs of tumor regression, indicating a diagnostic accuracy of pre-treatment CLN involvement of 85%. The small number of patients, the nonrandomized setting and the retrospective nature of this study limits our ability to reach definitive conclusions, because many factors can contribute to improved survival in patients treated with chemotherapy followed by additional surgery.

## CONCLUSIONS

Despite these limitations, this study confirmed that patients with carcinoma of the distal esophagus and CLN involvement have a poor prognosis. Radical esophagectomy in M1a patients with clinical response to chemotherapy is likely to be achieved and contributes to prolonged survival. Besides radical surgical resection, complete or major pathological re-

sponse to chemotherapy and a negative CLN status in the resection specimen are associated with long-term survival. Assessment of the CLN status after chemotherapy by EUS-guided FNA could be useful to further optimize selection of M1a patients who are most likely to benefit from additional surgery.

## ACKNOWLEGDEMENTS

We would like to acknowledge C.M. Vollebregt-Uiterwijk and N. Wijffels for assistance in collecting the data.

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# Chapter 6

## Chemotherapy followed by surgery versus surgery alone in patients with resectable esophageal squamous cell carcinoma: long-term results of a randomized controlled trial

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## ABSTRACT

**Introduction:** This is a randomized, controlled trial of preoperative chemotherapy in patients undergoing surgery for esophageal squamous cell carcinoma (ESCC). Patients were allocated to chemotherapy, consisting of 2-4 cycles of cisplatin and etoposide, followed by surgery (CS group) or surgery alone (S group). Initial results reported only in abstract form in 1997, demonstrated an advantage for overall survival in the CS group. The results of this trial have been updated and discussed in the timeframe in which this study was performed.

**Methods:** This trial recruited 169 patients with ESCC, 85 patients assigned to preoperative chemotherapy and 84 patients underwent immediate surgery. The primary study endpoint was overall survival (OS), secondary endpoints were disease free survival (DFS) and pattern of failure. Survival has been determined from Kaplan-Meier curves and treatment comparisons made with the log-rank test.

**Results:** There were 148 deaths, 71 in the CS and 77 in the S group. Median OS time was 16 months in the CS group compared with 12 months in the S group; 2-year survival rates were 42% and 30%; and 5-year survival rates were 26% and 17%, respectively. Intention to treat analysis showed a significant overall survival benefit for patients in the CS group (*P*=0.03, by the log-rank test; hazard ratio [HR] 0.71; 95%CI 0.51-0.98). DFS (from landmark time of 6 months after date of randomisation) was also better in the CS-group than in the S group (*P*=0.02, by the log-rank test; HR 0.72; 95%CI 0.52-1.0). No difference in failure pattern was observed between both treatment arms.

**Conclusions:** Preoperative chemotherapy with a combination of etoposide and cisplatin significantly improved overall survival in patients with ESCC.

## INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) accounts for most cases of esophageal cancer worldwide (1, 2). Even after complete surgical dissection, the prognosis of patients with ESCC is poor, with 5-year survival rates of 20 to 30%. Factors that contribute to this dismal prognosis include presence of locally advanced disease and undetected metastatic cancer at diagnosis. Because of the high rates of locoregional and distant failure, there is much interest in the combination of systemic chemotherapy and local surgical treatment.

The potential benefits of preoperative chemotherapy include increasing the likelihood of curative resection by downstaging the tumor and rapidly improving tumor-related symptoms. It is also been thought that systemic chemotherapy could contribute to the eradication of micrometastases and circulating tumor cells. More recently, the importance of systemic disease control has been emphasized by new insights in the metastasizing process of cancer (3). For decades, the dissemination of cancer has been considered the final stage in a deteriorating process. Now, there is accumulating evidence that dissemination already can occur at an early stage of the disease (4). In theory, the use of preoperative chemotherapy may therefore have a positive impact on survival of patients with esophageal cancer. Here, we report the design and long-term results of a randomized controlled trial in patients with resectable ESCC, comparing preoperative chemotherapy with cisplatin and etoposide followed by surgery to surgery alone.

## MATERIALS AND METHODS

All eligible patients had histologically confirmed squamous cell carcinoma of the intra-thoracic esophagus. Patients were deemed resectable if the disease was clinically limited to the locoregional area (tumor stage 1, 2 or 3; any nodal stage and no metastases). Patients with carcinoma of the distal esophagus and suspected celiac lymph nodes involvement (M1a) were also considered eligible for surgery. Patients had to be below 80 years of age, in adequate physical condition (Karnofsky score >70) to undergo surgery and had to have adequate hepatic, renal and bone marrow function. Exclusion criteria were synchronous cancer, tumor localization in the cervical esophagus (upper border, <18 cm from the incisor teeth), severe cardiovascular or pulmonary disease. Patients with previous malignancies were eligible if more than 5 years had elapsed from diagnosis without evidence of tumor recurrence; exceptions were made for adequately treated basal cell cancer of the skin or carcinoma *in situ* of the cervix. Preoperative work-up included clinical examination, esophago-gastroscopy with biopsies, chest radiography, external ultrasonography of the cervical and upper abdominal region and computed tomography (CT) of the chest and abdomen. Radionuclide bone scans

were performed if indicated. Bronchoscopy was performed when the primary tumor was adjacent to the trachea or main stem bronchus and invasion was suspected.
Central randomization took place at the Erasmus University Medical Center in Rotterdam (by trial coordinator TCK). Random assignment was stratified by age (<50; 51-60; >60), gender (male; female), weight loss (kg) in the past four months (0-5; 6-10; >10) and length of the tumor (cm) as measured by esophago-gastroscopy (1-3; 4-6; 7-10; >10). Patients assigned to preoperative chemotherapy were treated with two cycles, followed by a clinical response evaluation. Response evaluation was done three to four weeks after the last cycle of chemotherapy. Clinical response after chemotherapy was evaluated by esophago-gastroscopy and CT of the chest and abdomen. Tumor responses were assessed according to the World Health Organization (WHO) criteria (5). Complete absence of any evidence of malignant disease, including negative biopsies from the former tumor area, was defined as complete response (CR). Partial response (PR) was defined as >50% reduction of tumor bulk, without the appearance of new lesions. Stable disease (SD) was defined as <50% reduction of tumor bulk, without the appearance of new lesions. Progressive disease (PD) was defined as >25% progression of tumor bulk or the appearance of new lesions. Patients with complete or partial responses received two additional courses of chemotherapy, whereas non-responding patients (stable disease or progressive disease) were referred for immediate surgery. Patients with progressive disease (T4 or M1 disease) were treated palliative and observed for survival. Patients, who were randomly assigned to undergo surgery alone, underwent the operation as soon as possible. Patients who received chemotherapy were operated 4 to 6 weeks after the last treatment cycle. The study protocol was approved by the ethics committee of all participating institutions and written informed consent was obtained from all patients.

**Chemotherapy**

Cisplatin, at a dose of 80 mg/m$^2$, was given intravenously over 4 hours on day one of each cycle preceded and followed by adequate hydration. Etoposide, at a dose of 100 mg/m$^2$, was administered intravenously over 2 hours on day 1 (before cisplatin) and day 2, followed by etoposide 200 mg/m$^2$ orally on days 3 and 5. This course was repeated in week 4. In case of clinical response, two subsequent courses of chemotherapy were administered in week 8 and 11. All patients received prophylactic anti-nausea treatment with 5-HT 3 receptor antagonists during chemotherapy. Treatment related toxicities were measured according to the WHO recommendations (5). Re-treatment with the next cycle was permitted only if the absolute neutrophil count was at least 3,500/mm$^3$, and the platelet count was at least 100,000/mm$^3$. A delay of treatment of up to 2 weeks was permitted. In patients with severe toxic renal or neurological effects (≥ WHO grade 3) chemotherapy was stopped and patients were referred for surgery.

**Surgery and pathological examination**

For carcinomas of the upper half of the intra-thoracic esophagus a right-sided thoracotomy was performed. For carcinomas of the lower half of the intra-thoracic esophagus a transhiatal esophagectomy was done. The tumor and its adjacent lymph nodes were dissected *en bloc*. The left gastric artery was transected at its origin, with resection of local lymph nodes. The continuity of the digestive tract was restored by means of gastric tube reconstruction or colonic interposition with a cervical anastomosis. The tumor stage after resection was classified according to the TNM classification of the International Union Against Cancer (6). Resections were classified as radical when microscopical examination revealed all margins to be free of tumor (R0). Resections were considered not radical, if microscopically examination showed tumor-positive circumferential margin (R1) or presence of macroscopic disease (R2).

**Follow-up**

All patients were followed at an interval of three to four months during the first year, every six months for the second year, and annually for up to 5 years post surgery. After 5 years, follow-up data were obtained by telephone from the patient or his/her family practitioner. Recurrence of disease was diagnosed on clinical grounds. However, whenever a relapse was suspected, radiologic, endoscopic, or histologic confirmation was sought for.

Loco-regional disease recurrence was defined as relapse at the primary site including the anastomosis or in regional lymph nodes. Distant disease recurrence was defined as distant lymph node sites or involvement of distant organs including lung, liver, bone, and subcutaneous tissue.

**Statistical analysis**

The planned number of patients to be entered in the study was 80 for each treatment arm. With these numbers of patients the statistical power should be sufficient (power = 0.8; significance 0.05) to detect an increase of the median survival from 10 to 18 months.

Overall survival (OS) was calculated from the date of random assignment to date of death from any cause and surviving patients were censored at the date they were last known to be alive. Disease-free survival (DFS) was calculated from a landmark time of 6 months after date of randomization to allow for the difference in timing of surgery between the two treatment groups (7). In this analysis, events including macroscopically incomplete resection, local and distant recurrence, and death arising within the first 6 months after random assignment were regarded as events at this landmark time. Survival curves are presented by the Kaplan-Meier method and treatment comparisons are by the log-rank test.

Statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). Hazard ratios (HR) were calculated with the use of a Cox regression model including treatment alone (primary analysis) and after adjustment for baseline stratification factors. Categorical data were compared with the use of chi-square test or Fisher's exact test, with a

test for trend over ordered categories. All statistical comparisons were made with two-tailed tests and *P* values <0.05 were reported as significant.

## RESULTS

Between January, 1989, and January, 1996, 169 patients from six Dutch University Hospitals (Rotterdam, Amsterdam, Utrecht, Groningen, Nijmegen and Maastricht) were randomly assigned to either chemotherapy followed by surgery (CS group, N=85) or surgery alone (S group, N=84; Figure 1). An additional number of nine patients were included to adjust for study drop-outs. The majority of patients (N=122) were included by the Erasmus Medical Center (EMC), Rotterdam. From all participating centers, the EMC is the only hospital that collected outcome data (prospectively) for all patients with esophageal cancer referred in time this study was performed. Between January, 1989, and January, 1996, 257 patients with ESCC were referred to the EMC. Of these, 183 patients were deemed eligible for surgical resection, of which 122 (67%) were included in this trial. The reasons why 61 (33%) patients were not randomized for this trial are not well documented.

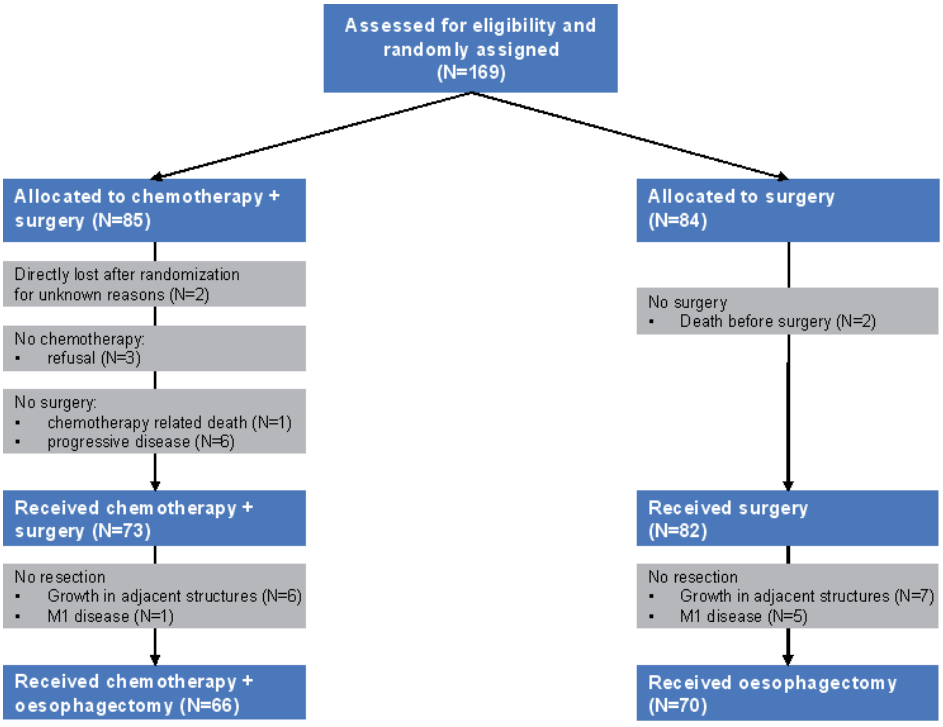


**Figure 1.** CONSORT Flow-diagram: random assignment, and compliance to the allocated treatment. CTX, chemotherapy

Table 1 show that the two groups were similar in terms of age, sex, and performance status. Distribution according to weight loss and size of the tumor was also balanced. One patient, allocated to preoperative chemotherapy, had a tumor located in the cervical part of the esophagus (the reason why this patient was included and randomized remains unclear, even after retrospective analysis of the patient's record). Preoperative staging by CT of the chest and the upper abdomen was performed in 149 patients (88%); two patients (1%) died before the planned CT scan; six patients (4%) were staged by endoscopic ultrasound, external ultrasonography of the cervical and upper abdominal region and chest radiography. From twelve patients (7%) no additional information on preoperative staging was available.

**Table 1.** Patient's characteristics

| Characteristics | Total (N=169) | CS group (N=85 ) | S group (N=84) | *P*-value* |
|---|---|---|---|---|
| Age, years | | | | 0,73 |
| <50 | 31 (18%) | 17 (20%) | 14 (17%) | |
| 51-60 | 54 (32%) | 25 (29%) | 29 (34%) | |
| >60 | 84 (50%) | 43 (51%) | 41 (49%) | |
| Median | 60 | 60 | 60 | |
| Range | 35 - 79 | 35 - 76 | 37 - 79 | |
| Sex | | | | 0,9 |
| Male | 126 (75%) | 63 (74%) | 63 (75%) | |
| Female | 43 (25%) | 22 (26%) | 21 (25%) | |
| Weight loss (% of normal weight) | | | | 0,24 |
| <5 | 56 (33%) | 30 (35%) | 26 (31%) | |
| 6-10 | 40 (24%) | 16 (19%) | 24 (29%) | |
| >10 | 51 (30%) | 30 (35%) | 21 (25%) | |
| Not recorded | 22 (13%) | 9 (11%) | 13 (15%) | |
| Tumor length (cm) | | | | 0,17 |
| <3 | 27 (16%) | 14 (17%) | 13 (16%) | |
| 4-6 | 69 (41%) | 36 (42%) | 33 (39%) | |
| 7-10 | 55 (32%) | 22 (26%) | 33 (39%) | |
| >10 | 6 (4%) | 5 (6%) | 1 (1%) | |
| Not recorded | 12 (7%) | 8 (9%) | 4 (5%) | |
| Location of the tumor | | | | 0,66 |
| Cervical | 1 (1%) | 1 (1%) | 0 | |
| Upper third | 7 (4%) | 3 (3%) | 4 (5%) | |
| Middle third | 76 (45%) | 38 (45%) | 38 (45%) | |
| Distal third | 71 (42%) | 34 (40%) | 37 (44%) | |
| Not recorded | 14 (8%) | 9 (11%) | 5 (6%) | |
| Karnofsky score* | | | | 0,53 |
| 70 - 80 | 125 (74%) | 60 (71%) | 65 (77%) | |
| 90 - 100 | 38 (22%) | 21 (24%) | 17 (20%) | |
| Not recorded | 6 (4%) | 4 (5%) | 2 (3%) | |

Abbreviations: *CS, Chemotherapy followed by surgery; S, Surgery alone*

* Comparisons were made by the chi-square test

**Chemotherapy**

Of the 85 patients assigned to preoperative chemotherapy, 80 (94%) received chemotherapy; 75 (88%) patients had two or more cycles and 5 patients (13%) received one cycle. The reasons why no chemotherapy or only one cycle was given were patient's refusal (N=3), death (N=1), tumor bleeding (N=3) and renal toxicity grade III (N=1). Two patients allocated to preoperative chemotherapy, were directly lost to follow-up after randomization. Tracing back the original patient's files was impossible; therefore, it is not clear if these two patients truly received chemotherapy followed by surgery.

Clinical response evaluation after two cycles of chemotherapy showed 43 patients with stable or progressive disease. Partial response to chemotherapy was observed in 32 patients. Of these, 30 patients received two additional cycles of chemotherapy; one received one additional cycle and one had three additional cycles of chemotherapy. Clinical response evaluation after the additional cycles of chemotherapy showed six patients with complete response; 20 patients had partial response; five showed stable disease and one had progressive disease. Detailed data on chemotherapy related toxicity is not available. In the prior phase II trial a high rate of grade III and IV nausea (38%) and vomiting (20%) was observed, probably due to the fact that 5-HT3 receptor blockers were rarely given throughout the study period (8). All patients in the current trial received prophylactic anti-nausea treatment with 5-HT 3 receptor antagonists during chemotherapy. No grade III or IV nausea and vomiting were observed. The major non-hematological toxicity (grade III) was alopecia. Hematological toxicity grade III was observed in 23 patients (one renal, twenty-two hematological). Eight patients had grade IV hematological toxicity.

**Outcome of surgery**

Surgery was performed in 76 CS and 82 S patients (Table 2). Median time from randomization to surgery was 14 days in the S group. In the CS group, the median time from randomization to surgery was 63 days (36-123) for patients who received two cycles of chemotherapy, and 114 days (54-165) for patients who received additional treatment cycles. Four patients (5%) in the CS group and three patients (4%) in S group died within 30-days after surgery.

Data on postoperative complications was available of 67/76 (88%) of patients in the CS group and 75/82 (91%) patient in the S group. The frequency of nonfatal postoperative events was closely similar in both groups (Table 2). However, pulmonary complications were significantly more observed in the CS group ($P$ =0.041).

Esophagectomy was performed in 91% (69/76) in the CS-group and 85% (70/82) in the S-group. In the CS group, six patients did not receive an esophagectomy because of tumor growth in adjacent structures (aorta or bronchial tree) and one had tumor positive celiac lymph nodes at laparotomy. In the S group, seven patients did not undergo surgical resection because of tumor encasement of the aorta or the bronchial tree and five due to tumor positive celiac lymph nodes at laparotomy. Of the 69 patients in the CS group who underwent

surgical resection, 71% had R0 resections, 25% had R1 resections, and 4% had R2 resections. Of the 70 patients in the S group who underwent surgical resection, 57% had R0 resections, 29% had R1 resections, and 14% had R2 resections. Although more patients in the CS group had R0 resections as compared with the S group, no significant differences was observed (*P*=0.09). However, there was a significant difference between the number of R2 resections in both treatment arm (*P*=0.04). Also the number of patients with lymph node involvement (N1 or M1a) did not differ between both treatment arms (43 and 46% in the CS group and S group, respectively). In the CS group, the pathological complete response rate (pT0N0M0) was 7%.

**Table 2.** Surgical details

| | Total (N=169) | CS group (N=85) | S group (N=84) | *P*-value |
|---|---|---|---|---|
| Surgery done | | | | 0,083 |
| Yes | 158 (93%) | 76 (90%) | 82 (98%) | |
| No | 9 (5%) | 7 (8%) | 2 (2%) | |
| Not recorded | 2 (2%) | 2 (2%) | 0 | |
| Reason no surgery undertaken | | | | |
| Died before surgery | 3 (2%) | 1 (1%) | 2 (2%) | |
| Progressive disease | | | | |
| Tumor unresectable | 3 (2%) | 3 (4%) | 0 | |
| Distant metastases | 3 (2%) | 3 (4%) | 0 | |
| Type of resection* | | | | 0,38 |
| Transhiatal | 113 (71%) | 55 (72%) | 58 (71%) | |
| Transthoracic | 20 (13%) | 9 (12%) | 11 (13%) | |
| Type not recorded | 5 (3%) | 4 (5%) | 1 (1%) | |
| Other | 1 (1%) | 1 (1%) | 0 | |
| No resection performed | 19 (12%) | 7 (10%) | 12 (15%) | |
| Postoperative deaths (within 30 days)* | 7 (4%) | 4 (5%) | 3 (4%) | 0,62 |
| Non-fatal postoperative complications* | | | | 0,64 |
| None | 68 (43%) | 30 (40%) | 38 (46%) | |
| Any | 67 (42%) | 33 (43%) | 34 (41%) | |
| Not recorded | 16 (10%) | 9 (12%) | 7 (9%) | |
| Type of non-fatal postoperative complications*,† | | | | |
| Pulmonary | 25 (16%) | 17 (23%) | 8 (10%) | 0,048 |
| Cardiac | 6 (4%) | 3 (4%) | 3 (4%) | 1,0 |
| Anastomotic | | | | |
| Subclinical | 10 (6%) | 5 (7%) | 5 (6%) | 1,0 |
| Clinical | 7 (4%) | 3 (4%) | 4 (5%) | 1,0 |
| Chylothorax | 7 (4%) | 4 (5%) | 3 (4%) | 0,70 |
| Bleeding | 5 (3%) | 3 (4%) | 2 (2%) | 0,67 |
| Vocal-cord injury | 22 (14%) | 10 (13%) | 12 (15%) | 0,82 |
| Other | 10 (6%) | 4 (5%) | 6 (7%) | 0,75 |

Abbreviations: *CS, Chemotherapy followed by surgery; S, Surgery alone*

* Percentages based on total patients undergoing surgery.

† Nonfatal postoperative events; comparisons were made by the Fisher's exact test

**Pattern of failure**

The outcomes of treatments were considered according to findings at operation and to patterns of disease progression (first disease-free survival event; Table 3). The rates of unresectable tumors or macroscopically incomplete resections were higher in the S group (*P*=0.23; *P*=0.05, respectively). The pattern of first disease progression was similar between both treatment groups; in particular there was no clear trend toward fewer patients with distant metastases as first site of relapse in the CS group. Ten patients treated with preoperative chemotherapy developed a second primary tumor; seven squamous cell carcinomas of head and neck, one pancreatic, one lung and one breast carcinoma. In contrast, four patients who underwent immediate surgical resection developed a second primary tumor, all squamous cell carcinomas of head and neck.

**Table 3.** Nature of first disease-free survival event

| Event | CS group (N=85) | S group (N=84) | *P*-value* |
|---|---|---|---|
| Disease free | 12 (14%) | 7 (8%) | 0,33 |
| No surgery performed | 7 (8%) | 2 (3%) | 0,17 |
| No resection performed | 7 (8%) | 12 (14%) | 0,23 |
| Macroscopic residual disease | 3 (3%) | 10 (12%) | 0,05 |
| 2nd Primary | 10 (12%) | 4 (5%) | 0,16 |
| Local recurrence | 16 (19%) | 21 (25%) | 0,36 |
| Distant metastases | 5 (6%) | 5 (6%) | 1 |
| Local recurrence and distant metastases | 9 (11%) | 10 (12%) | 0,81 |
| Death with cancer but site of failure not reported | 5 (6%) | 7 (8%) | 0,57 |
| Death from other or unspecified cause | 11 (13%) | 6 (7%) | 0,31 |

* Comparisons were made by the Fisher's exact test

**Overall and disease-free survival**

At the time of analysis, the median follow-up was 15 months in the CS group and 14 months in the S group. In an intention-to-treat survival analysis, two patients that were directly lost to follow-up were censored one month after the date of randomization. The OS of all randomized patients (N=169) on the intention to treat basis is shown in Figure 2. The median overall survival in the CS group was 16 months, and in the S group 12 months. Overall survival was better in the CS group than in the S group (*P*=0.03, by the log-rank test; HR 0.71; 95%CI [0.51-0.98]; Figure 2A). Survival at one year was 64% for those allocated to chemotherapy, 52% for those allocated to surgery alone; at two years 42% and 30%; 5-years, survival was 26% and 17%, respectively.

DFS is shown in Figure 2B. For disease free survival, 59 patients in the CS group and 40 patients in the S group remained for analysis after the landmark period of six months. In 6/59 (10%) patients in the CS group the date of disease recurrence was not documented. In these, the date of disease recurrence was estimated four months earlier than the date of death

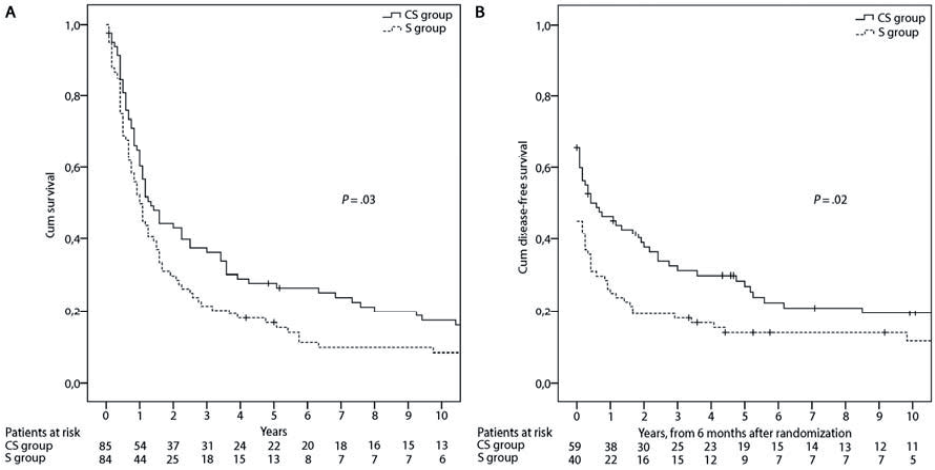


**Figure 2.**

**A)** Overall survival of all allocated patients. The distribution curves represent the results of an intention-to-treat survival analysis involving all patients. Patients who received preoperative chemotherapy had a median survival of 16 months; in comparison, patients who underwent only surgery had a median survival of 12 months ($P = 0.03$ by the log-rank test).

**B)** Disease-free survival of all patients from a landmark time of 6 months after date of randomisation ($P = 0.02$ by the log-rank test).

(in the CS group the median time between date of recurrence and date of death was four months). In the CS group, there is prolonged disease-free survival compared with the surgical resection alone group (*P*=0.02, by the log-rank test; HR 0.72; 95%CI [0.52-1.0]).

Overall survival according clinical response to preoperative chemotherapy showed that patients with clinical partial or complete response (those who received three or more cycles of therapy) had significantly better overall survival then those with stable or progressive disease (*P*=<0.001, by log-rank test; HR 0.38; 95%CI [0.23-0.65]).

Figure 3 shows no clear evidence that effect of chemotherapy varied in accordance with age, sex or length of the tumor. In this subgroup analysis, it appeared that patients with substantial weight loss (>10%) treated with preoperative chemotherapy had better overall survival as compared to those who received surgery alone. Possibly, patients allocated to chemotherapy and in a poor nutrition status (eg weight loss >10%) were more likely to receive nutritional support over a longer period of time as compared to patients that were allocated to surgery alone. This could have led to a better preoperative condition of patients who received chemotherapy, which could possibly contribute to improved overall survival. Furthermore, patients with a tumor located in the middle thoracic esophagus who received preoperative chemotherapy had better overall survival then patients who received surgery alone. The explanation for the observed survival benefit in this subgroup of patients is unclear.

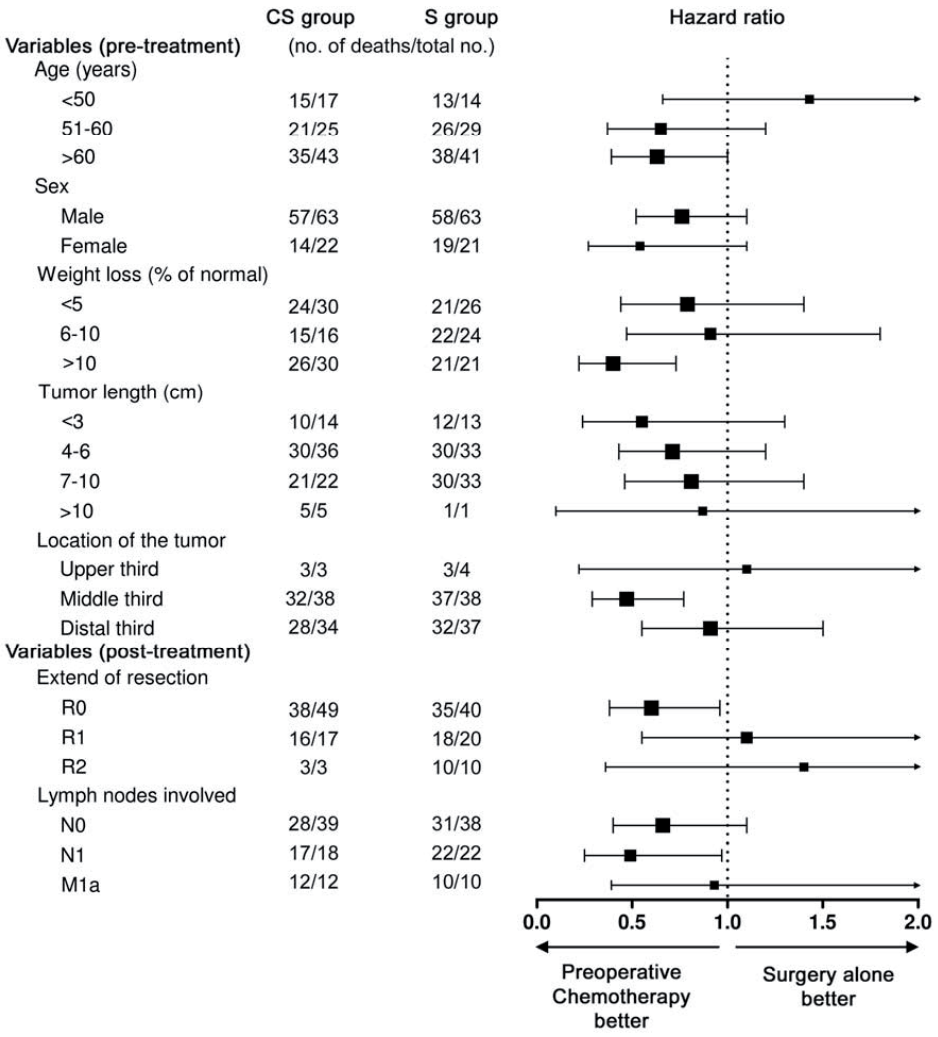


**Figure 3.** Survival by characteristics at randomisation and post-treatment. Centre of each square indicates hazard ratio, and area of square the amount of information. Lines on either side indicate 95 CI.

## DISCUSSION

The long-term results of this randomized controlled trial demonstrated a survival benefit for preoperative chemotherapy followed by surgery in patients with ESCC, when compared with surgery alone. This study has only been reported in abstract form, which hampers interpretation of our findings in context of other randomized trials (9). Why it took so long to report the design and results of this study is not completely understood. The main reasons are change

of personnel (the trial coordinator [TCK] moved to another hospital) and loss of interest in the used chemotherapeutic regime. Nevertheless, we believe that these results contribute to the ongoing debate about the optimal (preoperative) therapy for patients with ESCC.

The results of this study should be interpreted in the timeframe in which this study was performed. This study is one of the three largest randomized controlled trials among patients with ESCC treated with preoperative chemotherapy followed by surgery or surgery alone (10, 11). All these large randomized controlled trials were performed in the early '90s. The Medical Research Council (MRC) trial included most patients with esophageal cancer (533 esophageal adenocarcinoma (EAC) and 247 ESCC patients) and demonstrated a significant survival benefit for the use of preoperative chemotherapy (P=.004) (11, 12). Another large and well-conducted randomized controlled trial among patients with esophageal cancer (236 EAC and 204 ESCC patients), by the North American Intergroup (RTOG Trial 8911 or USA Intergroup 113 trial; further called the Intergroup trial), demonstrated no significant difference in survival between patients treated with preoperative chemotherapy and those who received surgery alone (10, 13). In the light of the results of both trials, we discuss the design and results of the present study.

As most randomized controlled trials performed in the early '90, this study reflects the methods for diagnosis, staging, treatment delivery and outcome measurement that indicate clinical practice during that period. In the present study, the majority of patients (88%) underwent preoperative staging by esophago-gastroscopy and CT scan of the chest and upper abdomen. The same preoperative staging methods were used in the Intergroup trial. In the MRC trial, however, there was no standardization of preoperative staging. These differences in preoperative staging could, by selection of different populations of ESCC patients, contribute to differences in results between the trials.

In the Intergroup trial as well as in the MRC trial the chemotherapeutic regime consists of cisplatin combined with fluorouracil; in the present study cisplatin was combined with etoposide. The ratio for this combination of chemotherapeutic agents was deducted from trials among patients with non-small-cell lung cancer in which this regime had showed to be safe and effective (14). Furthermore, a phase II trial in patients with advanced ESCC had shown that the response rate equals that of other cisplatin-based regimes and that the toxicity profile was mild (8). Patients without clinical response to chemotherapy received a total preoperative dose of 160 mg/m$^2$ cisplatin and 1000 mg/m$^2$ etoposide. The dose of cisplatin is similar as compared with the MRC trial (160 mg/m$^2$). Patients with clinical response to chemotherapy received total doses up to cisplatin 320 mg/m$^2$ and etoposide 2000 mg/m$^2$. In this subgroup of patients, the total preoperative dose of cisplatin was slightly higher as compared to the Intergroup trial (300 mg/m$^2$). The compliance to chemotherapy was 88% (patients who received two or more of the planned cycles of chemotherapy). This is similar to 90% of the patients that received both treatment cycles in the MRC trial, but differed from the Intergroup wherein 71% of the patients received all of three planned cycles of chemo-

therapy. It has been suggested that the higher dose of chemotherapy in the Intergroup trial was detrimental to patients who underwent esophagectomy. Other factors related to the chemotherapeutic regimes that could contribute to the differences in outcome between the three studies, are the use of chemoradiotherapy in a subset of patients in the MRC trial and the use of postoperative chemotherapy in a subgroup of patients in the Intergroup trial.

In our study the majority of patients underwent a transhiatal esophagectomy. This type of resection is associated with lower morbidity (and mortality) than a transthoracic resection ((15)). In the other trials, the type of surgical resection that has been performed is not clear (MRC trial) or the exact numbers are not described (Intergroup trial). The postoperative mortality rate (<30 days after surgery) in the current trial was 5% (4/76) in the CS group and 4% (3/82) in the S group and differed not among both groups. These rates are similar as those observed in the Intergroup trial, with 6% postoperative mortality in both treatment arms. In contrast, the MRC trial reported much higher postoperative mortality rate of 10% in the CS group and 11% in the S group.

In the present study surgery was performed in 89% and 98% of patients in the CS group and S group, respectively. Similar rates have been reported by the MRC trial, with surgery rates in the CS group of 92% and in the S group of 97%. In the Intergroup trial fewer patients underwent surgery, 80% of the CS group and 92% of the S group. The rate of microscopically tumor free resection margins (R0) in the CS group was 71%, as compared to 60% and 62% in the CS groups of the MRC and Intergroup trial, respectively. In the S group it was 57%, as compared to 54% and 59% in the S groups of the MRC and Intergroup trial, respectively. The difference in R0 resection rates between the CS group and the S group is likely to contribute to the observed survival benefit for patients treated with preoperative chemotherapy (as showed by the MRC trial; $P<0.001$), however, this difference was not statistical significant in the present study ($P=0.086$).

The median survival time of the CS group was 16 months, compared to 17 and 15 months in the MRC and Intergroup trial, respectively. The median survival time of the S group was 12 months, compared to 16 and 13 months in the Intergroup and MRC trial, respectively. It appears that the S group in our study had the worst survival outcome, but this may be due to patient selection. Both the MRC as Intergroup trial included more EAC than ESCC patients. Subgroup analysis of the MRC trial, including only ESCC patients, showed a median survival time of 11 months for patients who underwent surgery alone (12). Remarkably, in the subgroup analysis there is no significant survival benefit for ESCC patients treated with preoperative chemotherapy (P=0.1).

In line with the results of the MRC and Intergroup trial, there was no significant difference in pattern of failure between both treatment arms in our study. The rate of distant metastases was equal in both treatment groups. These findings provide no evidence for the general hypothesis that preoperative chemotherapy eliminates systemic micro-metastases. The results of this trial and the MRC trial suggest that the biologic effect of preoperative chemotherapy

seems to specifically influence the extent of surgery (12). In the present study, the incidence of incomplete resections was greater in the S group, but sites of first recurrence (local or distant) were similar. Furthermore, at 6 months, the DFS advantage is established for the CS group (Fig 2A) and remains consistent throughout follow-up as the survival curves remain approximately parallel. This suggests that the effect of preoperative chemotherapy is to reduce tumor volume and increase the potential for curative resection.

This study has its limitations. At first, the preoperative staging was hampered by the absence of endoscopic ultrasonography at the beginning of our trial. Therefore, the clinical T- and N-stage were not used as stratification parameters before randomization. Secondly, the missing data on two patients that underwent preoperative chemotherapy and the lack of some clinical characteristics of the patients reflect the difficulty of obtaining all data more than twenty years after the trial was performed. At third, it should be noticed that we selected patients who showed clinical response to chemotherapy based on esophago-gastroscopy and CT scan of the chest and upper abdomen, for additional cycles of chemotherapy. However, we did not correlate clinical response to pathological response. Therefore, selection of this subgroup could also reflect better prognostic characteristics of patients who respond to chemotherapy, rather than an effect of chemotherapy itself.

## CONCLUSIONS

In summary, this study reports a significant survival benefit for ESCC patients treated with preoperative chemotherapy. The chemotherapeutic regime used in this trial (etoposide and cisplatin) is not used anymore in treatment of patients with ESCC. Today, in our institution (EMC) the majority of patients with ESCC (and EAC) receive preoperative chemoradiotherapy (a combination of carboplatin and paclitaxel, and concurrent radiotherapy).

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# Chapter 7

## Functional polymorphisms associated with disease free survival in resected carcinoma of the esophagus

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## ABSTRACT

**Background:** The aim of this study was to determine whether clinical outcome after surgical resection of esophageal adenocarcinoma (EAC) or squamous cell carcinoma (ESCC) could be predicted by functional polymorphisms in different proto-oncogenes and tumor suppressor genes.

**Methods:** Six single nucleotide polymorphisms (SNPs) in the *AURKA* (rs2273535), *ERBB2* (rs1136201), *MDM2* (rs2279744), *CDH1* (rs5030625), *CDKN2A* (rs11515) and the *TP73* (rs2273953) gene were genotyped in a consecutive cohort of 346 esophageal cancer patients, who were underwent surgical resection with curative intent. Associations with disease free survival (DFS) were analyzed with Kaplan Meier curves and Cox regression, adjusting for potential confounders.

**Results:** Univariate analysis showed no significant associations between the tested polymorphisms and DFS in patients with EAC or ESCC. However, in a multivariate analysis, patients with EAC carrying the heterozygous *MDM2* (rs2279744) T/G genotype had significantly improved DFS compared with patients carrying the wild-type genotype (adjusted hazard ratio (AHR) 0.63, 95% confidence interval [CI] [0.45-0.88]). Patients with EAC harbouring the homozygous *CDH1* (rs5030625) GA/GA genotype had a significantly reduced survival as compared with patients carrying the wild-type genotype AHR 4.0, 95% CI [1.4-11].

**Conclusions:** In a large cohort of esophageal cancer patients, the *MDM2* T/G and *CDH1* GA/GA genotype confer risk of death in patients with EAC. These data suggests that inter-individual differences in germ-line DNA have an impact on DFS in patients with EAC.

## INTRODUCTION

Many therapeutic options are used to treat esophageal cancer, but traditionally surgery is used most frequently to obtain locoregional control and long-term survival (1, 2). Comprehensive preoperative staging has improved selection of patients for potentially curative surgery, however, many patients present with recurrent disease within two years after operation. The majority of these patients develop loco-regional recurrences, but also distant metastases (such as liver, lung, pleural and/or peritoneal disease recurrences) are common (3-5). Despite attempts to improve the outcome of patients with esophageal cancer, prognosis remains poor with a 5-year overall survival of 20-30% (5, 6).

Well-known prognostic factors for esophageal cancer are summarized in TNM staging (7, 8). Although TNM parameters have the advantage of simplicity, they do not seem to completely reflect the biologic diversity of esophageal cancer (9, 10). The true drivers of this clinical biologic diversity include the molecular aberrations of the cancer and the genetic make-up of the patient. In this respect, the study of host genetic variability offers worthwhile potential to identify individuals that may have the best chance of survival. Single nucleotide polymorphisms (SNPs) in the germ-line are the most common type of host genetic variations. Gene-related functional SNPs can potentially lead to differences in protein expression and/or function. In this way, SNPs in proto-oncogenes and tumor suppressor genes can potentially alter the risk for metastatic or aggressive tumor, resulting in differences in clinical outcome.

Altered expression of the AURKA, ERBB2, MDM2, CDH1, CDKN2A and the TP73 proteins has been correlated to disease progression and clinical outcome in patients with esophageal cancer (11-17). In addition, polymorphisms with effects on protein function have been identified in these proto-oncogenes and tumor suppressor genes (18-23). Based on these results, we postulated that functional SNPs in the *AURKA* (*AURKA*_NM_003600.2; rs2273535 c.449 T>A), *ERBB2* (*ERBB2*_NM004448.2; rs1136201 c.655 A>G), *MDM2* (*MDM2*_NM002392.2; rs2279744 309 T>G), *CDH1* (*CDH1*_NM004360.3; rs5030625 -347 G>GA), *CDKN2A* (*CDKN2A*_NM000077.3; rs11515 c.712 C>G), and the *TP73* (*TP73*_NM005427.1; rs2273953 81 G>A) genes may serve as molecular markers for clinical outcome in patients with EAC or ESCC who underwent surgical resection.

## PATIENTS AND METHODS

Between 1996 and 2001, a total of 632 consecutive patients with esophageal cancer were evaluated for surgery with curative intent at the Erasmus University Medical Center (Figure 1). Outcome for all patients with esophageal cancer referred to our hospital are collected prospectively and stored in a database by a data manager. The data collected encompassed all relevant diagnostic tests, scheduled treatments, and pathology. All patients were staged

using esophago-gastroscopy with biopsies, ultrasonography of the cervical and upper abdominal region and computed tomography (CT) of the thorax and abdomen. Endoscopic ultrasonography for evaluation of T-stage and nodal status was routinely performed.

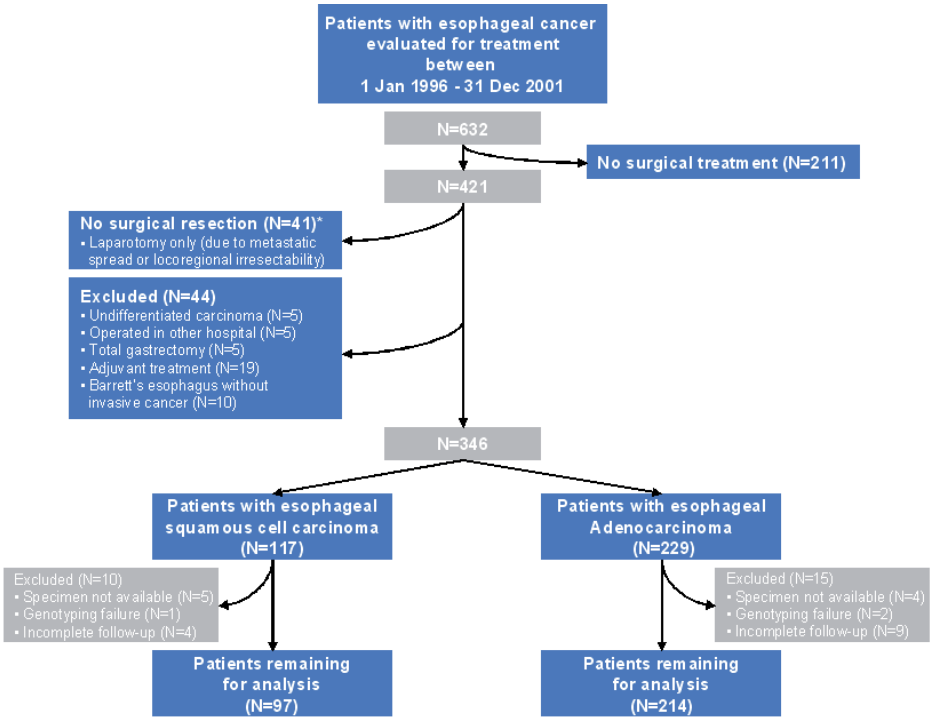


**Figure 1.** Flow-chart of patients with esophageal cancer referred to the ErasmusMC for treatment between January 1996 and December 2001. Patients excluded from the present study are shown.

### Surgery

For carcinomas of the upper half of the intra-thoracic esophagus a right sided thoracotomy was performed. For carcinomas of the lower half of the intra-thoracic esophagus a transhiatal esophagectomy was preferred. The tumor and its adjacent lymph nodes were dissected *en bloc*; however no extended lymph node dissection was performed. The continuity of the digestive tract was restored by means of a gastric tube reconstruction or colonic interposition with a cervical anastomosis. Resections were considered radical (R0) if microscopically examination revealed no tumor tissue at or less than 1 mm from the circumferential, proximal, or distal margins. Pathological staging was done according to the UICC 6$^{th}$ edition. The tumor stage after resection was classified according to the TNM classification of the International Union Against Cancer.

**SNP genotyping**

To determine the individual genotype for each SNP, genomic DNA was extracted from frozen or formalin fixed and paraffin-embedded tissues. Normal tissue was obtained from the resection specimens (i.e., tumor-negative lymph nodes or tumor-negative resection margins). All the archival tissue samples were used according to the code for adequate secondary use of tissue, code of conduct: "Proper Secondary Use of Human Tissue" established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org).

Polymerase chain reactions (PCR) were carried out in a volume of 15µl containing genomic DNA, 8.3µl $H_2O$, 5µl $Mg^{2+}$free buffer, 25mM $MgCl_2$, 0.3µl of 10mM deoxynucleotide triphosphates, 20pmol of each primer and 1U Taq polymerase (Promega, Madison WI, USA). PCR-conditions were standardized at 35 cycles of 95°C for 45 sec, 61°C for 45 sec, 72°C for 30 sec, with a 10 min extension at 72°C for 10 min following the last cycle. PCR primers for EACh SNP are shown in Table 1. For the polymorphism in *CDH1* (rs5030625) amplified PCR products were visualized on a denaturing polyacrylamide gel. For detection of the restriction length polymorphisms in *ERBB2* (rs1136201) and *AURKA* (rs2273535), PCR products were digested for 16h at the appropriate temperature with 10 units of restriction endonuclease BsmAI, MspI or APOI (Promega, Madison, WI, USA), respectively. The DNA fragments were separated using 3% agarose gels. The polymorphisms in *CDKN2A* (rs11515), *MDM2* (rs2279744) and *TP73* (rs2273953) were genotyped by bi-directional sequencing.

**Statistical analysis**

Data on follow-up were collected from the prospective database and the medical charts. All patients were followed at an interval of three to four months during the first year, every six months for the second year, and then at the end of each year until 5 years after treatment. Recurrence or disease progression was diagnosed on clinical grounds. Whenever a relapse was suspected, radiologic, endoscopic, or histologic confirmation was sought. Recurrent disease was classified as local–regional (occurring in the upper abdomen or mediastinum) or distant (including cervical recurrences).

Study end-point was disease free survival (DFS) that was defined as the time from surgery until recurrent disease or death from any cause. The Kaplan-Meier survival function and log-rank tests were used to assess clinical outcome in relation to patient's characteristics and individual polymorphisms. Cox proportional hazard ratios for patients with EAC were adjusted for weight loss prior to operation, tumor length, presence of Barrett's epithelium, radicality of resection and pathological tumor stage. For patients with ESCC, cox proportional hazard ratios were adjusted for location of tumor, resection type, post-operative TNM stage and radicality of resection. Statistical significance was set at the 5% level. We did not adjust for multiple testing since each gene - outcome was prespecified and of interest in itself.

**Table 1.** Description of the polymorphisms located in different onco- and tumor suppressor genes

| Gene | refSNP ID* | Change | Minor allele | Minor allele frequency† | Potential effect on protein function | Forward Primer | Reverse Primer |
|---|---|---|---|---|---|---|---|
| *AURKA* | rs2273535 | c.449 T>A (Phe>Ile) | A-allele | 0,25 | Preferentially amplified; faster growth of cultured cells (17) | 5′-TCCATTCTAGGCTACAGCTC-3′ | 5′-AAGAATTTGAAGGACACAAGAC-3′ |
| *ERBB2* | rs1136201 | c.655 A>G (Ile>Val) | G-allele | 0,16 | Increased dimerization, autophosphorylation of HER-2 and tyrosine kinase activity (18) | 5′-AGCCCTCTGACGTCCATC-3′ | 5′-CTGCAGCAGTCTCCGCATC-3′ |
| *MDM2* | rs2279744 | c.309 T>G | G-allele | ND | Associated with higher levels of MDM2 expression (19) | 5′-GCGGAGGTTTTGTTGGACTG-3′ | 5′-CTGAGTCAACCTGCCCACT-3′ |
| *CDH1* | rs5030625 | c.-347 G>GA | GA-allele | 0,14 | Associated with decreased transcriptional activity (20) | 5′-GGCCAGAGGACCGCTTGAG-3′ | 5′-GTTTGTTCGTTTTGGAGA-3′ |
| *CDKN2A* | rs11515 | c.712 C>G | G-allele | 0,13 | Potential detrimental effect on RNA stability (21) | 5′-CCCCGATTGAAAGAACCAGAGA-3′ | 5′-AGGACCTTCGGTGACTGATGAT-3′ |
| *TP73* | rs2273953 | c.-30 G>A | A-allele | 0,19 | Influences the p73 translation (22) | 5′-GAGCACGAGTTCCCAGGGTG-3′ | 5′-CCAAGCGCACTCACAGAGAG-3′ |

*Abbreviations:* ND, not determined

*refSNP ID (http://www.ncbi.nlm.nih.gov/SNP)

†Minor allele frequency according 102 controls of the SNP500CANCER cohort (http://snp500cancer.nci.nih.gov/snp.cfm)

## RESULTS

A total of 346 esophageal cancer patients underwent surgical resection with curative intent. Of these, 25 patients were excluded from the current follow-up study because no tissue samples were available (N=9), genotyping failure (N=3) or incomplete follow-up (N=13) (Figure 1). Of the 214 EAC and 97 ESCC patients remaining for analysis, the majority were male, 85% in EAC and 60% in ESCC. Median age at time of diagnosis was 64 and 61years, respectively. Of all patients with EAC, 84% underwent primary surgery and 16% received preoperative chemotherapy with or without radiotherapy. In contrast, 71% of patients with ESCC received preoperative chemotherapy with or without radiotherapy and 29% underwent primary surgical resection (Table 2).

### SNP genotyping

Genotyping was complete in 95% to 100% of EAC and ESCC patients. The genotype distributions did not deviate from HWE ($P$>0.05). The genotype distribution of each SNP is listed in Table 3. Tumor stage distributions were similar across all SNP genotypes, and there was no association between genotypes and age at diagnosis, sex, weight loss, smoking status or preoperative treatment.

### DFS and pattern of disease recurrence

The median DFS of EAC patients was 14 months (range 0.07-138 months), and for ESCC patients 16 months (range 0.5-148 months). At the time of analysis, 37 (17%) EAC and 27 (28%) ESCC patients were alive with a median disease free survival time of 93 (range 62-138 months) and 104 months (range 79-148 months), respectively. The pattern of disease recurrence is depicted in Table 3. Loco-regional recurrences were mediastinal or abdominal lymph node metastases, and recurrences in the gastric tube. Distant metastases were found in liver, lung, brain, bone, adrenal gland, pleura, peritoneum and skin. Recurrent disease after surgery was found in 138 (78%) EAC patients; 40 patients had loco-regional recurrence, 51 had distant metastasis and 46 had both loco-regional recurrence and distant metastasis. One patient had disease recurrence, but the site of failure was not recorded. Diseases recurrences were found in 51 ESCC patients; 28 patients had loco-regional recurrence, 10 had distant metastasis and 10 had both loco-regional recurrence and distant metastasis. Three patients had disease recurrence, but site of failure was not recorded.

### SNP genotype and DFS

Univariate analysis showed no significant associations between DFS in patients with EAC or ESCC and the genotype distributions of the *AURKA*, *ERBB2*, *MDM2*, *CDH1*, *CDKN2A* and the *TP73* gene polymorphisms (Table 4). However, in a multivariate analysis, patients with EAC

**Table 2.** Survival According to Patients' and Tumor Characteristics

| | Patients with EAC (N=214) | | | | Patients with ESCC (N=97) | | | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **No.** | **(%)** | **Median DFS** | ***P*-value** | **No.** | **(%)** | **Median DFS** | ***P*-value** |
| Age in years | | | | 0,23 | | | | 0,33 |
| <65 years | 111 | (52) | 19 | | 64 | (66) | 21 | |
| ≥65 years | 103 | (48) | 12 | | 33 | (34) | 12 | |
| Gender | | | | 0,41 | | | | 0,44 |
| Male | 182 | (85) | 16 | | 58 | (60) | 15 | |
| Female | 32 | (15) | 11 | | 39 | (40) | 20 | |
| Weight loss before operation | | | | 0,013 | | | | 0,47 |
| No loss or <5% | 127 | (59) | 19 | | 46 | (47) | 15 | |
| 5%-10% | 35 | (16) | 11 | | 31 | (32) | 27 | |
| >10% | 32 | (15) | 8 | | 17 | (18) | 10 | |
| Not recorded | 20 | (10) | 10 | | 3 | (3) | 5 | |
| Smoking status | | | | 0,98 | | | | 0,70 |
| Current smoker | 54 | (25) | 14 | | 43 | (44) | 16 | |
| No current smoker | 146 | (68) | 15 | | 47 | (49) | 20 | |
| Not recorded | 14 | (7) | 8 | | 7 | (7) | 4 | |
| Location of tumor | | | | 0,150 | | | | 0,008 |
| Upper one third thoracic esophagus | | | | | 3 | (3) | 4 | |
| Middle one third thoracic esophagus | 3 | (1) | 11 | | 39 | (40) | 12 | |
| Lower one third thoracic esophagus | 68 | (32) | 24 | | 45 | (47) | 20 | |
| GEJ | 86 | (40) | 13 | | 10 | (10) | 60 | |
| Gastric cardia | 57 | (27) | 12 | | | | | |
| Tumor length | | | | 0,028 | | | | 0,27 |
| 0-2 | 34 | (16) | 41 | | 8 | (8) | 37 | |
| 3-4 | 52 | (24) | 15 | | 24 | (25) | 10 | |
| 4-5 | 67 | (31) | 14 | | 31 | (32) | 24 | |
| ≥6 | 46 | (22) | 9 | | 25 | (26) | 9 | |
| Not recorded* | 15 | (7) | 16 | | 9 | (9) | 11 | |
| Barrett's epithelium | | | | 0,086 | | | | |
| No | 127 | (59) | 12 | | | | | |
| Yes | 87 | (41) | 24 | | | | | |
| Treatment | | | | 0,83 | | | | 0,18 |
| Surgery alone | 180 | (84) | 14 | | 28 | (29) | 8 | |
| Chemotherapy + Surgery | 23 | (11) | 15 | | 65 | (67) | 15 | |
| Chemoradiotherapy + Surgery | 11 | (5) | 21 | | 4 | (4) | Not reached | |
| Resection type | | | | 0,83 | | | | 0,042 |
| Transhiatal | 187 | (87) | 15 | | 53 | (55) | 37 | |
| Transthoracic | 27 | (13) | 9 | | 44 | (45) | 11 | |
| Postoperative UICC stage | | | | <0,001 | | | | <0,001 |
| Complete response | 8 | (4) | 40 | | 13 | (13) | Not reached | |
| I | 24 | (11) | 98 | | 13 | (13) | 86 | |
| IIA | 43 | (20) | 37 | | 34 | (35) | 12 | |
| IIB | 8 | (4) | 15 | | 4 | (4) | 26 | |
| III | 74 | (34) | 11 | | 17 | (18) | 8 | |
| IV | 57 | (27) | 7 | | 16 | (17) | 4 | |
| Radicality of resection | | | | <0,001 | | | | <0,001 |
| R0 | 141 | (66) | 34 | | 65 | (67) | 41 | |
| R1 | 70 | (33) | 7 | | 29 | (30) | 6 | |
| R2 | 3 | (1) | 9 | | 3 | (3) | 5 | |

Abbreviations: E*AC*, esophageal adenocarcinoma; ESCC, esophageal squamous cell carcinoma; *DFS*, disease free survival

**Table 3.** Polymorphisms and clinical outcome in patients with resected esophageal adenocarcinoma

| | Disease free survival in EAC patients | | | | Disease free survival in ESCC patients | | | |
|---|---|---|---|---|---|---|---|---|
| **Genotype** | **N** | **MPS (months)** | **Log-rank *P*** | **AHR [95% CI]*** | **N** | **MPS (months)** | **Log-rank *P*** | **AHR [95% CI]†** |
| ***AURKA***_rs2273535 | | | | | | | | |
| T/T | 129 | 15 | *0,83* | *Reference* | 62 | 12 | *0,72* | *Reference* |
| A/T | 75 | 14 | | 1,1 [0,76-1,4] | 29 | 20 | | 0,60 [0,17-2,1] |
| A/A | 9 | 21 | | 0,92 [0,42-2,0] | 5 | 55 | | 0,63 [0,18-2,1] |
| ***ERBB2***_rs1136201 | | | | | | | | |
| A/A | 113 | 14 | *0,25* | *Reference* | 66 | 12 | *0,66* | *Reference* |
| A/G | 86 | 15 | | 0,92 [0,67-1,3] | 23 | 26 | | 0,73 [0,41-1,3] |
| G/G | 14 | 12 | | 0,68 [0,33-1,4] | 6 | 8 | | 1,3 [0,49-3,2] |
| ***MDM2***_rs2279744 | | | | | | | | |
| T/T | 100 | 11 | *0,076* | *Reference* | 40 | 10 | *0,63* | *Reference* |
| T/G | 84 | 19 | | 0,63 [0,45-0,88] | 45 | 21 | | 0,98 [0,59-1,6] |
| G/G | 24 | 12 | | 0,95 [0,58-1,6] | 7 | 16 | | 0,81 [0,28-2,4] |
| ***CDH1***_rs5030625 | | | | | | | | |
| G/G | 166 | 17 | *0,14* | | 68 | 11 | *0,13* | *Reference* |
| G/GA | 41 | 11 | | 1,2 [0,78-1,7] | 18 | 27 | | 0,63 [0,32-1,3] |
| GA/GA | 4 | 7 | | 4,0 [1,4-11] | 1 | *Not rEAChed* | | |
| ***CDKN2A***_rs11515 | | | | | | | | |
| C/C | 162 | 13 | *0,79* | *Reference* | 74 | 12 | *0,67* | *Reference* |
| C/G | 47 | 19 | | 0,94 [0,65-1,3] | 20 | 20 | | 0,67 [0,36-1,3] |
| G/G | 4 | 19 | | 1,7 [0,52-5,6] | 1 | *Not rEAChed* | | |
| TP73_rs2273953 | | | | | | | | |
| G/G | 138 | 16 | *0,44* | *Reference* | 62 | 24 | *0,48* | *Reference* |
| G/A | 69 | 13 | | 0,98 [0,71-1,4] | 32 | 10 | | 1,1 [0,66-1,8] |
| A/A | 5 | 11 | | 1,1 [0,41-3,1] | 2 | 4 | | 1,7 [0,40-7,3] |

Abbreviations: *EAC*, esophageal adenocarcinoma; *ESCC*, esophageal squamous cell carcinoma; *AHR*, adjusted hazard ratio

* Adjusted hazard ratio for weight loss prior to operation, tumor length, presence of Barrett's epithelium, post-operative TNM stage and radicality of resection

† Adjusted hazard ratio for location of the tumor, type of resection, post-operative TNM stage and radicality of resection

**Table 4.** Pattern of failure

| | EAC (N=214) | ESCC (N=97) |
|---|---|---|
| Alive | 37 (17) | 27 (28) |
| Nature of first failure | | |
| Local recurrence | 40 (29) | 28 (55) |
| Distant metastases | 51 (37) | 10 (20) |
| Local recurrence and distant metastases | 46 (33) | 10 (20) |
| Disease recurrence but site of failure not reported | 1 (1) | 3 (5) |
| Total deaths | 177 (83) | 70 (72) |
| Cause of death | | |
| Cancer-related | 138 (78) | 51 (71) |
| Surgery-related | 14 (8) | 7 (11) |
| 2nd Primary | 5 (3) | 6 (8) |
| Death from other cause (not cancer related) | 20 (11) | 6 (8) |

Abbreviations: *EAC*, esophageal adenocarcinoma; ESCC, esophageal squamous cell carcinoma

carrying the heterozygous *MDM2* (rs2279744) T/G genotype had significantly improved DFS compared with patients carrying the wild-type T/T genotype (adjusted hazard ratio (AHR) 0.63, 95% confidence interval [CI] [0.45-0.88], P = 0.007). The post-operative TNM stage of the tumor and the radicality of resection were also found important factors for disease free survival (HR 1.4, 95% CI [1.2-1.6], *P* < 0.0001 and HR 2.3, 95%CI [1.7-3.1], P < 0.0001 respectively). Also, patients with EAC harboring the homozygous *CDH1* (rs5030625) GA/GA genotype had a significantly reduced survival as compared with patients carrying the wild-type G/G genotype AHR 4.0, 95% CI [1.4-11], P = 0.008. In multivariate analysis, the post-operative TNM stage of the tumor and the radicality of resection were found important factors for disease free survival (HR 1.4, 95% CI [1.2-1.5], P < 0.0001 and HR 2.4, 95%CI [1.7-3.2], P < 0.0001 respectively).

## DISCUSSION

In the present study, we determined the relationship between inter-individual DNA variations in six bona fide proto-oncogenes and tumor suppressor genes and DFS in a large cohort of Caucasian patients with esophageal cancer. After adjustment for potential confounders, the variant genotypes of SNPs located in the promoter region of the *MDM2* and *CDH1* gene were significantly associated with DFS in patients with EAC.

The results of the present study showed a significant survival benefit for patients harboring the *MDM2* T/G as compared with patients carrying the wild-type T/T genotype. The MDM2 protein is a nuclear phosphoprotein that binds and inhibits the tumor suppressor TP53 as part of an autoregulatory negative feedback loop. The most intensively characterized *MDM2* polymorphism is the T309G promoter SNP located in the first intron (*MDM2*_NM002392.2; rs2279744 309 T>G) (20). The G variant of this SNP is known to increase promoter-binding af-

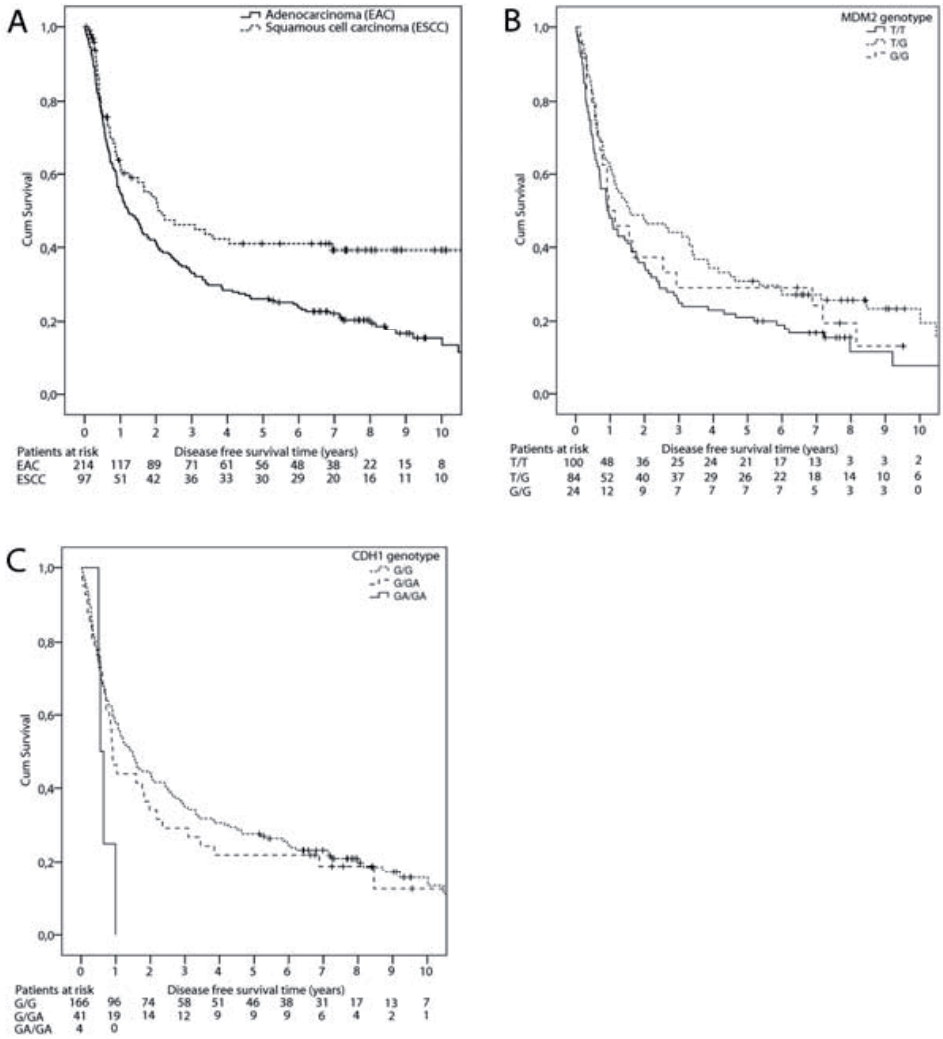


**Figure 2.**

**A)** Kaplan Meier analysis of disease free survival (DFS) in patients with esophageal cancer, by histological subgroup

**B)** Kaplan Meier analysis of disease free survival in patients with esophageal adenocarcinoma, by MDM2 (rs2279744)

**C)** Kaplan Meier analysis of disease free survival in patients with esophageal adenocarcinoma, by CDH1 genotype (rs5030625)

finity, leading to up-regulation of MDM2, and consequent inhibition and down-regulation of the p53 pathway. Therefore, it could be expected that the variant *MDM2* genotypes (T/G and G/G) are associated with adverse outcome in esophageal cancer patients (as showed in other cancer types) (24). However, the present study showed improved survival in patients with

the *MDM2* T/G genotype compared with the wild-type T/T genotype. A possible explanation for our findings is provided by a large study in breast cancer patients that reported strong interaction between the *MDM2* SNP status and tumor TP53 status, which appeared to modify the association between TP53 status and breast cancer survival (25). Among breast cancer patients with the wild-type *MDM2* genotype (T/T), a mutant TP53 status and aberrant TP53 expression in breast tumors was associated with poor survival. The tumor TP53 status was not associated with breast cancer survival among carriers of the variant *MDM2* allele (T/G or G/G). Since TP53 is the most frequently mutated gene in EAC, it could be hypothesized that the tumors of most patients with the T/T genotype harbor a TP53 mutation, which could lead to a reduced survival as observed in the present study. In a previously well-conducted study, the known *TP53* codon 72 Arg/Pro and *MDM2* polymorphisms were genotyped in 300 patients with EAC and 63 patients with ESCC (26). As in concordance with the results of the present study, patients with EAC harboring the *MDM2* T/G genotype had a borderline improved overall survival as compared with patients carrying the wild-type genotype (AHR for death 0.70 [0.50-0.99]; $P = 0.04$). But unlike the present study, the *MDM2* variant genotype did correlate with marked reduced survival in patients with ESCC. This could be due to differences in study samples size, population selection, tissue handling and genotyping methods.

In this study, patients carrying the CDH1 GA/GA genotype had a significantly reduced survival as compared with patients with the wild-type G/G genotype. However, it should be noted that only four EAC patients carried the GA/GA genotype, which may represents a chance finding. Nevertheless, this -347 G/GA insertion polymorphism located in the promoter of the cell-cell adhesion gene *CDH1* (*CDH1*_NM004360.3; rs5030625 -347 G>GA) has been reported to suppress *CDH1* gene expression and was found to be associated with familial gastric cancer and sporadic colorectal cancer (27). The GA-allele has been associated with significant suppression of CDH1 promoter activity in colorectal and gastric cancer cell lines (27). It can be hypothesized that the GA-allele might enhance the progression of esophageal cancer by reducing *CDH1* transcription resulting in a decrease in CDH1 protein expression and impairment of cell-cell adhesion. All four patients harboring the GA/GA genotype died of recurrent disease; three had loco-regional and distant metastasis; one had only locoregional disease recurrence.

To our knowledge this is one of the first studies that investigated the relationship between polymorphisms and esophageal cancer outcome. Here, we studied (only) six polymorphisms, whereas SNP-arrays can determine more than a million of SNPs in one sample. Although this technique is widely used on blood or fresh frozen samples, it is not very suitable for FFPE tissue samples (our series consisted primarily of FFPE samples). Therefore, collection of blood samples or fresh frozen tissues samples of esophageal cancer patients is necessary and should become standard procedure in order to perform genome-wide association studies. In this study, the majority of polymorphisms were not associated with disease free survival after esophagectomy. It could be well that our study, among a relatively large population (N=346)

of esophageal cancer patients, failed to observe a difference due to under powering. Since esophageal cancer has a relatively low incidence, consortia (of multiple hospitals) are needed to validate these associations.

Recurrent cancer is the leading cause of death in patients undergoing surgical resection (3-5). Although treatment options for esophageal cancer recurrences are limited, it could be proposed that early detection of recurrent disease is desirable because aggressive treatment may result in prolonged tumor-free survival or occasional cure. In this light, our findings could contribute to the identification of patients who are at high or low risk for the development of disease recurrences. It can also be proposed that patients with a certain genetic constitution that is associated with a high chance of (distant) disease recurrence should be given systemic adjuvant treatment after surgical resection. Furthermore, identification of polymorphisms associated with disease free survival could serve well as hypothesis generating for prospective studies that evaluate the prognostic significance of germ-line variants.

## CONCLUSIONS

In summary, our results indicate that two of six investigated functional polymorphisms are associated with disease free survival in patients who underwent esophagectomy for EAC. Patients with EAC carrying the heterozygous *MDM2* T/G genotype had two-fold reduced risk of disease recurrence and patients with the homozygous *CDH1* GA/GA had a four fold increased risk of disease recurrence. Additional prospective studies are necessary to validate both associations and to study the prognostic significance of both germ-line variants.

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# Part IV

## Summary, Conclusions and Epilogue

# Chapter 8

## Summary & Conclusions

## SUMMARY

Although the overall incidence and mortality of cancer in general in the world have decreased over the past two decades, both the incidence and mortality of esophageal cancer have increased. Despite improvements in diagnosis and treatment, the prognosis of patient with cancer of the esophagus remains poor, with 5-year survival rates of 10-25%. Nowadays, esophageal adenocarcinoma (EAC) account for most cases of esophageal cancer in the Western world. Most fundamental and translational studies on EAC focus on the identification of genetic alterations that underlie the metaplasia – dysplasia - adenocarcinoma sequence. This led to an increased knowledge on molecular mechanisms underlying EAC development; however translation of these results into improved patient's care and prognosis has been delayed.

The studies in the first part of this thesis intend to authenticate human EAC cell lines and to establish a reliable set of cell lines that can serve as tool for translational research and biomedical discovery. Studies in the second part address clinical aspects of esophageal cancer treatment.

**Human esophageal adenocarcinoma cell lines**

**Chapter 1** reviews the history EAC cell lines and their utility in translational research and biomedical discovery. In the past decade, the use of EAC cell lines has increased rapidly. One of the reasons for the widely use of these cell lines is the lack of appropriate animal models for EAC. Cell lines have proved useful in elucidating important signaling pathways (such as COX-2 and NF-kB pathway) in response to gastro-duodenal reflux. Furthermore, the EAC cell lines offer a robust model that can serve as a filter to fast track the most promising compounds and enables high-throughput science over short periods (such as c-MET and c-ERBB2 inhibitors). However, the veracity of experimental results relies on the correct derivation of the cell line. Literature findings indicate that up to one third of all cell lines have an origin other than that supposed. The most common cause is cross-contamination or misidentification of cell lines. Cross-contamination of cell lines can be the result of poor culture technique when a cell line culture is contaminated with another cell line. Alternatively, cross-contamination of cell lines can be due to clerical error by mislabeling of cell cultures or frozen stocks. In **Chapter 2** the authenticity of EAC cell line TE-7 was investigated. Examination of the TE-7 cell line xenograft revealed the histology of a squamous cell carcinoma. This prompted us to investigate all 15 TE-cell lines, by short tandem repeat (STR) profiling, mutation analyses and array-CGH. The results of these investigations revealed that the currently used TE-7 cell line is not an EAC but a squamous cell carcinoma cell line. Furthermore, cell line TE-7 shared the same genotype as TE-2, -3, -12 and -13. These five TE cell lines should be regarded as one single squamous cell carcinoma cell line of an unknown origin. Recent admixture of the cell

lines in our institute was excluded by an independent investigator (Dr. Y. Morita-Fujimura) who confirmed these results with the TE cell lines present in the cell line bank in Japan using a STR multiplex system. Since the TE-7 cell line is widely used for decades as a model for EAC we investigated independent TE-7 DNA samples obtained from different laboratories. The earliest passage TE-7 DNA (1986) was obtained from the Japanese Cell Resource Centre for Biomedical Research (Dr. Y. Morita-Fujimura). All these TE-7 samples shared the same allelic pattern indicating that cross-contamination has occurred at the site of origin or in an early exchange of cell lines between laboratories.

In **Chapter 3**, we verified the authenticity of all available EAC cell lines. Despite intensive efforts, only 14 permanent cell lines have been established: SEG-1, BIC-1, FLO-1, SK-GT-4, SK-GT-5, BE-3, KYAE-1, OE19, OE33, JH-EsoAd1, OACP4C, OACM5.1 and two newly established cell lines ESO26 and ESO51. In collaboration with the primary investigators who established the cell lines, the original EAC tissues for 13 of the 14 cell lines were traced in pathology archives and made available for study: only the original tissue for cell line BE-3 was not found. The availability of the primary tissues made it possible to authenticate these EAC cell lines by comparing the genotype of the cell line with the genotypes of patient's normal and tumor tissue. Using short tandem repeat (STR) profiling and *TP53* mutation analysis, we proved that the frequently used cell lines SEG-1 and BIC-1 (in more than 100 publications) and the SK-GT-5 cell line are in fact cell lines from other tumor types. Comparison of the genotypes of SEG-1, BIC-1, and SK-GT-5 with genotypes available from databases revealed that SEG-1 is lung carcinoma (large cell lung cancer) cell line H460 and BIC-1 is colorectal adenocarcinoma cell line SW620. The genotype and *TP53* mutation of the SK-GT-5 cell line matched with the tissue from which the cell line SK-GT-2 was derived indicating that cell line SK-GT-5 is actually the gastric fundus carcinoma cell line SK-GT-2. Experimental results based on these contaminated cell lines have led to ongoing clinical trials recruiting EAC patients, to more than 100 scientific publications and at least three NIH cancer research grants and 11 US patents, which emphasizes the importance of our findings. Even more important, we established a set of ten EAC cell lines that are derived from human EACs. All of these 10 verified EAC cell lines, together with their genotyping information, will be deposited in publicly available cell line repositories in the United States, Europe, and Japan, to promote and facilitate future solid research on EAC.

These verified EAC cell lines, together with a number of xenografts, are used in **Chapter 4** to screen for homozygous deletions (HDs) by genome wide single nucleotide polymorphism (SNP) arrays. In total, 61 HDs (range 1-6 per sample) were detected and confirmed by polymerase chain reaction. Besides HDs in common fragile sites and gene deserts, 27 HDs were located in gene containing regions. HDs were noted for known tumor suppressor genes involved in EAC carcinogenesis, including *CDKN2A*, *SMAD4* and *CDH3*/*CDH1*. To our knowledge, this is the first report that describes inactivation of *CDH3*/*CDH1* by homozygous deletion in EAC. Both genes play important roles in cell-cell adhesion. Loss of cell-cell adhesion by

deletion of *CDH3/CDH1* is characterized by the round or spindle shaped appearance of the tumor cells and the capacity of the cells to disseminate (the primary tumors of cell line KYAE-1 and xenograft P104x, metastasized to lung and liver, respectively). Furthermore, 22 new chromosomal regions were detected harboring potentially new tumor suppressor genes involved in EAC carcinogenesis. Two of these regions of homozygous loss, encompassing the *ITGAV* and *RUNX1* gene, were detected in multiple samples indicating a potentially role in the carcinogenesis of EAC. To exclude culturing artifacts, these last two deletions were confirmed by fluorescent in situ hybridization in the primary tumors of which the involved cell lines and xenografts were derived. These findings warrant further investigations on the role and function of these genes in EAC development.

**Clinical aspects of esophageal cancer treatment**

In **Chapter 5,** the clinical outcome of 38 patients who received chemotherapy for carcinoma of the distal esophagus with celiac lymph node involvement was evaluated. The median overall survival of these patients was 16 months. Patients who received chemotherapy alone (N=12) had a median survival of 10 months; patients who underwent additional surgery (N=26) had a median survival of 26 months (log-rank p<0.001). Radical esophagectomy in patients with celiac lymph nodes <1.5 cm and clinical response to chemotherapy is likely to be achieved and contributes to prolonged survival. Besides radical surgical resection, complete or major pathological response to chemotherapy and a negative CLN status in the resection specimen are associated with long-term survival. Assessment of the CLN status after chemotherapy by EUS-guided FNA could be useful to further optimize selection of M1a patients who are most likely to benefit from additional surgery.

**Chapter 6** describes the long-term results of a large randomized controlled trial among patients ESCC who were assigned to chemotherapy followed by surgery or surgery alone. Patients who received preoperative chemotherapy with a combination of etoposide and cisplatin had significantly improved survival as compared to patients who underwent surgery alone (*P*=0.03). In contrast with the general hypothesis that chemotherapy eliminates systemic micro-metastases (and thereby reducing the rate of distant metastasis), the results of this trial indicate that the effect of preoperative chemotherapy is to reduce tumor volume and increase the potential for curative resection. The relevance of this study (even after more than twenty years) is illustrated by the fact that the up-dated results directly have been incorporated into a new meta-analysis on survival after neoadjuvant chemo(radio)therapy by the Australasian Gastro-Intestinal Trials Group (ahead of publication in Lancet Oncology).

In **Chapter 7** we investigated the association between six functional polymorphisms, located in different proto-oncogenes and tumor suppressor genes, and clinical outcome after surgical resection of EAC or ESCC. In a multivariate analysis, patients with EAC carrying the heterozygous *MDM2* (rs2279744) T/G genotype had significantly improved disease free survival as compared with patients carrying the wild-type genotype. Patients with EAC harbouring

the homozygous *CDH1* (rs5030625) GA/GA genotype had a significantly reduced survival as compared with patients carrying the wild-type genotype. Additional prospective studies are necessary to validate both associations and to study the prognostic significance of both germ-line variants.

## CONCLUSIONS

1) Cell line TE-7 is not an EAC but a squamous cell carcinoma cell line (Chapter 2).

2) Cell lines TE-2, -3, -7, -12 and -13 shared the same genotype and should therefore be regarded as one single squamous cell carcinoma cell line of an unknown origin (Chapter 2).

3) Cell lines SEG-1 and BIC-1 (used in more than 100 publications) and the SK-GT-5 cell line are in fact cell lines from other tumor types (Chapter 3).

4) Cell lines FLO-1, KYAE-1, SK-GT-4, OE19, OE33, JH-EsoAd1, OACP4C, OACM5.1, ESO26, and ESO51 are derived from human EACs (Chapter 3).

5) Homozygous deletion is one of the mechanisms by which the *CDH1* and *CDH3* gene is inactivated in EAC (Chapter 4).

6) Homozygous deletions affecting the *ITGAV* and *RUNX1* gene implicate a tumor suppressor function of these genes involved in the carcinogenesis of EAC (Chapter 4).

7) Curative treatment of patients with carcinoma of the distal esophagus and celiac lymph node with preoperative chemotherapy is feasible and contributes in a subset of patients to prolonged survival (Chapter 5).

8) Preoperative chemotherapy with a combination of etoposide and cisplatin significantly improved overall survival in patients with ESCC (Chapter 6).

9) Inter-individual differences in germ-line DNA have an impact on disease free survival of patients with EAC (Chapter 7).

# Chapter 9

## General discussion & Future research

## HUMAN ESOPHAGEAL ADENOCARCINOMA CELL LINES

In the first part of this thesis, we focused on adenocarcinoma of the esophagus, the end-stage of the metaplasia - dysplasia - adenocarcinoma sequence. EACs harbour all genetic abnormalities that have been acquired during this multi-step process. The genetic aberrations identified in EACs could subsequently be investigated in earlier stages of the disease. In this context, cell lines could be powerful tools in the search for genetic alterations that are potentially involved in the carcinogenesis of EAC (as for example shown in chapter 4 of this thesis). These pure populations of tumor cells make detection of genetic changes including gene mutations, deletions, amplifications and translocations much easier. As reviewed in chapter 1 of this thesis, the use of EAC cell lines has emerged in the last five years and contributed to our knowledge about signaling pathways and the effects of different targeted therapies. However, there remains considerable skepticism in the scientific community about the validity resulting from the use of cell lines (1-4). Here, we will discuss four major concerns regarding the use of human cancer cells lines, namely genetic instability during long time culture, selective growth of subpopulations, lack of interaction with stroma and inflammatory cells, and cross-contamination or misidentification.

### Genetic instability

Genomic instability is a characteristic of probably all human cancers. In sporadic cancers, the molecular basis of genomic instability remains unclear. There are various forms of genomic instability. Most cancers have a form that is called chromosomal instability (CIN) which refers to the high rate by which chromosome structure and number changes over time in cancer cells compared with normal cells (6). Almost all EAC arise in association with CIN that leads to gains, partial loss of chromosomal regions and complete loss of chromosomes (7-9). Chromosomal instability might be caused by *TP53* mutations (10). Inactivation of *TP53* leads to centrosome duplication and these abnormally amplified centrosomes profoundly affect mitotic fidelity, resulting in unequal segregation of chromosomes. The presence of *TP53* mutations in all original tumors of which the EAC cell lines were derived, indicate that genomic instability is already present at time the cell lines were established. This inherent genomic instability and the considerably shorter population-doubling times of cell lines may result in an increased frequency of mutational changes during *in vitro* growth. Only a few studies have compared mutations identified in long-cultured cell lines with their corresponding tumor tissues and demonstrated that the mutation rate during prolonged cell culture is limited (11, 12). More recently, it has been shown that the process of cell culture *in vitro* does not lead to new clonal mutations into colorectal tumor cell lines (13). These findings are in line with observations in cell lines with high rates of ongoing structural and/or numerical chromosomal instability. These cell lines showed a relative stability of the consensus karyotype over many generations (14).

**Selective growth of subpopulations**

Cancer cell lines constantly generate variants with phenotypic and/or genotypic differences from the predominant population caused by exposure to different conditions (such as media, sera, trypsin, carbon dioxide levels, humidity and temperature). Similar to tumor cells *in vivo*, cells *in vitro* adapt their phenotype, by epigenetic (potentially reversible) or genetic mechanisms (irreversible), to the conditions to which they are exposed (15). Variants that are better adapted to the new conditions are likely to be selected.

Little is known about the principles guiding genetic evolution of established cancer cell lines (16). Based on the genetic heterogeneity of most *in vivo* tumors and ongoing genetic instability, it can be assumed that human cancer cell lines are genetically heterogeneous, consisting of multiple sub-clones that can be defined by their genomic profiles. This assumption is needed to understand how cancer cell lines maintain and may change in genotype during *in vitro* growth. Under standard culture conditions the fitness of cells having acquired novel chromosome aberrations, is too low to provide them a survival benefit as compared to the bulk of cell line population. This could also explain the more or less fixed chromosomal instability in tumor cell lines when cultured over time. However, in any situation when the proliferating population is reduced to a very small number of cells (infection, starvation, and other changes in growth conditions), any genetically distinct sub-population that normally would have a prevalence level too low for detection by genomic screening of the original bulk cell line can potentially re-grow into a new population with a different genomic profile than the original bulk. Although this phenomenon rarely occurs in generally fast proliferating cells, one example of a change in genotype is provided by breast cancer cell line MCF7. Analyses of different sub-lines from this cell line, obtained from three independent laboratories, showed that there was a wide genetic variation among the different lines with respect to genomic imbalances and expression profiles (17).

**Lack of interaction with stroma and inflammatory cells**

EACs are composed of an ecosystem of evolving subpopulations, competing and cooperating with each other and other cells in their microenvironment. Cells in culture lack the architectural and cellular complexity of *in vivo* tumors, which include inflammatory cells, vasculature, and other cellular and non-cellular components. Cell line xenografts facilitate research on the complex tumor cell –stromal interactions that facilitate tumor formation and progression. However, the requirement for immuno-compromised hosts, transplantation of human EAC cells into a foreign microenvironment and a lack of co-evolvement of the epithelial and stromal compartments of the tumor hamper translation of results into the human *in vivo* situation.

**Cross-contamination or misidentification**

In Chapter 2 & 3 of this thesis we verified all to us known EAC cell lines, by comparing the genotype of the cell lines with that of their corresponding original tissue. Despite these efforts, cross-contamination or misidentification of EAC cell lines is a continuous threat. To prevent use of cross-contaminated cell lines, the short tandem repeat (STR) profiles of all EAC cell lines provide a useful reference against which investigators can compare data. DNA profiling provides a simple, cheap and universal solution applicable to all human cell lines. With proper and regular monitoring for provenance and contamination, cell lines admixture can be largely prevented or detected and corrected before it causes major harm.

Genetic instability, artificial and selective growth of subpopulations, and lack of interaction with stromal components are inherent features of human cancer cell lines. These features should be taken into account when using cell lines as model for original tumors. The best prevention to overcome, at least partially, most of these concerns is to use early passage cell cultures. However, from most cell lines early passage cultures are not available and the available cell cultures have been passaged hundreds to thousands of times. So this precaution may be applied in the future with newly generated cell lines but does not apply to the currently available cell lines from the past.

**Future directions**

Although studies of cancer lines have made major contributions to our understanding of EAC biology and pathogenesis and to translational research, several issues need to be addressed:

1) More EAC cell lines need to be established, especially cell lines from more differentiated tumors.
2) Efforts should be undertaken to compare genetic alterations present in the cell lines with that in the original primary tumor. This could possibly add to the discussion on how close these EAC cell lines resemble the original *in vivo* tumors.
3) New culture systems need to be developed to study the interaction between tumor cells and non-malignant cells (for example tumor-derived fibroblasts).
4) Methods for the identification, isolation, and manipulation of the stem cell component of cultured tumor cells are needed.
5) A public database is needed to store data from genome-wide sequencing studies on these cell lines that are currently available and/or expected in the near future.

## CLINICAL ASPECTS OF ESOPHAGEAL CANCER TREATMENT

In the second part of this thesis, we addressed various aspects of esophageal cancer treatment (comprising squamous cell carcinoma and adenocarcinoma). Here, we focus on strategies

that translate molecular biology findings to clinical applications which may improve treatment and ultimately lead to prolonged survival of patients with cancer of the esophagus.

**Identifying patients at risk for esophageal cancer**

Individual variation in cancer risk has not only been related to environmental factors, including life style, but also to the genetic constitution. Sequence analysis of the human genome has disclosed a wide array of genetic variation, or polymorphisms, with the single nucleotide polymorphism (SNP) as the most common type. It has been speculated that combinations of polymorphisms or low-penetrance alleles can contribute to an individual's cancer risk. Genome wide association studies (GWAS) are proving adept at identifying common variants contributing to the inherited component of common diseases (18, 19). In this respect, genome wide association studies GWAS, like the Rotterdam Study (a prospective, population-based cohort study), that link genetic variation across the entire human genome to for example esophageal cancer, could potentially lead to the identification of set of polymorphisms that determine esophageal cancer risk. However, the number of esophageal cancer cases within these population-based studies is low (due to the relatively low incidence of esophageal cancer), which hampers the interpretation of differences in genotype distribution of SNPs between cases and controls. Therefore, consortia, like the Barrett's and Esophageal Adenocarcinoma Consortium (BEACON; http://tlvnet.net/beacon/) that combines data from the vast majority of the well-designed largely population-based studies in the world, are needed to determine a genetic risk profile for the development of esophageal cancer.

Population wide screening for patients with undiagnosed Barrett's esophagus could also contribute to early identification of patients at risk for esophageal adenocarcinoma. However, there are major feasibility and cost implications for the wide-scale application of screening using the "gold standard" endoscopy. Recently, a non-endoscopic screening test for Barrett's esophagus has been introduced (20, 21). A cytology sponge is compressed and encased in a gelatin capsule attached to a string. The capsule is swallowed and after a few minutes in the stomach, the liberated sponge is dragged back up the esophagus. Then the cytology sample is stained for trefoil factor 3, which is a strong diagnostic marker of esophageal columnar metaplastic cells (20). A pilot study in 96 controls and 36 BE patients found this test to have a sensitivity of 78% and a specificity of 94% for presence of Barrett's esophagus. Generalisation of these results requires a multicenter study to provide more robust estimates of diagnostic accuracy and to perform an in-depth cost effectiveness analysis.

**Improving patient selection for neoadjuvant chemo(radio)therapy**

Because not all patients with advanced esophageal cancer do benefit from preoperative chemo(radio)therapy, identification of subsets of patients who do or do not benefit from such a treatment is warranted. Identifying host genetic variations that contribute to drug efficacy and/or the risk of toxicity will provide a means with which to tailor therapy. Genetic

polymorphisms in genes involved in drug metabolism, drug targets, and DNA repair may contribute significantly to the variability of drug efficacy and toxicity (22). It is quite rare that a SNP result in significant changes in the ability to metabolize drugs. A combination of several SNPs in components of a 'biological' pathway or 'pharmacological' pathway might significantly influence therapeutic response. Till now, all pharmacogenetic studies on esophageal cancer are hypothesis driven and used a candidate gene approach that links the effects of only one or a few SNPs in one or more specific gene(s) to clinical outcome (23, 24). New pharmacogenetic studies using a whole-genome approach are needed and may be a major step towards individualized cancer therapy. Phase III studies, as for example the CROSS trial (25), could be used to link genome-wide variants (using SNP arrays) to toxicity or drug efficacy.

Understanding the molecular characteristics of a patient's tumor can increase the likelihood of selecting the treatment most likely to help. Somatic acquired mutations in the cancer genome are most likely to affect drug response. High throughput technology has allowed the complete sequencing of the cancer genome (26, 27), and may be used to investigate the association between genome wide acquired alterations and response to chemo(radio) therapy. This technique could be applied on preoperative tumor biopsies obtained from a cohort of patients that is randomized between surgery alone and preoperative chemo(radio) therapy (such as the CROSS trial). However, these biopsies do not contain the large amounts of high quality DNA that is needed. With great advances in the technique of genome-wide sequencing, it is to be expected that sequencing on lower amounts of DNA will become feasible in the near future (28).

Although gene-expression profiling of esophageal cancer patients has led to the establishment of a prognostic signature of four genes that is able to identify a cohort of patients with a 5-year survival of 58% (29), a reliable predictive gene expression signature for response to neoadjuvant treatment has not yet been identified. It seems that response to anticancer drugs is more difficult to predict by molecular tests than prognosis is. One of the main reasons for this difficulty is that resistance to anticancer agents can result from a variety of mechanisms. Probably, there is not a single gene-expression profile that correlates with resistance to a certain chemotherapeutic drug (30). However, in analogy with breast cancer (31), the esophageal cancer prognostic signature could also have a response prediction value.

**Discovery of new cancer drugs in patients with esophageal cancer**

An array of new high-throughput technologies (such as DNA sequencing, single-nucleotide polymorphism genotyping and gene expression microarrays) has been implemented in drug development efforts. This led to the identification of different cellular targets for treatment of esophageal cancer (as reviewed by (32)). However, a drawback of most of these approaches is that the data generated are mostly correlative and therefore do not directly identify the driver event among the many genetic alterations present in each cancer. Functional genetic

approaches, such as loss-of-function genetic screens, could contribute to the identification of completely new classes of drug targets for treatment of esophageal cancer (33). There are four approaches to finding novel drug targets through RNAi based genetic screens: pathway, phenotype, synthetic lethal and *in vivo* screens (34). These loss-of-function genetic screens in cultured cells (as for example esophageal cancer cell lines) use short hairpin RNA (shRNa) libraries to target a specific gene family or a subset of vectors comprising components of a molecular pathway of interest. shRNA are processed intracellularly into short duplex RNAs having short interfering RNA-like properties that lead to silencing of specific gene expression. A large population of cultured cells (as for example esophageal cancer cells) is infected with a shRNA library and divided into two populations. After sufficient numbers of cells are infected (in order to have the entire library of vectors represented) two replicate cell populations are created. One population is used as reference and left untreated, whereas the other population is treated with the stimulus of interest. After phenotypic selection has taken place a bar code identifier, specific for each shRNA, can be retrieved by PCR. Hybridization of the bar code identifier to a specific microarray reveals the abundance of shRNA in both the reference and experimental population. This approach could lead to the identification of druggable genes in esophageal cancer relevant pathways.

**Individualized cancer treatment for esophagel cancer patients**

Probably the most tailored and individualized form of translational drug development is a "personalized tumorgraft" (35). Tumors taken from patients are implanted directly into immunodeficient mice. Mice carrying the patient's tumor are then treated with several cancer drugs and drug combinations to help identify which treatment regimen is likely to be most effective for the specific cancer. This concept has been proven successful in patients with pancreatic cancer (36), but could also be applied in patients with esophageal cancer. However, it is time consuming (the total process takes 6 – 8) and very costly (commercial prize of personalized tumorgrafts for one individual costs about $100,000 (37)).

Very recently, a new initiative has been launched by the center for personalized cancer treatment (CPCT), a collaborative effort between the UMC Utrecht, Netherlands Cancer Institute, Amsterdam and the Erasmus MC, University Medical Center Rotterdam. The goal is to integrate exome sequencing of 2000 genes with clinical decision making. The mutational profile, which should be obtained within two weeks after a diagnostic biopsy has been taken, enables the clinician to determine which cancer therapy suits best for the individual patient. Also esophageal cancer patients will be included in this initiative. However, it is to be expected that it will take years to speed up the process of sequence analyses and to link mutation profiles to response on therapy.

## SUMMARY AND CONCLUSIONS

Genome wide sequencing will speed up the identification of genetic variants in the germline that can contribute to risk stratification for esophageal cancer. For example, comparison of these variants between a control cohort of asymptomatic individuals (perfectly matched for age, smoking, alcohol, body-mass index and race) and a population with Barrett's esophagus could result in the establishment of a genetic risk profile for Barrett's esophagus. This genetic signature could contribute to the identification of patients with undiagnosed Barrett's esophagus, which ultimately reduce the morality of EAC. In parallel, such a profile could also be established of patients with Barrett's esophagus at low risk of neoplastic progression. This allows the identification of the large proportion of people who are unlikely to develop EAC, allowing them to avoid or minimize worrisome, costly and risky endoscopic surveillance and interventions.

Genome wide sequencing shall also rapidly increase the number of genetic alterations detected in the esophageal cancer genome. It will be challenging to distinguish "driver" mutations underlying carcinogenesis of esophageal cancer from biologically neutral "passenger" alterations. Ultimately, identification of these driver mutations will result in the development of biomarkers that can contribute to early disease detection. New chemotherapeutic drugs will be developed targeting these driver mutations. Because the failure rate following current multimodality strategies remains unacceptably high, these targeting agents might be integrated rapidly into current clinical trial design. In addition, these essential genetic alterations in the esophageal cancer genome could also serve as predictive markers for response to chemo(radio)therapy and allow treatment to be tailored to individual patients rather than the current 'one fits all' approach.

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# Chapter 10

## Summary in Dutch / Samenvatting in het Nederlands

## SAMENVATTING

Slokdarmkanker is de zevende aan kanker gerelateerde doodsoorzaak in de westerse wereld. De twee belangrijkste histologische subtypen van slokdarmkanker zijn adeno- en plaveisecelcarcinomen. In Nederland worden per jaar ongeveer 1500 patiënten gediagnosticeerd met een tumor in de slokdarm (http://www.nationaalkompas.nl). De laatste decennia neemt de incidentie van slokdarmkanker snel toe. Dit is voornamelijk het gevolg van een stijging van het aantal patiënten met een adenocarcinoom van de gastro-oesophageale overgang (zowel het adenocarcinoom van distale slokdarm als de maagcardia). De belangrijkste risicofactor voor het ontwikkelen van een adenocarcinoom van de gastro-oesophageale overgang is (langdurige) reflux van maagzuur en duodenumsappen in de slokdarm. Dit kan uiteindelijk resulteren in een zogenaamde Barrett's slokdarm, waarbij het normale plaveiselcel epitheel van de slokdarm is vervangen door metaplastisch cilindrisch epitheel. Er zijn sterke aanwijzingen dat adenocarcinomen van de gastro-oesophageale overgang ontstaan uit dit (premaligne) Barrett epitheel door een opeenstapeling van genetische veranderingen die in de loop van de tijd plaatsvinden. De meeste fundamentele en translationele studies hebben als doel een beter inzicht te krijgen in de genetische veranderingen die ten grondslag liggen aan het adenocarcinoom van de slokdarm-maag overgang (het eindstadium van de "metaplasie – dysplasie – carcinoom" sequentie). Dit heeft geleid tot meer kennis over de moleculaire mechanismen die ten grondslag liggen aan het ontstaan van een adenocarcinoom van de slokdarm, maar translatie van deze resultaten in betere patiënten zorg en prognose is vooralsnog beperkt.

De studies in het eerste gedeelte van deze thesis hebben als doel de authenticiteit van alle slokdarm adenocarcinoom cellijnen te onderzoeken om zo een betrouwbare set cellijnen samen te stellen die vervolgens kunnen worden gebruikt voor fundamenteel en translationeel onderzoek. Studies in het tweede gedeelte van deze thesis richten zich op verschillende aspecten van behandeling van patiënten met slokdarmkanker.

**Slokdarm adenocarcinoom cellijnen**

Er bestaan een aantal strategieën om de genetische veranderingen, die ten grondslag liggen aan het ontstaan van tumoren, te bestuderen. Een van de manieren om meer inzicht te krijgen in welke genen betrokken zijn bij het de carcinogenese van adenocarcinomen van de slokdarm, is erfelijkheidsonderzoek (in analogie van het mamma- en coloncarcinoom). Echter het familiair voorkomen van slokdarm adenocarcinomen (al dan niet in combinatie met een Barrett's slokdarm) is zelden gerapporteerd. Een ander strategie is het bestuderen van diermodellen, maar tot op heden is geen geschikt diermodel beschikbaar. Voor onderzoek naar de genetische achtergrond van dit type kanker is men voornamelijk aangewezen op (gearchiveerd) patiënten materiaal. Een veel gebruikt onderzoeksmodel, dat een directe

afgeleide is van patiënten materiaal, zijn cellijnen. Cellijnen zijn zeer geschikte modellen om de biologische en moleculaire afwijkingen van kankercellen te onderzoeken. Een belangrijk voordeel van menselijke kankercellijnen is de beschikbaarheid van pure populaties kankercellen zonder bijmenging van normaal weefsel (dit vereenvoudigd de detectie van genetische afwijkingen). Een ander voordeel is dat cellijnen een oneindige bron van materiaal zijn. Daarnaast kunnen cellijnen dienen als model voor het testen van conventionele en nieuwe behandelingsmodaliteiten. Er kleven ook nadelen aan het gebruik van cellijnen, zoals de selectie van (kleine) subpopulaties van cellen die kan optreden tijdens het kweken waardoor de cellen niet meer representatief zijn voor de oorspronkelijke tumor. Daarnaast ontbreekt de interactie tussen tumorcellen en stromale cellen (zoals fibroblasten en macrofagen), die in de originele tumor wel aanwezig is.

In **Hoofdstuk 1** wordt een overzicht gegeven van de beschikbare slokdarm adenocarcinoom cellijnen. Tevens wordt de bijdrage die deze cellijnen hebben geleverd aan de huidige inzichten in de genetische achtergrond en de ontwikkeling van nieuwe behandelingsstrategieën beschouwd. Er zijn relatief weinig slokdarm adenocarcinoom cellijnen beschikbaar voor fundamenteel onderzoek. Hiervoor zijn een tweetal redenen. Ten eerste is het maken van cellijnen gebaseerd op trial en error, waarbij het continue zoeken is naar de ideale omstandigheden waaronder de kankercellen *in vitro* willen groeien. Het succes percentage is laag, waardoor het veel geld, tijd en inspanning kost om dergelijke cellijnen te maken. Ten tweede, de grote beschikbaarheid van tumorweefsel (uit receptiepreparaten) maakt dat onderzoekers veel minder gemotiveerd zijn om cellijnen te vervaardigen. In de afgelopen decennia hebben slokdarm adenocarcinoom cellijnen een belangrijke rol gespeeld bij functioneel onderzoek naar onco- en tumor suppressor genen zoals *ERBB2* en *c-MET*. Daarnaast spelen ze een cruciale rol bij het onderzoek naar de effecten van maagzuur en galzouten op tumor cellen. Zo is gebleken dat de arachidonzuur cascade (met als belangrijkste gen Cyclooxygenase-2 (COX-2) gen) geactiveerd is in tumorcellen die worden blootgesteld aan maagzuur en galzouten. De cellijnen vervullen ook een belangrijke rol bij de ontwikkeling van nieuwe "targeted therapies" voor het adenocarcinoom van de slokdarm (geneesmiddelen die zich richten op specifieke afwijkingen in tumorcellen), zoals trastuzumab (monoclonaal antilichaam tegen *ERBB2*), bortezomib (proteasoomremmer) en alvocidib (cycline-dependent kinase remmer).
Een belangrijke voorwaarde voor onderzoek met cellijnen is de authenticiteit van de gebruikte cellijnen, omdat een verkeerde identiteit van een cellijn kan leiden tot het trekken van verkeerde conclusies. Uit onderzoek is gebleken dat ongeveer 30% van alle cellijnen in de wereld zijn afkomstig van een ander tumor type dan wordt verondersteld. Een van de oorzaken is cross-contaminatie tussen cellijnen. Dit kan het resultaat zijn van een slechte kweek techniek, waarbij de ene cellijn wordt gecontamineerd met een andere cellijn, maar ook van een administratieve fout waardoor bijvoorbeeld cellijnen verkeerd worden gelabeld. In **Hoofdstuk 2 & 3** wordt de authenticiteit van alle slokdarm adenocarcinoom cellijnen

onderzocht. De belangrijkste technieken die hiervoor werden gebruikt zijn short tandem repeat (STR) profiling, xenografing en mutatie analyse. Met behulp STR profiling (ook wel DNA fingerprinting genoemd), een techniek die afkomstig is uit forensisch onderzoek, werd het genotype van de cellijnen bepaald. Het fenotype van de cellijnen werd onderzocht door cellen subcutaan te implanteren bij immuun-deficiënte naakte muis (ook wel xenografting genoemd). Beschreven mutaties werden gebruikt om verschillen dan wel overeenkomsten aan te tonen tussen cellijnen. In **Hoofdstuk 2** wordt de authenticiteit van slokdarm adenocarcinoom cellijn TE-7 onderzocht. Deze cellijn is onderdeel van een serie van 15 cellijnen welke, behoudens cellijn TE-7, allen afkomstig zijn van plaveiselcelcarcinomen van de slokdarm. Xenografting van cellijn TE-7 toonde niet het fenotype van een adenocarcinoom, maar dat van een plaveiselcelcarcinoom. Daarnaast bleek dat het genotype van cellijn TE-7 overeenkomt met dat van vier andere TE-cellijnen. Ook mutatie analyse van het *CYCLIND1* gen bevestigde dat vijf TE-cellijnen een dezelfde mutatie bezitten. Het gevonden genotype van cellijn TE-7 werd bevestigd door een aantal andere laboratoria in de wereld, die ook cellijn TE-7 gebruikten als *in vitro* model van het slokdarm adenocarcinoom. Op basis van deze gegevens kan worden geconcludeerd dat cellijn TE-7 niet representatief is voor een adenocarcinoom van de slokdarm. Een vroege passage van cellijn TE-7 toonde hetzelfde genotype als dat van de huidige TE-7 cellijn. Hieruit kan worden afgeleid dat cross-contaminatie waarschijnlijk al vroeg heeft plaatsgevonden, mogelijk al in het laboratorium waar de cellijn is gemaakt of in een vroege uitwisseling met andere laboratoria.

In **Hoofdstuk 3** wordt de authenticiteit van alle beschikbare slokdarm adenocarcinoom cellijnen onderzocht. In samenwerking met de makers van de cellijnen werd van bijna alle cellijnen het originele patiënten materiaal opgespoord. De beschikbaarheid van dit materiaal maakt het mogelijk het genotype van de cellijn te vergelijken met dat van de patiënt waar de cellijn van afgeleid is. Uit dit onderzoek blijkt dat twee veel gebruikte cellijnen SEG-1 en BIC-1, geen slokdarm adenocarcinoom cellijnen te zijn maar longkanker cellijn H460 en dikkedarm kanker cellijn SW620, respectievelijk. Daarnaast blijkt dat het genotype van SK-GT-5 overeenkomt met dat van maag adenocarcinoom cellijn SK-GT-2. De onderzoeker die cellijnen SEG-1 en BIC-1 heeft gemaakt bevestigde dat contaminatie heeft plaatsgevonden in de eerste passages van de cellijnen. Experimentele resultaten gebaseerd op het gebruik van deze gecontamineerde cellijnen heeft geleid tot klinische trials bij patiënten met een adenocarcinoom van de slokdarm. Daarnaast zijn zeker drie Amerikaanse onderzoeksbeurzen, 11 patenten en meer de 100 wetenschappelijke publicaties gebaseerd op werk met deze cellijnen. Van tien slokdarm adenocarcinoom cellijnen kon worden bevestigd dat ze daadwerkelijk zijn afgeleid van het originele patiënten materiaal. Onder deze cellijnen bevinden zich twee nieuwe cellijnen (ESO26 & ESO51). De gevalideerde cellijnen worden gedoneerd aan een internationale cellijn bank (ATCC) om het onderzoek naar slokdarm adenocarcinomen te stimuleren.

**Hoofdstuk 4** beschrijft het identificeren van homozygote deleties in de gevalideerde set slokdarm adenocarcinoom cellijnen en negen xenografts. Wanneer op beide chromosomen

een stuk erfelijk materiaal (DNA) weg is, spreekt men van een homozygote deletie (HDs). Gebieden die homozygoot gedeleteerd raken kunnen belangrijke aanwijzing zijn voor de lokalisatie van een tumor suppressor gen in dat betreffende gebied. Cellijnen en xenografts zijn pure populaties kankercellen (dit in tegenstelling tot patiënten materiaal dat naast tumorcellen ook een substantiële hoeveelheid normale cellen bevat) waardoor detectie van HDs wordt vereenvoudigd. Single nucleotide polymorphism (SNP) arrays werden gebruikt om HDs in kaart te brengen. In totaal werden 61 HDs gedetecteerd, waarvan er 26 HDs gelegen waren in zogenaamde breekbare regio's (gebieden die gevoelig zijn voor breuken in het DNA), acht HDs waren gelokaliseerd in gebieden zonder bekende genen. Van de 27 HDs in gebieden die een of meerdere genen bevatten, lagen er 22 in gebieden met potentieel nieuwe tumor suppressor genen. Twee van deze gebieden, die de genen *ITGAV* (coderend voor de integrin alpha chain V) en *RUNX1* (coderend voor runt-related transcription factor 1) gedeeltelijk omvatten, werden in meerdere sampels aangetoond. Deze twee deleties werden met behulp van fluorescence in situ hybridization (FISH) gevalideerd in het originele patiënten materiaal waarvan de van de cellijnen en de xenografts waren afgeleid. De potentiële rol van deze twee nieuwe tumor suppressor genen in de carcinogenese van het adenocarcinoom van de slokdarm verdient verder vervolgonderzoek.

**Klinische aspecten van slokdarmkanker behandeling**

De behandeling van patiënten met een slokdarm is complex en dient multidisciplinair te worden benaderd. Chirurgische resectie van de tumor is en blijft de belangrijkste hoeksteen voor curatieve behandeling voor patiënten zonder metastasen op afstand en/of lokaal irresectabele tumorgroei. Tegenwoordig worden de meeste patiënten met alleen lokale tumoruitbreiding behandeld met preoperatieve chemotherapie of chemoradiotherapie. Ondanks dat wereldwijd (en ook in Nederland) steeds meer gekozen wordt voor preoperatieve behandeling met chemoradiotherapie, worden patiënten in bijvoorbeeld het Verenigd Koninkrijk standaard behandeld met preoperatieve chemotherapie. De gedachte is dat preoperatieve chemotherapie niet alleen bijdraagt aan loco-regionale controle, maar dat door het systemische effect ook micrometastasen kunnen worden geëlimineerd en zo kan bijdragen aan een verbetering van overleving.

In **Hoofdstuk 5** wordt onderzocht wat de bijdrage is van preoperatieve chemotherapie bij patiënten met een tumor in de distale slokdarm en voor tumor verdachte lymfklieren nabij de truncus coeliacus. Volgens de huidige TNM classificatie worden deze lymfklieren geduid als metastasen op afstand (M1a). Er bestaat discussie of deze categorie patiënten nog in aanmerking komt voor in opzet curatieve behandeling. In een periode van acht jaar werden 38 patiënten met een tumor in het distale gedeelte van de slokdarm en voor tumor verdachte lymfklieren nabij de truncus coeliacus (met een diameter >1.5cm) behandeld met preoperatieve chemotherapie. De mediane overleving van alle patiënten was 16 maanden. In totaal kwamen 26 patiënten in aanmerking voor resectie, waarbij in 68% een microscopi-

sche radicale resectie werd bereikt. Histo-pathologisch onderzoek toonde bij 12 patiënten geen tumor aan in de lymfklier(en) nabij de truncus coeliacus. Bij acht van hen waren er duidelijk tekenen van tumor regressie (door chemotherapie). Deze acht patiënten hadden een significant betere overleving dan patiënten met tumor positieve coeliacus klieren in het resectiepreparaat. Samenvattend, patiënten met een tumor in de distale slokdarm en voor tumor verdachte lymfklieren nabij de truncus coeliacus hebben een slechte prognose. Preoperatieve chemotherapie kan tumor positieve lymfklieren nabij de truncus coeliacus steriliseren en daarmee bijdragen aan een verlengde overleving na chirurgische resectie. Het opnieuw stadieren van deze lymfklieren na chemotherapie, door bijvoorbeeld endo-echografisch geleide punctie, zou kunnen bijdragen aan het selecteren van patiënten die het meeste voordeel hebben van chirurgische resectie.

**Hoofdstuk 6** beschrijft de lange termijn resultaten van een gerandomiseerde studie waarbij patiënten met plaveiselcelcarcinoom van de slokdarm werden gerandomiseerd voor preoperatieve chemotherapie gevolgd door chirurgische resectie of alleen chirurgische resectie. Deze studie laat een significant overlevingsvoordeel zien voor patiënten die worden behandeld met preoperatieve chemotherapie ($P$=0.03). In tegenstelling tot de algemene veronderstelling dat chemotherapie systemische micrometastase elimineert, tonen de resultaten van deze studie, dat het effect van preoperatieve chemotherapie met name is gelegen in het reduceren van tumor-volume en dat daarmee de kans op een radicale resectie wordt vergroot. De resultaten van deze studie, die in de vroege jaren negentig is uitgevoerd, zijn alleen in abstract vorm beschreven. De vele citaties van dit abstract en de voort durende discussie over de waarde van preoperatieve chemotherapie in patiënten met slokdarmkanker maken dat publicatie van de studieopzet en de uiteindelijke resultaten nog steeds relevant zijn.

In **Hoofdstuk 7** wordt de relatie onderzocht tussen zes SNPs in verschillende onco- en tumorsuppressor genen en ziekte vrije overleving bij patiënten die chirurgische resectie ondergingen in verband met een adeno- of plaveiselcelcarcinoom van de slokdarm. De genen waarin de onderzochte polymorphismen gelokaliseerd zijn, zijn allen betrokken bij de carcinogenese van slokdarmkanker. De SNPs die werden geselecteerd, *AURKA* (rs2273535), *ERBB2* (rs1136201), *MDM2* (rs2279744), *CDH1* (rs5030625), *CDKN2A* (rs11515) en *TP73* (rs2273953), leiden tot functionele veranderingen in genexpressie. In deze retrospectieve studie werden 346 patiënten, die tussen 1996 en 2001 een resectie van de slokdarm ondergingen in verband met slokdarmkanker, gegenotypeerd. Muli-variate analyse (met correctie voor preoperatief gewichtsverlies, tumor lengte, aanwezigheid van Barrett's epitheel, radicaliteit van de resectie en het pathologische TNM stadium) toonde aan dat patiënten met een adenocarcinoom van de slokdarm die het heterozygote *MDM2* T/G genotype bezitten een significant langere ziekte-vrije overleving hebben dan patiënten met het wild-type *MDM2* (T/T) genotype. Patienten met een adenocarcinoom van de slokdarm en het homozygote *CDH1* GA/GA genotype hadden een significant kortere ziekte-vrije overleving vergeleken met patiënten met het

wild-type *CDH1* G/G genotype. Deze studie laat zien dat de genetische constitutie van de patiënt ook medebepalend kan zijn voor de klinische uitkomst na na chirugische resectie. Ook hiervoor geldt dat het niet waarschijnlijk is dat een variant, maar een combinatie en een veelvoud van verschillende varianten, mede een bijdrage levert aan ziekte-vrije overleving. Additionele prospectieve studies zijn nodig om de beschreven associaties te valideren.

## CONCLUSIES

1) Cellijn TE-7 is geen slokdarm adenocarcinoom cellijn, maar een plaveiselcelcarcinoom cellijn (Hoofdstuk 2).

2) Cellijn TE-3, -3, -7, -12 en -13 hebben hetzelfde genotype en moeten daarom worden beschouwd als een plaveiselcel carcinoom cellijn van onbekende oorsprong (Hoofdstuk 2).

3) Cellijnen SEG-1, BIC-1 en SK-GT-5 zijn cellijnen van andere tumortypen (Hoofdstuk 3).

4) Cellijnen FLO-1, KYAE-1, SK-GT-4, OE19, OE33, JH-EsoAd1, OACP4C, OACM5.1, ESO26, and ESO51 zijn afkomstig van adenocarcinomen van de slokdarm (Hoofdstuk 3).

5) Homozygote deleties zijn een van de mechanismen waarop de genen *CDH1* en *CDH3* kunnen worden geïnactiveerd in adenocarcinomen van de slokdarm (Hoofdstuk 4).

6) Homozygote deleties van gebieden waarin de genen *ITGAV* en *RUNX1* gelegen zijn wijzen op een mogelijke tumor suppressor rol van beide genen in de ontwikkeling van een adenocarcinoom van de slokdarm (Hoofdstuk 4).

7) Patienten met een carcinoom in het distale deel van de slokdarm met uitzaaiingen naar lymfklieren rond de truncus coeliacus komen in aanmerking voor in opzet curatieve behandeling (Hoofdstuk 5).

8) Preoperatieve chemotherapie met een combinatie van etoposide en cisplatinum verbetert de overleving van patiënten met een plaveiselcelcarcinoom van de slokdarm significant (Hoofdstuk 6).

9) Individuele verschillen in germ-line DNA hebben impact op de ziekte-vrije overleving van patiënten met een adenocarcinoom van de slokdarm (Hoofdstuk 7).

# Appendices

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Dr Dinjens, zeer gewaardeerde co-promotor, beste Winand, dank voor je geweldige begeleiding. Je bent een fantastisch mens, altijd enthousiast, stimulerend en inspirerend. De relaxte en ongedwongen sfeer op het laboratorium maakte dat ik mij er direct thuis voelde. In deze ambiance voelde ik mij ook altijd vrij om zeer laagdrempelig met nieuwe ideeën te komen, die altijd in alle openheid werden besproken en vaak ook leidde tot mooie resultaten. Met jouw geweldige contactuele eigenschappen heb je een enorm netwerk opgebouwd waarvan je ook mij hebt laten profiteren. Heel veel dank hiervoor. Ondanks het feit dat je een "DNA-boer" bent, heb je ook veel oog voor ontwikkeling van andere velden van onderzoek. Jouw zorgvuldige wetenschappelijke manier van werken, namelijk het links en rechtsom bevestigen van je resultaten, neem ik graag van jouw over. Ontzettend bedankt voor drie onvergetelijke jaren van onderzoek! Hopelijk kunnen wij onze samenwerking op de een of andere manier blijven continueren.

De leden van de kleine commissie, prof. dr. Fodde, prof. dr. van Lanschot en prof. dr. Kuipers, dank voor uw bereidheid om dit dit proefschrift op zijn wetenschappelijke waarde te beoordelen en voor het geven van waardevol commentaar. De leden van de grote commissie, de hooggeleerden prof. dr. Bosman, prof. dr. Trapman en dr. Wijnhoven, dank voor uw bereidheid om zitting te nemen in de grote commissie.

Daarnaast wil ik de afdeling heelkunde van het Erasmus MC bedanken. Professor van Lanschot, uw komst naar het Erasmus MC heeft een extra impuls gegeven aan het onderzoek naar slokdarmkanker. De rechtdoorzee benadering van u waardeer ik zeer. Dr Wijnhoven, beste Bas, dank dat je als deskundige in mijn promotie commissie wilt plaatsnemen. Het is een eer te participeren in een onderzoekslijn waarin jij ook ooit bent begonnen. In dit kader wil ik ook dr Koppert bedanken, beste Linetta, dank voor je nuttige adviezen vooral in het begin van mijn onderzoekstijd. Beste Anneke, dank voor je begeleiding bij het administratieve gedeelte van de laatste loodjes. Beste Conny, dank voor het altijd snel reageren op alle vragen die ik je stelde omtrent de Rotterdamse slokdarm database.

Ook wil ik de afdeling pathologie bedanken. Allereerste alle medewerkers van het Dinjens laboratorium. Beste Ronald, jij verdient ook een ere plaats in dit dankwoord. Zonder jou inzet was dit boekje er (nog) niet geweest. Jouw technische kennis en kunde waren onmisbaar. Als ik weer iets (te) snel af wilde hebben was je altijd bereid om te helpen. Vooral je kennis van CGH, SNP-arrays en FISH is goud waard! Jou en Brechtje moet ik ook bedanken voor de wekelijkse slokdarmwerkbespreking, waarin alle resultaten en onderzoeksplannen de revue passeerden. Erik Jan, beste kamergenoot, dank voor de gezellige jaren. Jij staat altijd open voor een praatje of discussie over van alles en nog wat. Hoe jij je hoofd boven weet te houden in deze moeilijke tijden, verdient diep respect. Verder ben ik dank verschuldigd aan Erwin, Peggy, Lotte, Ina, Kees, en iedereen die ik nu vergeet. Ludo, dank voor al die blokjes die je voor mij hebt gesneden, je bent onnavolgbaar. Dank voor alle gezelligheid. Ook de medewerkers van de weefselbank, beste Peter, Monique en Bas, jullie hebben een grote bijdrage geleverd aan de illustraties in dit proefschrift door vele coupes voor mij in te scannen. Dank hiervoor! Beste dr. van Dekken, beste Herman, inmiddels al een tijdje vertrokken naar het Amsterdamse, maar ik ben je dank verschuldigd voor het beoordelen van alle coupes.

Beste collega's van het St. Antonius Ziekenhuis Nieuwegein, wat een fantastische jaren hebben we al gehad. Maatschap Interne Geneeskunde, beste dr. Geers, dank dat u mij de tijd heeft gegund om in mijn opleidingstijd dit proefschrift af te kunnen schrijven. Hulde voor jullie wetenschappelijke belangstelling. Gelukkig mag ik mij de komende twee jaar nog Antoniaan noemen!

Beste Bram, dank voor je lange trouwe vriendschap. Wat een mooie tijd hebben we samen gehad in het Groningse. Dit is een van mijn mijlpalen waar ik je graag bij wil betrekken. Super dat je mijn paranimf wil zijn!

Lieve Eric en Sylvia, ik had mij geen grotere betrokkenheid van mijn schoonouders kunnen wensen. Dank voor jullie nimmer afnemende belangstelling, maar bovenal jullie gezelligheid. Beste Olav, Nicole en Hugo, dank voor jullie warme belangstelling en gezelligheid.

Lieve Anneke en Sjoerd, lieve broer en zus, dank voor al jullie steun welke ik niet met een pen kan beschrijven. In dit kader mag ik natuurlijk Fabienne, Sebastiaan en Juna ook niet vergeten te bedanken voor jullie grenzeloze gastvrijheid en hartelijkheid.

Lieve Ray, lieve pap, je hebt me altijd gesteund in al mijn keuzes ook in het schrijven van dit proefschrift, dank hiervoor. Lieve Annemarie, lieve mam, dank voor alles!

Lieve Hester, jou ben ik het meeste dank verschuldigd. De uren die je alleen hebt doorgebracht toen ik boven deze artikelen zat te schrijven, kun je nooit meer inhalen. Eindelijk is het zover: het boekje is af! Daarnaast verdien je alle credits voor het runnen van ons gezinnetje. Lieve Isabel en Esmee, mijn twee kleine meiden, jullie vader heeft vanaf nu alle tijd voor jullie. Ik heb jullie lief!

## LIST OF PUBLICATIONS

Boonstra JJ, van der Velden AW, Beerens EC, van Marion R, Morita-Fujimura Y, Matsui Y, Nishihira T, Tselepis C, Hainaut P, Lowe AW, Beverloo BH, van Dekken H, Tilanus HW, Dinjens WN. Mistaken identity of widely used esophageal adenocarcinoma cell line TE-7. *Cancer Res*. 2007;67:7996-8001.

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Boonstra JJ, van Marion R, Beer DG, Lin L, Chaves P, Ribeiro C, Pereira AD, Roque L, Darnton SJ, Altorki NK, Schrump DS, Klimstra DS, Tang LH, Eshleman JR, Alvarez H, Shimada Y, van Dekken H, Tilanus HW, Dinjens WN. Verification and unmasking of widely used human esophageal adenocarcinoma cell lines. *J Natl Cancer Inst*. 2010;102:271-4.

Boonstra JJ, van Marion R, Tilanus HW, Dinjens WN. Functional polymorphisms associated with disease-free survival in resected carcinoma of the esophagus. *J Gastrointest Surg*. 2011;15:48-56.

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Boonstra JJ, Tilanus HW, Dinjens WNM. Translational research on esophageal adenocarcinoma: from cell line to clinic. *Submitted*.

Boonstra JJ, van Marion R, Douben HJCW., Lanchbury JS, Timms KM, Abkevich V, Tilanus HW, de Klein A, Dinjens WNM. Mapping of homozygous deletions in verified esophageal adenocarcinoma cell lines and xenografts. *Submitted*.

## CURRICULUM VITAE

Jurjen Boonstra werd geboren op 31 mei 1981 te Holten. In 1999 behaalde hij zijn VWO diploma op scholengemeenschap de Waerdenborch te Holten. Datzelfde jaar werd hij uitgeloot voor de studie Geneeskunde en startte met de studie Farmacie te Groningen. Na een aantal maanden kwam hij erachter dat zijn interesse toch echt bij patiënten ligt en niet bij het fabriceren van pillen, waarna hij zijn studie opgaf en ging werken (als verkoper in een buitensportzaak). Het jaar erop (2000) werd hij alsnog ingeloot voor de studie Geneeskunde aan de Rijksuniversiteit Groningen. Zijn keuze co-schap doorliep hij in het Erasmus Medisch Centrum Rotterdam op de afdeling gastro-intestinale chirurgie onder leiding van prof. dr. H. W. Tilanus. In augustus 2006 behaalde hij zijn doctoraal en arts-examen (dit laatste cum laude). Vanaf september 2006 t/m december 2009 was hij werkzaam als arts-onderzoeker op de afdeling heelkunde en pathologie (prof. dr. H. W. Tilanus en dr. W. N. M. Dinjens). Tijdens dit promotietraject verrichte hij twee maanden onderzoek naar de herkomst van slokdarm adenocarcinoom cellijnen in het laboratorium van de afdeling pathologie in het Memorial Sloan Kettering Cancer Center, New York (dr. L. Tang en prof. dr. D. Klimstra). Gedurende de jaren groeide zijn voorkeur voor een meer beschouwend specialisme. Gezien zijn grote interesse in maag-, darm-, en leverziekten (MDL), koos hij ervoor om zich verder te specialiseren tot MDL-arts. Sinds 1 januari 2010 is hij gestart met zijn opleiding in het St. Antonius Ziekenhuis te Nieuwegein (dr. T. Geers/dr. R. Timmer) en zet zijn laatste 2 jaar van zijn MDL-opleiding voort in het UMC Utrecht (prof. dr. P. D. Siersema).

## PHD PORTFOLIO

Name PhD student: J. J. Boonstra
Erasmus MC Department: Surgery/Pathology
Research School: Molecular medicine

PhD period: 2006-2011
Promotor(s): Prof. Dr. H. W. Tilanus
Supervisor: Dr. W. N. M. Dinjens

### 1. PhD training

| | Year | Workload (Hours/ECTS) |
|---|---|---|
| **General courses** | | |
| - Stralingshygiëne, deskundigheidsniveau 5B | 2006 | 4.0 ECTS |
| - NIHES "Prognosis Research" | 2007 | 4.0 ECTS |
| - NIHES "Survival Analysis for Clinicians" | 2008 | 4.0 ECTS |
| **Presentations** | | |
| - Various presentation at research meetings of the department of surgery and pathology | 2007-2010 | |
| **Presentations (inter)national conferences** | | |
| - European Society of Surgical Reseach, Rotterdam "Mistaken identity of widely used esophageal carcinoma cell lines" | 2007 | 1.0 ECTS |
| - International Society for Diseases of the Esophagus, Dublin "Single Nucleotide Polymorphisms and Susceptibility for Cancer of the Esophagus and the Gastro-Esophageal Junction" | 2007 | 1.0 ECTS |
| - 12th Molecular Medicine Day, Rotterdam "Mistaken identity of widely used esophageal adenocarcinoma cell lines TE-7" | 2008 | 1.0 ECTS |
| - Nederlandse Vereniging voor Gastroenterologie, Veldhoven "The MDM2 promoter SNP285C/SNP309G haplotype is associated with susceptibility for oesophageal squamous cell carcinoma and related to poor overall survival after surgical resection" | 2009 | 1.0 ECTS |
| **Poster presentations** | | |
| - Frontiers in Gastrointestinal Cancer, Keystone Symposia, Beijing "Single nucleotide polymorphisms and susceptibilty for cancer of the esophagus and the gastro-esophageal junction" | 2007 | 1.0 ECTS |

| | | |
|---|---|---|
| - Frontiers in Gastrointestinal Cancer: Keystone Symposia, Beijing<br>"Mistaken identity of widely used esophageal adenocarcinoma cell lines TE-7" | 2007 | 1.0 ECTS |
| - World Congress of the International Society for the Diseases of the Esophagus, Budapest<br>"Activation of the Mammalian Target of Rapamycin (mTOR) in Esophageal Adenocarcinoma" | 2008 | 1.0 ECTS |
| - United Gastroenterology Week, Wenen<br>"Mistaken identity of widely used oesophageal adenocarcinoma cell lines" | 2008 | 1.0 ECTS |
| - United Gastroenterology Week, Londen<br>"The MDM2 promoter SNP285C/SNP309G haplotype is associated with susceptibility for oesophageal squamous cell carcinoma and related to poor overall survival after surgical resection" | 2009 | 1.0 ECTS |
| - United Gastroenterology Week, Londen<br>"Inhibition of mTOR in oesophageal adenocarcinoma cell lines seems to be more effective in cell lines with aberrations in the PI3K/PTEN/mTOR pathway" | 2009 | 1.0 ECTS |
| - United Gastroenterology Week, Barcelona<br>"Screening for homozygous deletions in verified oesophageal adenocarcinoma cell lines and xenografts" | 2010 | 1.0 ECTS |
| - Nederlandse Vereniging voor Gastroenterologie, Veldhoven<br>"Verification and Unmasking of Human Esophageal Adenocarcinoma Cell Lines" | 2009 | 1.0 ECTS |

**Other**

| | | |
|---|---|---|
| - Research stage at the Memorial Sloan Kettering Cancer Center (Prof. dr. Klimstra, Dr L. Tang), New York | 2008 | |

**2. Teaching**

| **Other** | **Year** | **Workload** (Hours/ECTS) |
|---|---|---|
| - Supervising second year medical students<br>Elective "Kanker: van kliniek tot diagnostiek" | 2007 t/m 2009 | 6x0.5 = 3.0 ECTS |
| - Supervising third year medical students<br>"Research elective" | 2008-2009 | 3.0 ECTS |

## TABLES

**Appendix Table 1.** Short tandem repeat profile of eight short tandem repeat (STR) loci in the original tissues from which the cell lines were derived*

| | No. of repeats at each locus | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Short tandem repeat locus | | | | | | | |
| **Primary tissue** | **D21S11** | **TH01** | **D3S1358** | **FGA** | **TPOX** | **D8S1179** | **vWA** | **D5S818** |
| FLO-1 | 30, 32.2 | 6, 9.3 | 15 | 21 | 9, 11 | 13 | 16 | 12, 14 |
| KYAE-1 | NA | 6, 9 | 15, 16 | 18 | 8 | NA | 14 | 10, 13 |
| OE19 | 28, 30 | 8, 9 | 15, 18 | 23, 26 | 8 | 13, 15 | 17, 18 | 11, 14 |
| OE33 | NA | 7, 8 | 18 | 22, 23 | 8, 11 | 10, 12 | 14, 17 | 11 |
| OACM5.1 | NA | 6, 9.3 | 16, 17 | 22 | 8 | 13, 14 | 19 | 12 |
| OACP4C | NA | 6, 9 | 14, 18 | 20 | 9, 11 | 13, 14 | 16 | 9, 12 |
| SK-GT-4 | NA | 6, 9.3 | 16, 17 | 22, 23 | 8, 10 | NA | 17 | 12 |
| JH-EsoAd1* | NA | 6, 7 | NA | NA | 8, 9 | NA | 18, 19 | 11 |
| ESO26 | 30, 31.2 | 9 | 15 | 21 | 7, 9 | 13, 14 | 14, 18 | 11, 12 |
| ESO51 | 30, 33.2 | 9 | 15, 16 | 21, 22 | 8, 11 | 10, 11, 12 | 14, 15 | 11, 13 |

*JH-EsoAd1 repeat number at additonal STR loci; CSF1PO: 9, 10; Amelogenin: X, Y; D13S317: 11; D16S539: 10, 12; D7S820: 10,12. NA = no available data due to technical failure.

**Appendix Table 2.** Short tandem repeat profile of eight STR loci and *TP53* mutations in the original tissue from which the cell lines SK-GT-2 and SK-GT-5 are derived

| | Short tandem repeat locus | | | | | | | | ***TP53* mutation** |
|---|---|---|---|---|---|---|---|---|---|
| **Primary Tissue** | **D21S11** | **TH01** | **D3S1358** | **FGA** | **TPOX** | **D8S1179** | **vWA** | **D5S818** | |
| SK-GT-2 | 29, 32,2 | 8, 9 | 15, 17 | 25, 26 | 9, 12 | 13, 15 | 15, 18 | 10, 12 | c.524G>A |
| SK-GT-5 | 28, 32.2 | 9 | 15, 17 | 21, 22 | 7, 8 | 10, 13 | 17 | 12 | c.916C>T |

Indicated are the repeat numbers at each locus

The currently used EAC cell line SK-GT-5 (see supplementary table 1) is in fact the gastric fundus carcinoma cell line SK-GT-2

**Appendix Table 3.** Clinical characteristics of verified human esophageal adenocarcinoma cell lines

| | Cell Line | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Characteristics** | **FLO-1** | **KYAE-1** | **OE19** | **OE33** | **OACM5.1** | **OACP4C** | **SK-GT-4** | **JH-EsoAd1** | **ESO26** | **ESO51** |
| *Patient* | | | | | | | | | | |
| Sex | Male | Male | Male | Female | Female | Male | Male | Male | Male | Male |
| Age | 68 | 60 | 72 | 73 | 47 | 55 | 89 | 66 | 56 | 74 |
| Etnicity | Caucasian | Asian | Caucasian | Caucasian | Caucasian | Caucasian | Caucasian | Caucasian | Caucasian | Caucasian |
| Neoadjuvant treatment | No | CTX/RTX | No | No | No | No | No | No | No | No |
| *Tumor* | | | | | | | | | | |
| Barrett | No | No | No | Yes | Yes | No | Yes | Yes | No | Yes |
| Tumor location | Distal EAC | Distal EAC | Cardia AC | Distal EAC | Distal EAC | Cardia AC | Distal EAC | Distal EAC | Cardia AC | Distal EAC |
| Nodal involvement | Yes | Yes | Yes | No | Yes | Yes | Yes | No | Yes | Yes |
| Distant metastases | No | Yes (Lung) | Unknown | Unknown | Yes (Pleura) | Yes (Pleura) | Unknown | Unknown | Yes (Pleura) | Unknown |
| pTNM | pT2N1M0 | pT4N1M1 | pT3N1Mx | pT3N0Mx | pT3N1M1 | pT3N1M1 | T2N1Mx | pT3N0Mx | pT4N1M1 | pT3N1Mx |
| *Cell line* | | | | | | | | | | |
| Year of establishment | 1991 | 2001 | 1993 | 1993 | 1996 | 1996 | 1989 | 1997 | 2000 | 2000 |
| Site of origin | Primary Tumor | Pleural Effusion | Primary Tumor | Primary Tumor | Metastatic Lymph Node | Primary Tumor | Primary Tumor | Primary Tumor | Primary Tumor | Primary Tumor |
| Xenograft | No | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| *TP53* mutation | c.830G>T | c.455C>T | c.929dupA | c.404G>A | c.164delC c.743G>A | c.574C>T | c.298C>T | c.797G>A | c.742C>T | c.524G>A |

Identical *TP53* mutations (Genbank accession number AF307851.1) were observed in all cell lines and their corresponding primary tissues.
Abbreviations: CTX/RTX, Chemoradiotherapy; distal EAC, Distal Esophageal Adenocarcinoma; Cardia AC, Cardia Adenocarcinoma.

**Appendix Table 4.** Reports in which only cell lines SEG-1 and/or BIC-1 have been used, without esophageal adenocarcinoma (EAC) cell lines and or human

| Authors | Publication date | Cell line(s) | Esophagus related cell line(s) | Other cell line(s) | Xenograft | Study title |
|---|---|---|---|---|---|---|
| Avissar, *et al.* | 2009 | SEG-1 | | | | Bile acid or in combination with acid induces CDX2 expression through activation of the EGFR |
| Wang, *et al.* | 2008 | SEG-1 | | | | TAE226, a dual inhibitor for FAK and IGF-IR, has inhibitory effects on mTOR signaling in EA cells |
| Delgado, *et al.* | 2008 | SEG-1 | | | | Sorafenib triggers antiproliferative and pro-apoptotic signals in human EA cells |
| Wu, *et al.* | 2008 | SEG-1 | | | | Deoxycholic acid induces the overexpression of intestinal mucin, MUC2, via NF-kB signaling pathway in human EA cells |
| Sun, *et al.* | 2008 | SEG-1 | ECA-109 | | | Antiproliferation and apoptosis induction of paeonol in human esophageal cancer cell lines |
| Pataer, *et al.* | 2008 | SEG-1; BIC-1 | | A549; H1299 | | Adenoviral endoplasmic reticulum-targeted mda-7/interleukin-24 vector enhances human cancer cell killing |
| Keswani, *et al.* | 2008 | SEG-1 | | | | Sorafenib inhibits MAPK-mediated proliferation in a Barrett's esophageal adenocarcinoma |
| Zhang, *et al.* | 2008 | SEG-1 | TE-2 | | | Treatment of radioresistant stem-like esophageal cancer cells by an apoptotic gene-armed, telomerase-specific oncolytic adenovirus |
| Kresty, *et al.* | 2008 | SEG-1 | | | | Cranberry proanthocyanidins induce apoptosis and inhibit acid-induced proliferation of human esophageal adenocarcinoma cells |
| McFadden, *et al.* | 2008 | SEG-1; BIC-1 | | | | Corn-derived carbohydrate inositol hexaphosphate inhibits Barrett's adenocarcinoma growth by pro-apoptotic mechanisms |
| Wang, *et al.* | 2008 | SEG-1 | | NIH3T3 | SEG-1/NIH3T3 | The adenocarcinoma-associated antigen, AGR2, promotes tumor growth, cell migration, and cellular transformation |
| Si, *et al.* | 2008 | SEG-1 | | | | STAT5 mediates PAF-induced NADPH oxidase NOX5-S expression in Barrett's esophageal adenocarcinoma cells |
| Baguma-Nibasheka, *et al.* | 2007 | SEG-1; BIC-1 | | | | Selective cyclooxygenase-2 inhibition suppresses basic fibroblast growth factor expression in human esophageal adenocarcinoma |
| Levy, *et al.* | 2007 | SEG-1 | | HaCaT; 293T; NIH3T3 | | Arkadia activates Smad3/Smad4-dependent transcription by triggering signal-induced SnoN degradation |
| Hu, *et al.* | 2007 | SEG-1 | HET-1A ; HKESC-1; HKESC-2 | | | The pathogenesis of Barrett's esophagus: secondary bile acids upregulate intestinal differentiation factor CDX2 expression in esophageal cells |

| Authors | Publication date | Cell line(s) | Esophagus related cell line(s) | Other cell line(s) | Xenograft | Study title |
|---|---|---|---|---|---|---|
| Hu, *et al.* | 2007 | SEG-1 | HET-1A ; HKESC-1; HKESC-2 | | | Pathogenesis of Barrett esophagus: deoxycholic acid up-regulates goblet-specific gene MUC2 in concert with CDX2 in human esophageal cells |
| Dvorak, *et al.** | 2007 | SEG-1 | HET-1A; CP-D | | | Bile acids in combination with low pH induce oxidative stress and oxidative DNA damage: relevance to the pathogenesis of Barrett's oesophagus |
| Si, *et al.* | 2007 | SEG-1 | hTERT NSE | | | NADPH oxidase NOX5-S mediates acid-induced cyclooxygenase-2 expression via activation of NF-kappaB in Barrett's esophageal adenocarcinoma cells |
| Chao, *et al.* | 2007 | SEG-1 | | | SEG-1 | 3'-deoxy-3'-(18)F-fluorothymidine (FLT) positron emission tomography for early prediction of response to chemoradiotherapy-a clinical application model of esophageal cancer |
| Dvorak, *et al.* | 2006 | SEG-1 | HET-1A | | | Esophageal acid exposure at pH < or = 2 is more common in Barrett's esophagus patients and is associated with oxidative stress |
| Raju, *et al.* | 2006 | SEG-1 | | | | Improvement of esophageal adenocarcinoma cell and xenograft responses to radiation by targeting cyclin-dependent kinases |
| | 2006 | SEG-1 | | | | Early detection of chemoradioresponse in esophageal carcinoma by 3'-deoxy-3'-3H-fluorothymidine using preclinical tumor models |
| Fu, *et al.* | 2006 | SEG-1 | HET-1A | | | cAMP-response element-binding protein mediates acid-induced NADPH oxidase NOX5-S expression in Barrett esophageal adenocarcinoma cells |
| Li, *et al.* | 2006 | SEG-1; BIC-1 | | | | Enhanced sensitivity to chemotherapy in esophageal cancer through inhibition of NF-kappaB |
| Jaiswal, *et al.* | 2006 | SEG-1 | hTERT NSE | | | Bile salt exposure increases proliferation through p38 and ERK MAPK pathways in a non-neoplastic Barrett's cell line |
| Kassis, *et al.* | 2006 | SEG-1; SK-GT-5 | | A549; CALU-6;H2373; H2052 | | Depletion of DNA methyltransferase 1 and/or DNA methyltransferase 3b mediates growth arrest and apoptosis in lung and esophageal cancer and malignant pleural mesothelioma cells |
| Sarosi, *et al.* | 2005 | SEG-1 | | | | Acid increases MAPK-mediated proliferation in Barrett's esophageal adenocarcinoma cells via intracellular acidification through a Cl-/HCO3- exchanger |
| von Holzen, *et al.* | 2005 | SEG-1; BIC-1 | TT | | | Role for the double-stranded RNA-activated protein kinase PKR in Ad-TNF-alpha gene therapy in esophageal cancer |
| Shammas, *et al.* | 2005 | SEG-1 | | | | Telomerase inhibition by siRNA causes senescence and apoptosis in Barrett's adenocarcinoma cells: mechanism and therapeutic potential |

| Authors | Publication date | Cell line(s) | Esophagus related cell line(s) | Other cell line(s) | Xenograft | Study title |
|---|---|---|---|---|---|---|
| Vona-Davis, *et al.* | 2005 | SEG-1; BIC-1 | | | | MAPK and PI3K inhibition reduces proliferation of Barrett's adenocarcinoma in vitro |
| Vona-Davis, *et al.* | 2004 | SEG-1 | | | | Proteomic analysis of SEG-1 human Barrett's-associated esophageal adenocarcinoma cells treated with keyhole limpet hemocyanin |
| McFadden, *et al.* | 2004 | SEG-1; BIC-1 | | | | Peptide YY inhibits the growth of Barrett's esophageal adenocarcinoma in vitro |
| Souza, *et al.* | 2004 | SEG-1 | | | | Acid increases proliferation via ERK and p38 MAPK-mediated increases in cyclooxygenase-2 in Barrett's adenocarcinoma cells |
| Torquati, *et al.* | 2004 | SEG-1 | | | | RUNX3 inhibits cell proliferation and induces apoptosis by reinstating transforming growth factor beta responsiveness in esophageal adenocarcinoma cells |
| Jaiswal, *et al.* | 2004 | SEG-1 | | | | Bile salt exposure causes phosphatidyl-inositol-3-kinase-mediated proliferation in a Barrett's adenocarcinoma cell line |
| Moore, *et al.* | 2004 | SEG-1; BIC-1 | SK-GT-4 | AGS-B; AR42J; CHO; GB | | Gastrin stimulates receptor-mediated proliferation of human esophageal adenocarcinoma cells |
| Vona-Davis, *et al.* | 2004 | SEG-1; BIC-1 | KYSE-150; KYSE-410 | | | Antiproliferative and apoptotic effects of rofecoxib on esophageal cancer in vitro |
| Shammas, *et al.* | 2004 | SEG-1; BIC-1 | | | | Growth arrest, apoptosis, and telomere shortening of Barrett's-associated adenocarcinoma cells by a telomerase inhibitor |
| Morgan, *et al.* | 2004 | SEG-1 | | | | In vitro acid exposure has a differential effect on apoptotic and proliferative pathways in a Barrett's adenocarcinoma cell line |
| Somasundar, *et al.* | 2003 | SEG-1; BIC-1 | | | | Leptin stimulates esophageal adenocarcinoma growth by nonapoptotic mechanisms |
| McFadden, *et al.* | 2003 | SEG-1; BIC-1 | | | | Keyhole limpet hemocyanin, a novel immune stimulant with promising anticancer activity in Barrett's esophageal adenocarcinoma |
| Joe, *et al.* | 2003 | SEG-1; BIC-1 | HCE-7 | | | Exisulind and CP248 induce growth inhibition and apoptosis in human esophageal adenocarcinoma and squamous carcinoma cells |
| Gupta, *et al.* | 2003 | SEG-1; BIC-1 | | | | Selective gene expression using a DF3/MUC1 promoter in a human esophageal adenocarcinoma model |
| Gupta, *et al.* | 2002 | SEG-1 | | | SEG-1 | Combined gene therapy and ionizing radiation is a novel approach to treat human esophageal adenocarcinoma |

| Authors | Publication date | Cell line(s) | Esophagus related cell line(s) | Other cell line(s) | Xenograft | Study title |
|---|---|---|---|---|---|---|
| Joe, *et al.* | 2002 | SEG-1; BIC-1 | | SW480; MCF7; HCE7; HL60 | | Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines |
| Mauceri, *et al.* | 2001 | SEG-1 | SQ-20B | | SEG-1; SQ-20B | Treatment of head and neck and esophageal xenografts employing Alimta and concurrent ionizing radiation |
| Fang, *et al.* | 2001 | BIC-1 | | | | Translocation breakpoints in FHIT and FRA3B in both homologs of chromosome 3 in an esophageal adenocarcinoma |
| Salloum, *et al.* | 2000 | SEG-1 | SQ-20B | LLC; U373 MG | U373 MG | NM-3, an isocoumarin, increases the antitumor effects of radiotherapy without toxicity |
| Gorski, *et al.* | 1999 | SEG-1 | SQ-20B | LLC; U1 | SEG-1 | Blockage of the vascular endothelial growth factor stress response increases the antitumor effects of ionizing radiation |

* Also human tissue samples were used

Esophagus related cell lines:
HET-1A: Non-cancer derived, SV40 immortalized squamous epithelial cell line (established by Harris et al.)
hTERT NSE: hTERT immortalized squamous epithelial cell line: hTERT NSE (established by Spechler et al.)
CP-A; CP-C; CP-D: hTRT immortalized Barrett's esophagus derived cell lines (established by Rabinovitch et al.)

Esophageal squamous cell carcinoma cell lines:
KYSE-series; TE-series; ECA-109; HKESC-1; HKESC-2; TT; HCE-7; SQ-20B

**Appendix Table 5.** Reports in which SEG-1 and or BIC-1 have been used in combination with other esophageal adenocarcinoma cell lines and or human tissues

| Authors | Publication date | Cell line(s) | EAC cell line(s) | Esophagus related cell lines | Patient tissue samples | Study title |
|---|---|---|---|---|---|---|
| Beales, *et al.* | 2009 | BIC-1 | OE33 | CP-? | | Glycine-extended gastrin inhibits apoptosis in Barrett's esophageal and EA cells through JAK2/STAT3 activation |
| van Dekken, *et al.* | 2008 | SEG-1 | OE19; OE33; BE-3; ESO-51; KYAE-1; P4CE; OACM5.1; SKGT-4; SKGT-5 | | EAC; GAC | Molecular dissection of the chromosome band 7q21 amplicon in gastroesophageal junction adenocarcinomas identifies cyclin-dependent kinase 6 at both genomic and protein expression levels |
| Shammas, *et al.*† | 2008 | SEG-1: BIC-1 | FLO-1 | | Normal; Barrett; EAC | Telomere maintenance in laser capture microdissection-purified Barrett's adenocarcinoma cells and effect of telomerase inhibition in vivo |
| Boult, *et al.* | 2008 | SEG-1 | | | Barrett; EAC | Oesophageal adenocarcinoma is associated with a deregulation in the MYC/MAX/MAD network |
| Breton, *et al.* | 2008 | SEG-1; BIC-1 | FLO-1; OE33 | KYSE-30; OE21; HET1A; CP-A; CP-C; CP-D | | Proteomic screening of a cell line model of esophageal carcinogenesis identifies cathepsin D and aldo-keto reductase 1C2 and 1B10 dysregulation in Barrett's esophagus and esophageal adenocarcinoma |
| Ogunwobi, *et al.** | 2008 | BIC-1 | FLO; OE33; OE19 | | | Globular adiponectin, acting via adiponectin receptor-1, inhibits leptin-stimulated oesophageal adenocarcinoma cell proliferation |
| Ogunwobi, *et al.* | 2008 | BIC-1 | OE33 | | | Statins inhibit proliferation and induce apoptosis in Barrett's esophageal adenocarcinoma cells |
| Yen, *et al.* | 2008 | SEG-1 | BE-3; SK-GT-4 | CP-A; CP-C | | Bile acid exposure up-regulates tuberous sclerosis complex 1/mammalian target of rapamycin pathway in Barrett's associated esophageal adenocarcinoma |
| Jethwa, *et al.*‡ | 2008 | SEG-1 | OE33 | TE-7 | Normal; Barrett; EAC | Overexpression of Slug is associated with malignant progression of esophageal adenocarcinoma |
| Clément, *et al.*‡ | 2008 | SEG-1; BIC-1 | OE19; OE33; | TE-7 | Normal; Barrett; EAC | Epigenetic alteration of the Wnt inhibitory factor-1 promoter occurs early in the carcinogenesis of Barrett's esophagus |
| Boult, *et al.* | 2008 | SEG-1 | OE33 | | ESCC | Overexpression of cellular iron import proteins is associated with malignant progression of esophageal adenocarcinoma |

| Authors | Publication date | Cell line(s) | EAC cell line(s) | Esophagus related cell lines | Patient tissue samples | Study title |
|---|---|---|---|---|---|---|
| Watts, *et al.*‡ | 2007 | SEG-1; BIC-1 | | TE-7 | Normal; Barrett | Identification of Fn14/TWEAK receptor as a potential therapeutic target in esophageal adenocarcinoma |
| Zhang, *et al.* | 2007 | SEG-1 | | | EAC | Alternative splicing of the FGF antisense gene: differential subcellular localization in human tissues and esophageal adenocarcinoma |
| Jin, *et al.* | 2007 | SEG-1; BIC-1 | OE33 | KYSE-series | EAC; ESCC | Hypermethylation of tachykinin-1 is a potential biomarker in human esophageal cancer |
| van Duin, *et al.* | 2007 | SEG-1; BIC-1 | OE19; OE33; BE-3; ESO-26; ESO-51; FLO-1; KYAE-1; P4CE; OACM5.1; SK-GT-4; SK-GT-5 | | | High-resolution array comparative genomic hybridization of chromosome 8q: evaluation of putative progression markers for gastroesophageal junction adenocarcinomas |
| Jin, *et al.* | 2007 | SEG-1; BIC-1 | OE33 | KYSE-series | Normal; Barrett; EAC; ESCC | Hypermethylation of the nel-like 1 gene is a common and early event and is associated with poor prognosis in early-stage esophageal adenocarcinoma |
| Hao, *et al.*‡ | 2007 | SEG-1 | OE33 | OE21; TE-7 | Barrett | Gene expression changes associated with Barrett's esophagus and Barrett's-associated adenocarcinoma cell lines after acid or bile salt exposure |
| Liu, *et al.* | 2007 | SEG-1; BIC-1 | FLO1; SKGT4; BE3 | HET-1A | | Regulation of Cdx2 expression by promoter methylation, and effects of Cdx2 transfection on morphology and gene expression of human esophageal epithelial cells |
| Lin, *et al.*‡ | 2006 | SEG-1; BIC-1 | FLO-1; OE33; H80-T; L20-T; BA1 | HET-1A; S95-B | Normal; Barrett; EAC | Expression and effect of inhibition of the ubiquitin-conjugating enzyme E2C on esophageal adenocarcinoma |
| Watson, *et al.** | 2006 | SEG-1; BIC-1 | FLO-1 | | | Inhibition of c-Met as a therapeutic strategy for esophageal adenocarcinoma |
| Sims-Mourtada, *et al.*† | 2006 | SEG-1; BIC-1 | SKGT4; BE-3 | | EAC | Hedgehog: an attribute to tumor regrowth after chemoradiotherapy and a target to improve radiation response |
| Hamilton, *et al.* | 2006 | SEG-1; BIC-1 | OE33 | KYSE-series | Barrett; EAC | Reprimo methylation is a potential biomarker of Barrett's-Associated esophageal neoplastic progression |

| Authors | Publication date | Cell line(s) | EAC cell line(s) | Esophagus related cell lines | Patient tissue samples | Study title |
|---|---|---|---|---|---|---|
| Rees, *et al.*‡ | 2006 | BIC-1 | OE33 | TE-7 | EAC | In vivo and in vitro evidence for transforming growth factor-beta1-mediated epithelial to mesenchymal transition in esophageal adenocarcinoma |
| Su, *et al.*‡ | 2006 | SEG-1; BIC-1 | OE19 | KYSE-30; OE-21; H5E973; TE-7 | | Comparative genomic hybridization of esophageal adenocarcinoma and squamous cell carcinoma cell lines |
| Hao, *et al.*‡ | 2006 | SEG-1 | OE33 | OE-21; TE-7 | Barrett; EAC | Gene expression profiling reveals stromal genes expressed in common between Barrett's esophagus and adenocarcinoma |
| Chang, *et al.*† | 2006 | SEG-1 | SK-GT-5; SK-GT-4 | | | Tumor-specific apoptotic gene targeting overcomes radiation resistance in esophageal adenocarcinoma |
| Lin, *et al.* | 2006 | SEG-1 | FLO-1 | HET-1A | EAC | Multiple forms of genetic instability within a 2-Mb chromosomal segment of 3q26.3-q27 are associated with development of esophageal adenocarcinoma |
| Younes, *et al.* | 2006 | SEG-1; BIC-1 | | | EAC | Functional expression of TRAIL receptors TRAIL-R1 and TRAIL-R2 in esophageal adenocarcinoma |
| Watson, *et al.*† | 2006 | SEG-1; BIC-1 | FLO-1 | | | Ad-IRF-1 induces apoptosis in esophageal adenocarcinoma |
| Miller, *et al.* | 2006 | SEG-1; BIC-1 | FLO-1; OE33 | | EAC | Genomic amplification of MET with boundaries within fragile site FRA7G and upregulation of MET pathways in esophageal adenocarcinoma |
| Abdalla, *et al.* | 2005 | SEG-1; BIC-1 | OE33 | OE-21 | Barrett; EAC | Effect of inflammation on cyclooxygenase (COX)-2 expression in benign and malignant oesophageal cells |
| Zou, *et al.* | 2005 | SEG-1; BIC-1 | OE33 | | Normal; Barrett; EAC | Aberrant methylation of secreted frizzled-related protein genes in esophageal adenocarcinoma and Barrett's esophagus |
| Onwuegbusi, *et al.*‡ | 2005 | SEG-1; BIC-1 | FLO-1; OE33 | CP-A; CP-C; CP-D; TE-7 | Barrett; EAC | Impaired transforming growth factor beta signalling in Barrett's carcinogenesis due to frequent SMAD4 inactivation |
| Schulmann, *et al.* | 2005 | BIC-1 | | | Barrett; EAC | Inactivation of p16, RUNX3, and HPP1 occurs early in Barrett's-associated neoplastic progression and predicts progression risk |

| Authors | Publication date | Cell line(s) | EAC cell line(s) | Esophagus related cell lines | Patient tissue samples | Study title |
|---|---|---|---|---|---|---|
| Darnton, *et al.* | 2005 | SEG-1; BIC-1 | FLO-1; OE33 | | EAC | Tissue inhibitor of metalloproteinase-3 (TIMP-3) gene is methylated in the development of esophageal adenocarcinoma: loss of expression correlates with poor prognosis |
| Hansel, *et al.* | 2005 | SEG-1; BIC-1 | KYAE-1; OE33 | | Barrett; EAC | CDC2/CDK1 expression in esophageal adenocarcinoma and precursor lesions serves as a diagnostic and cancer progression marker and potential novel drug target |
| Zou, *et al.* | 2005 | SEG-1; BIC-1 | OE33 | | Normal; Barrett; EAC | Frequent methylation of eyes absent 4 gene in Barrett's esophagus and esophageal adenocarcinoma |
| Mahidhara, *et al.* | 2005 | SEG-1; BIC-1 | FLO-1 | HET-1A | EAC | Altered trafficking of Fas and subsequent resistance to Fas-mediated apoptosis occurs by a wild-type p53 independent mechanism in esophageal adenocarcinoma |
| Herrera, *et al.* | 2005 | SEG-1; BIC-1 | FLO-1 | HET-1A | EAC | The HGF receptor c-Met is overexpressed in esophageal adenocarcinoma |
| Lin, *et al.*‡ | 2004 | SEG-1; BIC-1 | FLO-1; OE33; H80-T; L20-T; BA1 | HET-1A ; S95-B | EAC | Melanoma-associated antigens in esophageal adenocarcinoma: identification of novel MAGE-A10 splice variants |
| Lin, *et al.*‡ | 2004 | SEG-1; BIC-1 | FLO-1; OE33; H80-T; L20-T; BA1 | HET-1A; S95-B | EAC | L-type amino acid transporter-1 overexpression and melphalan sensitivity in Barrett's adenocarcinoma |
| Kim, *et al.* | 2003 | SEG-1; BIC-1 | OE33 | KYSE-?; OE-21 | | Transforming growth factor-beta is an endogenous radioresistance factor in the esophageal adenocarcinoma cell line OE-33 |
| Miller, *et al.* | 2003 | SEG-1; BIC-1 | FLO-1 | HET-1A | EAC | Gene amplification in esophageal adenocarcinomas and Barrett's with high-grade dysplasia |
| Souza, *et al.* | 2002 | SEG-1 | | | Barrett | Acid exposure activates the mitogen-activated protein kinase pathways in Barrett's esophagus |
| Arlt, *et al.** | 2002 | SEG-1; BIC-1 | FLO-1 | | | Molecular characterization of FRAXB and comparative common fragile site instability in cancer cells |
| Weiser, *et al.* | 2001 | SK-GT-5 | BE-3 | TE-series | | Induction of MAGE-3 expression in lung and esophageal cancer cells |

| Authors | Publication date | Cell line(s) | EAC cell line(s) | Esophagus related cell lines | Patient tissue samples | Study title |
|---|---|---|---|---|---|---|
| Souza, *et al.* | 2000 | SEG-1; BIC-1 | FLO-1 | | | Selective inhibition of cyclooxygenase-2 suppresses growth and induces apoptosis in human esophageal adenocarcinoma cells |
| Aggarwal, *et al.* | 2000 | SEG-1; BIC-1 | FLO-1 | | Normal; Barrett; EAC | Indomethacin-induced apoptosis in esophageal adenocarcinoma cells involves upregulation of Bax and translocation of mitochondrial cytochrome C independent of COX-2 expression |
| Compton, *et al.* | 1999 | SEG-1; BIC-1 | FLO-1 | HET-1A | Normal; Barrett | Induction of glutathione s-transferase-pi in Barrett's metaplasia and Barrett's adenocarcinoma cell lines |
| Soldes, *et al.* | 1999 | SEG-1; BIC-1 | FLO-1 | HET-1A | Barrett; EAC | Differential expression of Hsp27 in normal oesophagus, Barrett's metaplasia and oesophageal adenocarcinomas |
| Schrump, *et al.* | 1998 | SK-GT-5 | SK-GT-4; SK-GT-2 | HCE-4 | | Flavopiridol mediates cell cycle arrest and apoptosis in esophageal cancer cells |
| Hughes, *et al.* | 1997 | SEG-1; BIC-1 | FLO-1 | | EAC | Fas/APO-1 (CD95) is not translocated to the cell membrane in esophageal adenocarcinoma |

Abbreviations: EAC, Esophageal adenocarcinoma; ESCC, Esophageal squamous cell carcinoma; GAC, Gastric adenocarcinoma

* Also cell line(s) from other species or cancer types were used
† Also SEG-1 xenografts were used
‡ Cell lines H80-T; L20-T established by Beer et al. do not grow in vitro (personal communication)
‡ Cell line BA-1 established by dr Rutten does not grow in vitro and origin is unknown (personal communication)
‡ Cell line TE-7 is not an esophageal adenocarcinoma cell line, but a squamous cell carcinoma cell line of unknown origin (as reported by Boonstra, *et al.*)

Esophagus related cell lines:
HET-1A: Non-cancer derived, SV40 immortalized squamous epithelial cell line (established by Harris et al.)
hTERT NSE: hTERT immortalized squamous epithelial cell line: hTERT NSE (established by Spechler et al.)
CP-A; CP-C; CP-D: hTRT immortalized Barrett's esophagus derived cell lines (established by Rabinovitch et al.)
S95-B; E6/E7 retroviral immortalized Barrett's esophagus derived cell line

Esophageal squamous cell carcinoma cell lines:
KYSE-series; TE-series; OE21; H5E973; HCE-4

**Appendix Table 6.** Homozygous deletion affecting common fragile sites

| Cytoband | Start | Stop | Size (bp) | Sample | Fragile site | Genes |
|---|---|---|---|---|---|---|
| 3p14.2 | 60254740 | 61191150 | 936410 | ESO26; ESO51; JH-EsoAd1; KYAE-1; P100x; P101x; P102x; P117x; P140x; P171x; P175x; P58x | FRA3B | *FHIT* |
| 3p14.2 | 60409270 | 60437190 | 27920 | OACM5.1 | FRA3B | *FHIT* |
| 3p14.2 | 60145820 | 60489260 | 343440 | OE33 | FRA3B | *FHIT* |
| 4q22.1 | 91661770 | 91808390 | 146620 | FLO-1; P100x | FRA4F | *FAM190A/TMSL3* |
| 4q22.1 | 92239940 | 92562840 | 322900 | P100x | FRA4F | *FAM190A/TMSL3* |
| 7q36.3 | 157136920 | 157283380 | 146460 | P101x | FRA7I | *PTPRN2* |
| 16q23.1 | 77137560 | 77411730 | 274170 | ESO26; KYAE-1; OE33; P100x | FRA16D | *WWOX* |
| 20p12.1 | 14729690 | 14794610 | 64920 | SK-GT-4; ESO26; P101x | FRA20B | *MACROD2 (C20orf133)* |
| 20p12.1 | 14791900 | 15129670 | 337770 | FLO-1 | FRA20B | *MACROD2 (C20orf133)* |

**Appendix Table 7.** Homozygous deletion affecting regions without genes

| Cytoband | Start | Stop | Size (bp) | Sample |
|---|---|---|---|---|
| 1q31.2 | 191517870 | 191604510 | 86640 | KYAE-1 |
| 4q22.3 | 97309470 | 97470920 | 161450 | P101x |
| 8q21.3 | 90003095 | 90183062 | 179967 | P4CE |
| 9p21.2 | 26480277 | 26744888 | 264611 | P58x |
| 12q21.2 | 75058720 | 75205180 | 146460 | OE33 |
| 16q21 | 61996620 | 62338360 | 341740 | KYAE-1 |
| 17p12 | 14272780 | 14596780 | 324000 | FLO-1 |
| 21q21.1 | 15579170 | 15795570 | 216400 | OE19 |