ADENOCARCINOMAS OF THE GASTRO-OESOPHAGEAL JUNCTION :

FROM GENE TO CLINIC

B.P.L. WIJNHOVEN

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ADENOCARCINOMAS OF THE GASTRO-OESOPHAGEAL JUNCTION : FROM GENE TO CLINIC

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PREFACE

Worldwide, adenocarcinomas of the gastro-oesophageal junction, *i.e.* distal oesophagus and gastric cardia, show an increase in incidence over the past three decades. These carcinomas have a poor prognosis, with 5-year survival rates of about 25% after surgery with curative intent. The exact cause of this rise is not known yet. Several risk factors have been identified, of which longstanding gastro-oesophageal reflux is probably the most important one. The reflux of duodenal and gastric contents into the oesophagus leads to oesophagitis and ultimately premalignant metaplastic epithelium, also known as Barrett's oesophagus, which can develop in a subset of patients. Adenocarcinomas are thought to arise from this precursor lesion through a multistep process of malignant changes.
Understanding the genetic alterations that occur during the progression of Barrett's oesophagus into invasive carcinoma is important. Information about abnormalities in (proto-) oncogenes and tumour suppressor genes can aid the clinician in stratifying patients' risk of progressing to adenocarcinoma. Moreover, novel molecular biomarkers can lead to a better prognostication of patients with adenocarcinoma of the gastro-oesophageal junction. It may identify patients who are at high risk for tumour metastases or recurrence and who might or might not benefit from (curative) surgical intervention or (palliative) chemotherapy.

This thesis focusses on epidemiologic, clinical and genetic aspects of adenocarcinomas of the gastro-oesophageal junction. In collaboration with the Eindhoven Cancer Registry (Integraal Kankercentrum Zuid-West Nederland) the epidemiology of adenocarcinomas of the gastro-oesophageal junction was studied. Data from the department of surgery, University Hospital Rotterdam-Dijkzigt on patients operated on adenocarcinomas of the gastro-oesophageal junction were used for clinical assessments. Most of the laboratory experiments described in this thesis were conducted at the department of pathology, Erasmus University Rotterdam. Finally, the author of this thesis was able to perform 6 weeks of research in the cell adhesion laboratory of the department of pathology, University of Bristol, Bristol, United Kingdom.

B.P.L. Wijnhoven
Rotterdam, June 2001

CONTENTS

Disease is very old, and nothing about it has changed. It is we who change, as we learn to recognise what was formerly imperceptible.

Jean Martin Charcot (1825-1893)

To Marjolein and Juul
To my parents

OUTLINE OF THE THESIS

Adenocarcinomas of the gastro-oesophageal junction are thought to arise from premalignant Barrett's epithelium. Barrett's epithelium is columnar epithelium that has replaced the normal squamous cell lining of the oesophagus. This metaplastic change is driven by duodeno-gastro-oesophageal reflux, which leads to oesophagitis and ultimately, in some patients, to Barrett's epithelium. The development of Barrett's carcinoma involves multiple genetic changes.
In **PART I**, the general introduction of this thesis, **CHAPTER 1** reviews our current knowledge on these genetic changes involved in the progression from Barrett's oesophagus to adenocarcinoma.
Over the past decades, many researchers focused on the role of cell-cell adhesion in carcinogenesis. The E-cadherin-catenin complex is thought to be the most important regulator of tight cell-cell adhesion in normal tissues, and perturbation of this complex is associated with malignancy. There is evidence that dysfunction of the E-cadherin-catenin complex also plays an important role in the pathogenesis of adenocarcinomas of the gastro-oesophageal junction. In **CHAPTER 2**, the literature on the role of the E-cadherin-catenin complex in human cancer and the possible clinical implications are discussed. This chapter serves as an introduction to Part IV (chapters 7-10).

PART II of the thesis deals with epidemiological and clinical aspects of adenocarcinomas of the gastro-oesophageal junction. Worldwide population based studies suggest that the incidence of oesophageal and gastric cardia adenocarcinomas is rising. This is especially true for the USA, where the rise in incidence surpasses that of any other cancer. However, it is still not entirely clear whether this rise reflects a true increase. There are several pitfalls: the decrease in the number of unspecified tumours and the increase use of diagnostic tools might bias the observed trends. In **CHAPTER 3**, we assessed these issues by studying time trends in mortality and incidence rates for oesophageal and gastric cancer according to subsite and histology in the Southeast of the Netherlands since 1978.
According to the current staging criteria established by the Union Internationale Contre le Cancer (UICC) and the American Joint Committee on Cancer (AJCC), carcinoma of the cardia is classified as gastric cancer and carcinoma of the (distal) oesophagus is classified as oesophageal cancer. However, there are several epidemiological, clinical and genetic studies that suggest common risk factors and a similar pheno- and genotype for adenocarcinomas arising from the distal oesophagus and gastric cardia. In **CHAPTER 4** the question is addressed whether these tumours should be regarded and staged as one clinical entity. Pathology, pathological tumour node metastasis (pTNM) stage and 5-years survival data are presented. Multivariate analysis was performed to evaluate independent factors predicting survival.

So far, little is known about the cellular and molecular mechanisms underlying the origin of adenocarcinomas of the gastro-oesophageal junction. This is partly caused by the lack of sufficient *in vivo* and *in vitro* model systems. Tumour xenografts are useful models to study the *in vivo* behaviour of tumours. Furthermore, human tumour xenografts are a source of tumour cells without admixture of normal human cells. This facilitates the analysis of tumour specific molecular alterations. To date only few *in vitro* growing human oesophageal adenocarcinoma cell lines are available.

PART III, CHAPTER 5 describes the establishment and characterisation of xenografts and cell lines from primary adenocarcinomas of the gastro-oesophageal junction and lymph node metastasis.

The generation of new cell lines is labour intensive and tedious work. Moreover, the risks of contamination with other cell lines and/or viruses are high. In **CHAPTER 6** we show that two recent established and characterised human oesophageal adenocarcinoma cell lines from the United Kingdom are in fact admixtures of the human colon carcinoma cell line HCT-116.

PART IV of the thesis includes chapters 7, 8, 9, and 10 and describes the role of the E-cadherin-catenin cell-cell adhesion complex in adenocarcinomas of the gastro-oesophageal junction.

As reviewed in the introduction (Chapter 2), immunohistochemical studies in human cancers have frequently shown that changes in E-cadherin-catenin expression appear to be an important step in the development and progression of a malignant tumour. However, the mechanism responsible for decreased expression of E-cadherin remained elusive. In **CHAPTER 7**, we screened adenocarcinomas of the gastro-oesophageal junction for E-cadherin gene mutations and loss of heterozygosity (LOH) of the E-cadherin gene.

Besides E-cadherin its intra-cellular partners, the catenins, are important for tight cell-cell adhesion. Not only decreased membranous expression, but also increased cytoplasmic and nuclear expression of β-catenin is seen in cancers. In colorectal carcinomas, mutations in the β-catenin gene or adenomatous polyposis coli (APC) gene are responsible for this aberrant expression, which is indicative for the role of β-catenin in cell signalling. In **CHAPTER 8** it is hypothesized that genetic alterations of the β-catenin gene also contribute to the pathogenesis of oesophageal adenocarcinomas. Mutational screening of exon 3 of the β-catenin gene has been performed in a large series of carcinomas. In selected tumours we also screened the mutation cluster region of the APC gene for aberrations.

Recently, another catenin-like molecule, p120-catenin ($p120^{ctn}$) has been identified as being associated with E-cadherin at the cell junctions. However, the exact function of $p120^{ctn}$ and its role in malignancy is not clear yet. Therefore, as outlined in **CHAPTER 9**, we studied the *in vivo* expression of $p120^{ctn}$ in adenocarcinomas of the gastro-oesophageal junction. In addition we evaluated the relationship between

expression and clinicopathological features in order to examine the application of $p120^{ctn}$ as a prognostic marker.
To further elucidate the role of the E-cadherin-catenin complex in adenocarcinomas of the gastro-oesophageal junction, the expression of E-cadherin and catenins and their biochemical distribution between the membrane bound and cytoskeleton bound fraction was assessed using fractional protein extraction and Western blot analysis **(CHAPTER 10)**. The spatial distribution of the proteins between the cell membrane, cytoplasm and nucleus was assessed using immunohistochemistry.

PART V describes the search for novel tumour suppressor genes in adenocarcinomas of the gastro-oesophageal junction. Tumour suppressor genes are cellular genes. In a normal cell, these genes inhibit cell growth and stimulate differentiation. In carcinomas, their inhibitory function is lost.
CHAPTER 11 focuses on the short arm of chromosome 3 (3p). Candidate genes within this region are the Von Hippel-Lindau (VHL) gene and the Human Peroxisome Proliferator-Activated Receptor gamma (PPARγ) genes. Mutational screening and loss of heterozygosity (LOH) analysis of the 3p region is studied in order to disclose the role of these two tumour suppressor genes in adenocarcinomas of the gastro-oesophageal junction, as has been suggested in the literature.
In **CHAPTER 12** evidence is presented for the existence of a novel candidate tumour suppressor gene on the long arm of chromosome 14 at 14q31.1-32.11 that plays an important role in the Barrett's carcinoma progression. We performed a detailed LOH analysis with 22 polymorphic microsatellite markers on 37 tumour samples and identified a minimal region of overlapping deletion.

Finally, **CHAPTER 13, PART VI** is a summary of the preceding chapters and the major conclusions are drawn. In the epilogue, **CHAPTER 14**, the consequences of the results of the thesis are briefly discussed, recommendations for future research are provided and prospects for clinical application are considered.

PART I

GENERAL INTRODUCTION

CHAPTER 1

THE MOLECULAR BIOLOGY OF BARRETT'S ADENOCARCINOMA

BPL Wijnhoven, HW Tilanus and WNM Dinjens

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PRÉCIS

The genetic pathway involved in the progression from premalignant Barrett's oesophagus to oesophageal adenocarcinoma still remains largely unknown. Studying the molecular biology of Barrett's oesophagus enables better understanding of the mechanisms of malignant degeneration and might ultimately lead to targeted cancer prevention and/or therapeutic interventions.

INTRODUCTION

Barrett's adenocarcinoma

Since 1970 the incidence of adenocarcinomas of the oesophagus has increased in many countries, at a rate that exceeds that of any other malignancy (1-5). It is now generally accepted that oesophageal adenocarcinomas develop from a premalignant lesion of the oesophagus, also referred to as Barrett's oesophagus. Barrett's oesophagus is a metaplastic change of the normal squamous cell epithelium of the oesophagus to a columnar type due to longstanding gastro-oesophageal reflux. Three subtypes of Barrett's epithelium have been described, but the specialised intestinal type is the only subtype clearly associated with malignant transformation (6,7). The risk of oesophageal adenocarcinoma in patients with Barrett's oesophagus appears to be about 30-125 fold higher than that in the general population, with an estimated incidence of 1 in 180 patient-years (8-11). Although high grade dysplasia of Barrett's oesophagus is generally considered a precursor to invasive carcinoma, the assessment of novel biomarkers and better understanding of the pathophysiology of Barrett's adenocarcinoma may help to identify those patients at increased risk for malignant transformation. Furthermore, elucidating the genetic alterations leading to malignant transformation may someday lead to its prevention.

Genetic alterations in human cancers

It is generally accepted that a multistep process of genetic and epigenetic alterations causes the transformation of a normal cell into a malignant tumour cell. These alterations render the cell independent of regulated proliferative and cell death pathways and deliver the cells with proliferative, invasive and metastasising capacities. As a consequence, a malignant tumour is generated composed of cells with an increased proliferative activity, a prolonged lifespan and with metastasising capacity. At least five to ten genetic alterations are necessary to generate the malignant phenotype and most tumours are characterised by genomic instability facilitating the accumulation of mutations. The genomic instability occurs in two different forms; one characterised by microsatellite instability and the other by chromosomal instability (12). The targets of the genomic instability are four classes of genes:

1. Proto-oncogenes. These are dominant genes that all act in signal transduction from extracellular stimuli to the nucleus or in regulation of gene expression, and have a role in cell proliferation or inhibition of apoptosis. Upon activation of proto-oncogenes, by mutation, amplification, translocation, etc. these genes turn into oncogenes with unregulated, constitutive activity. This results in excessive stimulation of cell proliferation or preventing apoptosis and both contribute to tumour formation.
2. Tumour suppressor genes. Tumour suppressor genes are normal cellular genes, which primarily are involved in cell proliferation, apoptosis, cell adhesion and gene expression regulation. These are recessive genes, which implies that both gene copies need to be inactivated to contribute to tumourigenesis. Functional inactivation of tumour suppressor genes can be caused by genetic as well as by epigenetic phenomena: mutation, deletion of (part of) the gene and epigenetic silencing through promoter methylation.

3. Mismatch repair genes. Genetic instability can be caused by impairment in DNA repair. This deficiency is recognised in tumours by microsatellite instability (MSI) or the replication error (RER) phenotype. The genes PMS1, PMS2, MLH1, MSH2, MSH6 and the recently discovered MBD4 (MED1) are all associated with microsatellite instability (13,14). In tumours with underlying defects contractions or expansions of short repeat sequences (microsatellites) can be found (15). The mismatch repair deficiency leads to a genome wide accumulation of mutations, also in proto-oncogenes and tumour suppressor genes, and contributes as such in tumourigenesis.
4. Mitotic checkpoint genes. An inactivating mutation in one copy of these genes has a dominant effect on the phenotype (dominant-negative). To date eight human genes with a role in mitotic checkpoint control have been discovered (16). Inactivation of mitotic checkpoint genes results in chromosomal instability and an abnormal chromosome number (aneuploidy)(16). Mutation analysis of the human mitotic checkpoint genes in aneuploid cancers revealed only few alterations. It is therefore anticipated that genes yet to be discovered are responsible for most of the checkpoint defects found in aneuploid cancers.

Genomic instability, at the nucleotide or at the chromosomal level, ultimately leads to the activation of proto-oncogenes and inactivation of tumour suppressor genes. There are no proto-oncogenes or tumour suppressor genes that are activated or deleted in all cancers. Even comparable cancers from the same organ and cell type never share alterations in the same oncogenes and tumour suppressor genes completely. Although clear from a conceptual point of view, the relevant genetic alterations underlying comparable tumours, like oesophageal adenocarcinomas, will show variation in the genes involved.

CELL PROLIFERATION AND APOPTOSIS

Dividing normal cell populations maintain the balance between cell proliferation and cell loss. This is important for maintaining a constant number of cells within a tissue. If there is increased proliferation, decreased apoptosis or both, uncontrolled growth occurs and this may result in tumour formation (17).

Cell proliferation

In order to assess the amount and distribution of cell proliferation in paraffin-embedded tissues, monoclonal antibodies have been developed to detect cell cycle modulators. Several studies used a monoclonal Ki-67 (MIB-1) and PCNA antibody to study the proliferative properties in Barrett's oesophagus and adenocarcinomas. An increased number of proliferating cells and an expansion of the proliferative compartment have been demonstrated in Barrett's oesophagus and adenocarcinoma. PCNA immunostaining is mainly seen in the basal cells of the neck/foveolar epithelial compartment of the glands in Barrett's oesophagus. However, in mucosa with high-grade dysplasia, the proliferative compartment extended upwards into the superficial layers of the glands (18-20). Ki-67 staining pattern also correlated with the histologic

findings in Barrett's oesophagus: the number and localisation of Ki-67 positive nuclei was significantly different between non-, low and high-grade dysplastic Barrett's and adenocarcinoma (18,21-24).

Apoptosis

Apoptosis, or programmed cell death, is one of the mechanisms responsible for cell loss. Furthermore, apoptosis provides a protective mechanism by removing senescent, DNA-damaged, or diseased cells that could either interfere with normal function or lead to neoplastic proliferation. Apoptosis itself can be detected by use of immunohistochemical detection of DNA fragmentation as markers for apoptosis. An increase in apoptotic rate with increasing histologic severity in intestinal metaplasia/dysplasia and carcinoma has been noted (25), whereas others found few apoptotic cells in Barrett's high-grade dysplasia and adenocarcinoma (26,27).

The Fas/APO-1 (CD95) gene encodes a transmembrane protein that is involved in apoptosis. Loss of its expression during carcinogenesis can result in the interruption of the apoptotic pathway. Hughes *et al.* found that expression of Fas on the cell surface by oesophageal adenocarcinomas is reduced or absent whereas high levels of Fas mRNA were detected in these tumours (28). Furthermore, they demonstrated in an oesophageal adenocarcinoma cancer cell line that wild-type Fas protein is retained in the cytoplasm. Apparently, retention of wild-type Fas protein within the cytoplasm may represent the mechanism by which malignant cells evade Fas-mediated apoptosis (28).

The bcl-2 proto-oncogene encodes a protein that blocks apoptosis (29). Bcl-2 expression is increased in reflux oesophagitis, non dysplastic Barrett's and low grade dysplastic Barrett's epithelium (70-100% of cases), but low or virtually absent in high grade dysplasia (0-25 % of cases) and carcinomas (0-40% of cases) (18,23,27,30). Apparently, inhibition of apoptosis by overexpression of bcl-2 protein occurs early in the dysplasia-carcinoma sequence of Barrett's oesophagus. The resulting prolongation of cell survival may promote neoplastic progression. As malignancy appears, cells acquire other ways of avoiding apoptosis.

It can be concluded that gradually increased and spatially distinguished cell proliferation is a well-established permanent alteration, whereas the role of apoptosis and bcl-2 seems less certain. The assessment of the ratio of proliferation to apoptosis may well be more important than the isolated assessment of either, and this may well be a useful and sensitive marker of neoplastic change in Barrett's oesophagus (31).

Telomerase

During normal somatic cell division, telomeres shorten. In contrast, immortalised and carcinoma cells show no loss of telomere length during cell division. Telomerase is a ribonucleoprotein complex that synthesises telomeric DNA located at the chromosomal ends, thereby maintaining telomere length. The increase in telomerase activity that accompanies most neoplastic and many preneoplastic conditions may permit the emergence of a population of immortalised cells, thereby facilitating the subsequent accumulation of genetic mutations (32). By using in situ hybridisation (ISH), Morales *et al.* detected only weak levels of telomerase RNA in cells of the basal layer of normal squamous, oesophageal epithelium, representing the population of

stem cells. In contrast, moderate to strong telomerase RNA expression, similar to the zone of proliferation, was seen in Barrett's metaplasia (70% of the cases), in 90% of the low-grade dysplasias, and in 100% of the high-grade dysplasias and oesophageal adenocarcinomas (33). Interestingly, cardiac and fundic type Barrett's mucosa, which is not associated with an increased risk of adenocarcinoma, demonstrated no telomerase RNA (33).

DNA CONTENT AND CHROMOSOMAL ABNORMALITIES

DNA content/aneuploidy

A normal cell has a chromosome number of 2N, for which the term *diploid* is applied. Cells reproduce by duplicating their content (4N) and then dividing in two. This mammalian cell division cycle is divided into several distinct phases (Figure 1).

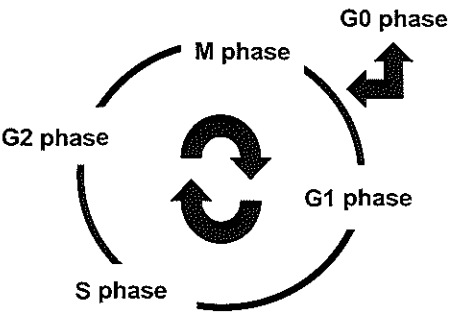


FIGURE 1. *Schematic representation of the cell cycle. Mitosis (M phase) is the process of nuclear division. Replication of DNA occurs in the S (=synthesis) phase. The interval between M phase and S phase is called the G1 (=gap) phase and the interval between the end of the S phase and the beginning of the m phase the G2 phase. Cells can exit the cell cycle and enter the G0 Phase, which is the quiescent state.*

A cell with numerical aberrations is designated as *aneuploid*. When a suspension of single cells or nuclei is stained with quantitative fluorescent DNA dye, the amount of fluorescence is directly proportional to the amount of DNA in each cell. By using this technique, known as flow cytometry, it has been shown that the evolution from normal oesophagus to premalignant Barrett's metaplasia is frequently associated with abnormal DNA content (aneuploidy) and increased G2/M fraction of the metaplastic cells (34-36). Moreover, abnormal DNA content shows a correlation with the histologic diagnosis of dysplasia and carcinoma (37,38). Flow cytometry also detects a subset of patients whose biopsies are histologically indefinite or negative for dysplasia and carcinoma, but who have DNA content abnormalities similar to those otherwise seen only in dysplasia and carcinoma (36,39). Therefore, this technique (in combination with histology) may be of use in screening patients with Barrett's oesophagus for early signs of malignant change (40). Indeed, prospective studies indicate that both aneuploidy and dysplasia may be prognostic factors for malignant

transformation in Barrett's epithelium (35,40,41): 70% of the patients with aneuploidy or increased G2/tetraploid fractions in biopsy specimens obtained during initial endoscopic evaluation developed high grade dysplasia or cancer, whereas none of the patients without flow cytometric abnormalities on initial evaluation showed progression to invasive carcinoma or high-grade dysplasia (35). Others report that histologic dysplasia and aneuploidy are often discordant (42). Most discordance between several studies can be explained by methodological differences.
Several studies report that Barrett's adenocarcinomas with abnormal nuclear DNA content are associated with increased lymph node metastases, advanced stage disease and poorer survival (43-46). Others did not find a relationship between ploidy status and clinicopathological parameters (47,48).
It is suggested that chromosome instability, tetraploidization, and asymmetrical chromosome segregation during cell division leading to aneuploidy are the result of deregulated cell cycle genes with multiple functions that normally exert active checks on the cell cycle processes including apoptosis and chromosome stability, e.g. p53 and k-ras genes (49). This is supported by the finding that in patients with Barrett's oesophagus demonstrating allelic loss of 17p (p53 gene locus) and p53 mutations in diploid cells, develop increased 4N (G2/tetraploid) fractions and subsequently aneuploidy (50,51). As already discussed in the introduction, chromosomal instability is associated by defects in mitotic checkpoint genes (16).

Chromosomal abnormalities

In Barrett's oesophagus and Barrett's cancer chromosomal alterations have also been described based on karyotyping and in situ hybridisation (ISH) studies. The most consistent numerical chromosomal abnormalities found in cytogenetic studies of dysplastic Barrett's mucosa and adenocarcinoma is loss of the Y-chromosome (52-54). In oesophageal adenocarcinoma Y-chromosome loss is found in 31 to 93% of the tumours. In one study, the frequency of Y-chromosome loss in Barrett's mucosa increased along with grade of dysplasia (24). Although Barrett's oesophagus and Barrett's adenocarcinoma occur more commonly in men, no specific onco- or tumour suppressor genes have been assigned to the Y-chromosome. Perhaps, as genetic instability increases during malignant transformation of Barrett's oesophagus, Y-chromosome loss randomly occurs. Karyotyping revealed frequent structural rearrangements in oesophageal adenocarcinomas in the 1p, 3q, 11p-13, and 22p regions (52,55). Furthermore, trisomies for chromosomes 5 and 7 and translocations involving chromosome 3 and 6 in Barrett's oesophagus have been described (54). Other frequent numerical aberrations in oesophageal adenocarcinomas are over representation of chromosomes 6, 7, 8, 11, 12, 14 and 20 and loss of chromosomes 4, 17, 18 and 21 (52,53,55-57). In a subset of cases in which premalignant lesions were examined, aneusomy of chromosomes 6, 7, 11 and 12 was found to be an early change, frequently present in both Barrett's oesophagus and dysplastic regions. It remains to be determined whether any of these abnormalities are predictive markers of progression to malignancy.

MICROSATELLITE INSTABILITY

A form of genetic instability that has recently been identified is microsatellite instability. Microsatellites are mostly highly polymorph short, tandem repeat DNA sequences. They are abundantly and evenly distributed throughout the genome. Microsatellite instability (MSI) is caused by a failure of the DNA mismatch repair (MMR) system to repair errors that occur during the replication of DNA and is characterised by the accumulation of single nucleotide mutations and alterations in length of the microsatellites. This widespread MSI is a characteristic feature of tumours from Hereditary Non-Polyposis Coli Cancer (HNPCC) kindreds (58).
Meltzer *et al.* reported microsatellite instability at 1 or more chromosomal loci of the 5 dinucleotide microsatellite repeats tested in 2/28 (7%) patients with Barrett's metaplasia and 8/36 (22%) oesophageal adenocarcinomas (59). Among 25 flow cytometry sorted adenocarcinomas, instability occurred in 8 (32%). In 4 of these 8 positive cases, the diploid component of the tumour showed instability suggesting that the instability may develop as an early event in Barrett's associated neoplastic progression (59). However, there remains confusion as how to define the phenomenon of MSI, specifically, how many markers should be used, and how many must display instability before the tumour is defined as being MSI. According to more stringent definition of MSI, Gleeson *et al.* found that MSI is infrequent in Barrett's adenocarcinomas and adenocarcinomas of the gastric cardia (60,61). Several studies have confirmed the low prevalence of MSI, between 5-10%, in oesophageal adenocarcinomas (62-66). Interestingly, Wu *et al.* found a trend towards an improved survival for oesophageal adenocarcinomas demonstrating MSI (66). It is not yet known which mismatch repair genes are responsible for the microsatellite instability observed in Barrett's adenocarcinoma.

TABLE 1. *Summary of chromosomal regions which show frequent LOH (>40% of oesophageal adenocarcinomas)*

Chromosome arm	*No. of tumours with LOH/informative cases (%)*	*Minimal area of loss*	*Candidate genes*	*References*
3p	14/22 (64)	3p24-26	VHL, PPARγ	(65)
3q	11/17 (65)	3qter	Unknown	(67,68)
4q	57/81 (70%)	4q21.1-22	Unknown	(67,69,70)
		4q32-33	"	
		4q35	"	
5q	10/22 (45)	5q11.2-13.3	MSH3	(65,67,71-73)
	99/138 (72)	5q21-22	APC	
			MCC	
			IRF-1	
5pq	4/9 (44)	5p12	Unknown	(74)
	2/4 (50)	5q31.1	"	
6q	10/17 (56)	6qter	Unknown	(67)
9p	81/131 (61)	9p21.1-22	p15	(65,67,75-78)
			p16	
			IFNA	
9q	8/17 (47)	9qter	Unknown	(67)
11p	8/15 (53)	11p15	P57KIP2,	(65)
	14/23 (61)	11p15.5	TSG101,WT2,	
			H19	
12p	8/17 (47)	12pter	Unknown	(67)
12q	11/17 (76)	12qter	Unknown	(67)
13q	62/133 (47)		Rb	(64,65,79-82)
16q	31/48 (65)	16q22	E-cadherin	(83)
17p	124/166 (75)	17p11.2-13.3	OVCA1/2,	(64,67,70,79,81,84,85)
			HIC1	
			TP53	
17q	81/131 (63)	17q11.2-12	NF1, CSF3,	(65,84,86,87)
		17q21	erbB-2, ITB4,	
		17q24-25	BRCA1	
		17q25-ter	GH, TOC	
			Unknown	
18q	40/68 (59)	18q22.1	DCC, DPC4	(64,65,70)
			Smad-2	

IFNA, interferon-alfa gene; NF1, neurofibromatosis gene; CSF3, colony stimulating factor 3; erbB-2, member of epidermal growth factor receptor family; ITB4, integrin-beta 4 ; MSH3, DNA mismatch repair gene; Rb, retinoblastoma; VHL, Von Hippel Lindau gene; PPARγ, human peroxisome proliferator-activated receptor-gamma; MCC, mutated in colorectal cancer; APC, adenomatous polyposis coli; IRF-1, interferon regulatory factor 1; TOC, tylosis oesophageal cancer gene; DCC, deleted in colorectal carcinoma; DPC4, deleted in pancreatic carcinoma, locus 4; TP53, p53 tumour suppressor gene; BRCA1, breast cancer gene; WT2, Wilms's tumour suppressor gene; H19, gene involved in genomic imprinting; p57KIP-2, a cyclin dependent kinase inhibitor; HIC1, hypermethylated in cancer; OVCA1/2, ovarian cancer tumour suppressor genes 1 and 2; GH, growth hormone gene; Smad-2, signal transduction molecule involved in TGF-beta signalling pathway.

TUMOUR SUPPRESSOR GENES

Microsatellite allelotyping or loss of heterozygosity (LOH) analysis is a useful technique to define chromosomal regions of deletion in carcinomas. The LOH analysis uses the polymorphic microsatellite repeats (as mentioned above). These microsatellites are present on all chromosomes and differ in size in the population and usually between the paternal and maternal chromosome of an individual. PCR amplification of the microsatellites followed by size separation can identify chromosomal arms or regions that are lost in the tumour compared to normal tissue from the same individual. Frequent loss of one allele involving a chromosomal arm or locus suggests the presence, at or near that locus, of a tumour suppressor gene. Several groups have evaluated chromosomal regions for LOH in Barrett's oesophagus-associated neoplasia (Table 1). We will discuss the most common areas of chromosomal loss with their target genes.

Chromosome 3p: FHIT, VHL and PPARγ

Fragile sites are genomic regions that predispose for structural chromosome aberrations such as translocations or deletions. It is hypothesised that genes at fragile sites are altered by chromosome rearrangements and thus contribute to neoplastic growth. Chromosome band 3p14.3, encompassing the most inducible common fragile region, has been cloned and the Fragile Histidine Triad (FHIT) gene characterised. In Barrett's oesophagus and associated adenocarcinomas, alterations of the FHIT transcripts (FHIT mRNA lacking one or more exons or homo- or hemizygous deletions of the gene) were observed in 86 and 93%, respectively (88). Another study found low rates of alterations in the FHIT open reading frame in primary oesophageal cancers, although lack of expression of FHIT transcripts was common in oesophageal cancer cell lines (89). However, aberrant FHIT transcripts have also been detected in normal, non-cancerous tissues of the gastrointestinal tract, questioning the role of FHIT as a tumour suppressor (90). Its apparent involvement might simply reflect its location within an unstable region of the genome and FHIT might not be causally related to the tumourigenesis of the oesophagus.

Other candidate tumour suppressor genes on 3p are the von Hippel Lindau (VHL) gene and the gene encoding the human peroxisome proliferation activated receptor-gamma (PPARγ). Both genes are mutated in VHL disease and colon carcinomas, respectively (91,92).

Chromosome 5q: MCC and APC

LOH at the MCC locus occurred in 63% of the patients with oesophageal carcinoma (72). So far, no reports have been published on MCC mutation analysis in oesophageal adenocarcinomas. Observations from colorectal and gastric cancers suggests that, despite the high frequency of LOH of the MCC gene, mutation of the retained MCC allele is uncommon (93,94). This suggests that MCC does not function as a tumour suppressor gene in gastrointestinal malignancies.

The APC gene is also a target of LOH on chromosome 5q21-22. LOH of the APC locus on 5q has been found in oesophageal adenocarcinomas as well as in the surrounding high-grade dysplastic Barrett's epithelium. Furthermore, the patterns of

allelic loss of the APC gene were identical in all stages of neoplastic progression, suggesting the emergence of a clonal population of cells (72). But LOH has not been found in Barrett's metaplasia or low-grade Barrett's dysplasia (77). While APC mutations were found frequently in colorectal cancers, a very low rate of APC mutations in oesophageal cancers was detected (77,95,96), although in most studies not the whole coding sequence of the gene was screened for mutations. This raised the possibility that a gene distinct from APC may be the target of the frequent LOH on 5q. Deletion of the APC locus may just be the result of large deletions on 5q and may not be important in oesophageal carcinogenesis (95). The Interferon Regulatory Factor1 (IRF-1) gene or other gene(s) on 5q31.1 may be the true target of frequent deletions on 5q that may play an important role in the pathogenesis of the majority of oesophageal carcinomas (95).

Chromosome 9p: p16

The p16 gene (MTS1, CDKN2A) encodes a 16kDa protein. It forms complexes with the cyclin dependent kinases (CDK) CDK4 and CDK6, inhibiting their ability to phosphorylate the retinoblastoma (Rb) protein. Unphosphorylated Rb prevents the cell from entering the cell cycle (S phase). Thus inactivation of this gene may lead to uncontrolled cell growth. The p16 gene is located on chromosome 9p at 9p21, a locus at which frequent allelic loss occurs in oesophageal adenocarcinomas (75,77). Point mutations in exons 1 and 2 of the p16 gene are rare (ca. 5%) in oesophageal adenocarcinomas whereas p16 mutations were found more frequent in squamous cell carcinomas (76,89,97,98). Barrett *et al.* reported a higher prevalence (23%) of p16 gene mutations in adenocarcinomas with LOH of 9p21 (78). However, in this study only aneuploid cell populations were investigated, which is not representative for oesophageal carcinomas in general and thus might explain the higher prevalence of p16 gene mutations. It is possible that p16 is inactivated by different mechanisms. Gonzalez *et al.* report homozygous deletions of the p16 gene in 3 of 12 (25%) oesophageal adenocarcinomas (77). However, these genetic changes were not present in patients with non-dysplastic Barrett's oesophagus. Two studies showed that p16 promoter methylation (with or without p16 LOH) is a common mechanism of p16 inactivation during neoplastic progression in Barrett's oesophagus, and is already present in non-dysplastic premalignant Barrett's epithelium (99,100). P16 inactivation may indeed be a useful biomarker to stratify patients' risk of progression of Barrett's metaplasia to oesophageal cancer (101).

Another tumour suppressor gene on 9p, p15 (MTS2/CDKN2B), a close homologue of p16 and located 20kb centromeric, is extremely rare altered in various types of human cancers, including oesophageal adenocarcinomas (78,97).

Chromosome 13q: retinoblastoma

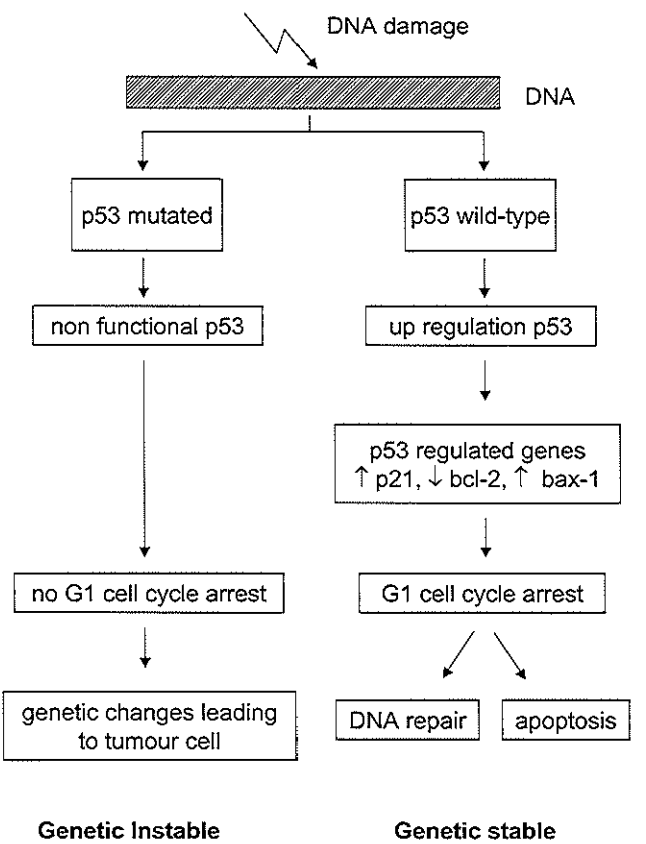
The protein coded for by the normal retinoblastoma (Rb) gene is a critical regulatory molecule in the G1 phase of the cell cycle. Mutations in Rb result in uncontrollable cell proliferation and predispose to numerous human tumours. LOH of 13q has been demonstrated in oesophageal adenocarcinomas(80), and this was associated with an unfavourable survival rate(82). There are no reports on mutation analysis of the Rb gene in oesophageal adenocarcinomas, but loss of normal Rb protein expression was

observed as the Barrett's metaplasia progressed to dysplasia and carcinoma, indicating accumulation of unstainable aberrant protein (25,102). LOH of the Rb gene and Rb protein expression, as detected by immunohistochemistry, however, were not significantly correlated.

Chromosome 17p: p53

Mutations and deletions of the p53 gene are the most common genetic lesions in human cancers. The p53 protein functions in a homotetrameric complex as a transcription factor that induces expression of genes that facilitate cell cycle arrest, DNA repair and apoptosis (Figure 2).

FIGURE 2. *P53 and cell cycle regulation. Upon DNA damage there is upregulation of wild-type p53 protein. This leads to increased transcription of p53 regulated genes (e.g. p21, bax-1,bcl-2) which inhibit the cell cycle. This facilitates DNA repair or the cell enters the apoptotic pathway. In this way p53 provides genomic stability. Mutations in the p53 gene render the p53 protein inactive and the damaged DNA is transmitted.*



One mutant p53 protein in the tetrameric p53 complex abolishes the function of the entire complex. Furthermore, most mutant proteins have a much longer half-life than the wild type protein. This implicates that when a cell harbours one inactivating p53 mutation, the concentration of this protein will increase relative to the product of the wild type allele and the activity of the wild type protein will be inhibited by complexing with the mutant protein (dominant-negative). The prolonged half-life of the mutant p53 protein and the concomitant increased cellular p53 concentration makes visualisation by immunohistochemistry possible. Studies on Barrett's oesophagus report a very low percentage of metaplasia cases with p53 protein accumulation (21,103-108). P53 accumulation increases in low grade dysplasia to high grade dysplasia from less than 10 to more than 70% respectively (105,106,108-110). From numerous immunohistochemical studies it is clear that in more than 50% of oesophageal adenocarcinomas pronounced p53 overexpression is present (104,106-108,110-113). With molecular techniques to detect p53 gene alterations, such as single strand conformation polymorphism (SSCP) analysis, sequencing and loss of heterozygosity (LOH) analysis, occasional cases of metaplasia and low grade dysplasia with p53 mutations have been found (66,114,115). In high grade dysplastic Barrett's epithelium and invasive adenocarcinoma the prevalence of p53 mutations exceeds 40% and p53 locus LOH in these conditions is generally found at even higher rates (50,66,101,111,112,114-116). In high grade dysplasia and in oesophageal adenocarcinoma p53 mutations have been found even in diploid cell subpopulations from aneuploid tumours (101,112). Furthermore, allelic loss of chromosome 17p often occurs before the loss of 5q during neoplastic progression (108,117). These findings suggest that p53 mutation is an early event in oesophageal adenocarcinogenesis.
In conclusion, there is overwhelming evidence that p53 gene alterations are early and frequent events in oesophageal adenocarcinomas and that this gene is associated with malignant transformation of Barrett's oesophagus. Although prognostic significance of p53 alterations has been suggested, p53 abnormality alone is not sufficient to predict progression to cancer or disease outcome (118-120).

Chromosome 18q: DPC4 and DCC

Allelotype analysis has shown that allelic loss of 18q was frequent in oesophageal adenocarcinomas. However, mutational analysis of the deleted in pancreatic cancer (DPC4) gene did not reveal any mutations (121). Therefore, DPC4 is unlikely to be the target gene on 18q21 and other candidates such as deleted in colorectal cancer (DCC) or as yet unidentified target genes can be involved.

PROTO-ONCOGENES

The proto-oncogenes encode a group of proteins that are involved in signal transduction (from the plasma membrane to the nucleus) or the regulation of gene expression. Mutated proto-oncogenes are called oncogenes, because most mutations activate the proteins, which results in excessive stimulation of growth (Figure 3.).

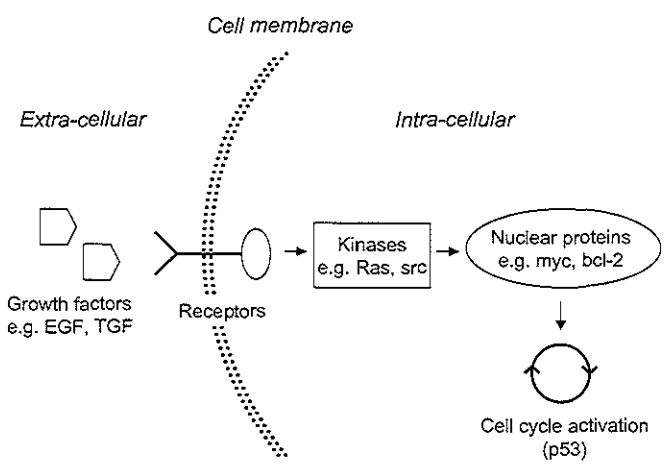
Growth factors and their receptors

EGF, TGF-α and EGFR

An important family of growth factors is the one that bind to the epidermal growth factor receptor (EGFR), including epidermal growth factor (EGF) and transforming growth factor-α (TGF-α). EGF has a stimulatory effect on epithelial cell proliferation in the gastrointestinal tract and has been demonstrated to be overexpressed in oesophageal carcinomas. Although EGF is also expressed in Barrett's oesophagitis, the expression of EGF does not discriminate between dysplastic and neoplastic epithelium (122). Overexpression of its receptor EGFR in the oesophagus did correlate with the degree of mucosal dysplasia and the occurrence of adenocarcinoma, suggesting that high expression levels may reflect increased malignant transformation potential in Barrett's mucosa (22,122-124). Al-Kasspooles *et al.* found EGFR gene amplification in 30% of the oesophageal adenocarcinomas and also in Barrett's metaplasia, but no correlation with the level of EGFR expression by immunohistochemistry (125).

Transforming growth factor-α (TGF-α) is structurally and functionally related to EGF. TGF-α expression is increased in metaplastic, dysplastic and neoplastic tissue of the oesophagus compared to normal mucosa(122). The degree of abnormal expression becomes more marked as dysplasia increases and correlates with the proliferative indices in Barrett's oesophagus (19,122,126). In conclusion, EGF/TGF-α and their receptor EGFR are important in the progression of normal oesophageal squamous epithelium to metaplasia, dysplasia and finally carcinoma and possibly associated with autocrine growth regulation in normal gastrointestinal mucosa and neoplasia (127).

FIGURE 3. *Schematic representation of signal transduction form the cell membrane to the nucleus and the proteins (merely proto-oncogene products) involved. Activation (e.g. mutations) of the genes encoding growth factors, their receptors or the signal transduction pathway genes (Ras, src, myc, bcl-2)can lead to constitutive activation of the cell cycle. Abbreviations: EGF, epidermal growth factor; TGF, transfroming growth factor.*



c-erbB2

The c-erbB2 proto-oncogene (HER2/neu; chromosome 17q21) encodes a transmembrane glycoprotein with intrinsic tyrosine kinase activity that is homologous to, but distinct from the EGFR. C-erbB2 protein overexpression and/or amplification of the c-erbB2 receptor gene occur in approximately 10-70% of oesophageal adenocarcinomas (43,122,128-132). Overexpression of c-erbB2 was not demonstrated in dysplastic Barrett's epithelium suggesting that it is a late event in the dysplasia-carcinoma sequence (129). C-erbB2 overexpression in adenocarcinomas correlated significantly with tumour invasion, lymph node involvement, distant metastasis and status of residual tumour after resection (43,132).

FGF

The fibroblast growth factors (FGFs) are potent mitogens that possess angiogenic properties and the capability to regulate growth and differentiation of various cell types. The expression of acidic and basic FGF (aFGF, bFGF) has also been studied in oesophageal adenocarcinoma and Barrett's metaplasia. FGF is generally sequentially accumulated in the progression from metaplasia to neoplasia. Oesophageal adenocarcinomas and high grade dysplastic Barrett's epithelium showed enhanced expression of aFGF mRNA and protein (immunohistochemistry) but not of bFGF, when compared to low grade dysplasia and normal control epithelium (133,134).

TGF-β

In contrast to TGF-α, TGF-β is a potent inhibitor of cell proliferation, an inducer of differentiation in epithelial cells of the intestine *in vitro*, and a suppressor of genomic instability (135). There is some evidence that the TGF-β signalling pathway is involved in the initiation and progression of oesophageal adenocarcinomas. First, TGF-β is expressed in non-dysplastic Barrett's epithelium, as well as oesophageal adenocarcinomas (136,137). Second, inactivating mutations occur in MADR2, an important component of the signalling pathway for TGF-β, in colon cancers (138). The chromosomal localisation of the MADR2 gene is 18q21, which frequently shows LOH in oesophageal adenocarcinomas. Finally, loss of expression of the functional receptor for TGF-β (TGF-β receptor type II) appears to be associated with Barrett's oesophagus and oesophageal adenocarcinomas (137,139,140). The exact role of TGF-β and its receptor in Barrett's adenocarcinomas needs to be clarified.

RAS-family

The ras families of proto-oncogenes (H, K and N) encode specific proteins, which appear to be essential components in normal cell division and differentiation. Ras proteins act as signal-transducing molecules in the cytoplasm. Ras has not been shown to be mutated in most studies on Barrett's oesophagus and oesophageal adenocarcinomas (141-143). On the other hand, increased H-ras expression in Barrett's carcinoma and amplification of the K-ras gene in oesophageal adenocarcinomas has been reported (144-146). Two studies reported, for the first time, point mutations in K-ras in Barrett's oesophagus and in oesophageal adenocarcinomas, but these were rare (147,148). In conclusion, activation of the Ras proto-oncogenes

seems to be of little importance in Barrett's adenocarcinomas contrary to that observed in other carcinomas of the gastrointestinal tract.

c-myc

The c-myc gene is located on chromosome 8q24 and encodes a nuclear protein that is thought to regulate the transcription of other genes important for cell growth (149). Activation of c-myc gene may contribute to tumour progression by preventing cells from entering the G0-resting phase. Studies suggest that c-myc is the target gene of the chromosome 8q high level amplifications found in oesophageal adenocarcinomas (57,150-152). By using in situ hybridisation technique, Abdelatif *et al.* found enhanced c-myc expression in dysplastic Barrett's epithelium and adenocarcinomas, but not in non-dysplastic Barrett's mucosa (146). On the contrary, c-myc could not be detected immunohistochemically in oesophageal adenocarcinomas or in Barrett's epithelium (144). Presently, it is unclear whether amplification or mutation of c-myc play a significant role in the malignant progression of Barrett's oesophagus, but it appears that it is of limited prognostic value in human oesophageal carcinomas (153).

Src

The cellular oncogene c-src and its viral homologue v-src encode 60kDa, cytoplasmic, membrane-associated, protein-tyrosine kinases. A close correlation exists between elevated specific kinase activity and cell transformation. Src may deregulate cell-adhesion by anchorage-dependent growth control, thereby maintaining cells in the proliferative state (154). The src activity was found to be 3 to 4 fold higher in Barrett's epithelium and 6 fold higher in oesophageal adenocarcinomas than in control tissues (155). Moreover, Jankowski *et al.* found that 20% of the oesophageal adenocarcinomas and Barrett's oesophagus expressed src (144). These data suggest a role for src in the malignant transformation of Barrett's oesophagus and warrants further investigation.

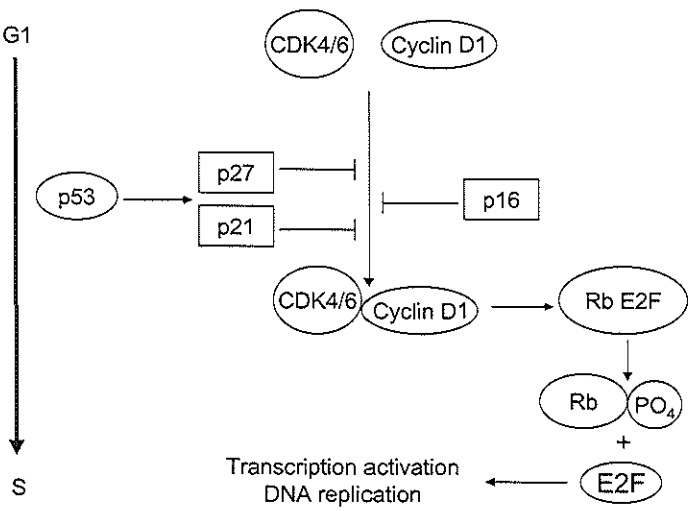
Prostaglandins

Cyclo-oxygenase (COX) catalyses the rate-limiting step in prostaglandin (PG) synthesis. There are two different isoforms of COX, referred to as COX-1 and COX-2. COX-1 is constitutively expressed and involved for example in cytoprotection of the gastric mucosa. In contrast, COX-2 is normally absent in most tissues, but can be induced by pro-inflammatory or mitogenic stimuli. COX-2 is involved in a large number of processes fundamental for tumour development: apoptosis, cell adhesion, invasion and metastasis and angiogenesis (156). Chronic oesophagitis is associated with the excessive mucosal production of prostaglandin E2 (PGE2) and bile acids stimulate COX-2 expression in oesophageal cells *in vitro* (157,158). Furthermore, COX-2 is expressed (determined by immunohistochemistry and/or RT-PCR or western blotting) in 70-80% of the oesophageal adenocarcinomas and also in corresponding Barrett's metaplasia (159,160). Inhibition of COX-2 in oesophageal cancer cell lines induced apoptotic cell death and reduced proliferative activity and synthesis of PGE2 (159,160). Among regular aspirin users, a 40-50% or even higher reduction in oesophageal cancer risk was found (161,162). These data indicate that the chemopreventive potential of NSAIDs in oesophageal adenocarcinomas, by repressing the induction of COX-2 enzymes in oesophagitis and Barrett's metaplasia, deserves further attention.

GENES INVOLVED IN CONTROLLING THE CELL CYCLE

The progression of cells through the cell cycle is governed by genes encoding proteins transmitting positive (e.g. activated cyclins and cyclin-dependent kinases (CDKs) and negative (e.g. inhibitors of CDK) signals (Figure 4).

FIGURE 4. *Genes involved in cell cycle progression and inhibition. Cell cycle progression from G1 into S phase requires activation of the cyclin dependent kinases (CDK4/6) in association with cyclin D1. This active complex phosphorylates the retinoblastoma protein (Rb). Phosphorylated Rb releases Rb-bound transcription factors (E2F-family). Free E2Fs transactivate genes that are essential for entry in the S phase and DNA replication. At the G1 checkpoint there are also negative regulatory signals controlling the cell cycle, namely inhibitors (p16, p21 and p27) of activated cyclin-CDK complexes.*



Cyclins form a family of proteins that complex with CDKs. Phosphorylation of the Rb protein by cyclin D1-CDK4/6 is correlated with the transition across the G1 checkpoint. Cyclin D1-CDK4/6 activity is regulated by phosphorylation events and by CDK inhibitors (CDKI), which bind to the cyclin-CDK complex and inhibit its activity. This cyclin-CDK inhibition impairs Rb phosphorylation and thereby prevents the cell from entering the cell cycle (S phase). The CDKIs p15 and p16 have been already discussed. Another group of CDKIs is the Cip/Kip family and includes p21, p27 and p57.

Cyclin D1

Cyclin D1 abnormalities, either gene amplification or overexpression, lead to constitutive activation of the cyclin D1-CDK4/6 pathway. Increased nuclear expression of cyclin D1 is observed in 22-64% of the oesophageal adenocarcinomas, and is already present in Barrett's metaplasia (82,163-165). The increased expression of cyclin D1 is especially frequent in the intestinal-type lesions and in tumours with early T-stage (163,166). Amplification of cyclin D1 gene was observed in 16-26% of the oesophageal adenocarcinomas (73,166). However, cyclin D1 immunoreactivity was not always associated with gene amplification (82,167). Therefore, additional regulatory mechanism of protein expression probably exists.

$P27^{Kip-1}$

The $p27^{Kip1}$ (p27) gene is located on chromosome 12p13. Overexpression of p27 induces a block during G1 in the cell cycle. Singh *et al.* found p27 protein expression and p27 mRNA to be increased in intensity and distributed throughout the glands of high-grade dysplastic Barrett's epithelium, indicating transcriptionally upregulation of p27 (168). In contrast, low p27 protein expression but elevated levels of p27 mRNA were found in 83% of oesophageal adenocarcinomas, possibly due to post-transcriptional regulation of the gene. In addition to nuclear staining, cytoplasmic staining of p27 was noted in 48% and 26% of dysplasia and carcinomas, respectively (168). Loss of nuclear and/or cytoplasmic staining for p27 correlated with higher histological grade, depth of invasion, presence of lymph node metastasis and shorter survival (168). These findings suggest that the cell cycle inhibitor p27 may be overexpressed to counteract proliferative stimuli in Barrett's associated dysplasia. Loss of p27 or altered subcellular localisation as the process becomes invasive suggests an important role for this CDKI in preventing progression of Barrett's oesophagus to adenocarcinoma (136).

$P21^{WAF1/CIP1}$

The G1-S phase of the cell cycle can also be down regulated by inhibition of CDKs by $p21^{WAF1/CIP1}$ (p21). Nuclear expression of p21 is upregulated by the wild-type p53 tumour suppressor, but not mutated p53 (169). P21 expression was elevated in Barrett's tissue classified as indefinite for dysplasia, low-grade dysplasia, high grade dysplasia and in Barrett's adenocarcinoma, but not in Barrett's epithelium negative for dysplasia (170). No relationship between p21 and p53 staining in oesophageal adenocarcinomas was found, indicating that there are also p53 independent pathways for the upregulation of p21 (103,170). The elevated nuclear p21 expression in Barrett's oesophagus and adenocarcinoma does, most likely, not represent mutated protein (171). p21 expression was significantly associated with prognosis: patients with p21 positive tumours showed a better prognosis than patients with p21 negative tumours (165).

CELL-CELL ADHESION

It has long been known that cell-cell adhesion is generally reduced in human cancers. Reduced cell-cell adhesiveness removes contact inhibition of proliferation, thus allowing escape from growth control signals. Moreover, invasion and metastases, which are life-threatening properties of malignant tumours, are considered to be later, but critically important carcinogenic steps.

E-cadherin-catenin complex

The E-cadherin-catenin complex is the prime mediator of calcium-dependent cell-cell adhesion in normal epithelial cells. In non-malignant epithelia, E-cadherin and the catenins show a membranous localisation at intercellular borders. In Barrett's adenocarcinomas, reduced membranous expression of E-cadherin as well as the catenins is observed in 60-80% of the tumours (172-175). Moreover, reduced expression of E-cadherin and alfa- and beta-catenin correlated significantly with unfavourable tumour stage, tumour grade, lymph node metastases and survival (175). Reduced expression has also been shown to be associated with higher degrees of dysplasia in Barrett's oesophagus (173,176). This suggests that E-cadherin-catenin complex may be useful as a marker for neoplastic progression from Barrett's metaplasia to adenocarcinoma and metastases. No mutations could be detected in oesophageal adenocarcinomas despite frequent LOH of the E-cadherin locus at 16q22 (83).

Besides establishing cell-cell adhesion, beta-catenin has been shown recently to function in cell signalling (177). Under normal conditions, beta-catenin is bound to the cytoplasmic tail of E-cadherin. Free, unbound beta-catenin in the cytoplasm is kept low by rapid degradation of unbound beta-catenin. In order to be degraded, beta-catenin is phosphorylated by a protein complex, of which the adenomatous polyposis coli (APC) protein is one of the members. Inactivation of APC leads to an increase in cellular free beta-catenin that enters the nucleus of the cell, directly binds to transcription factors and activates gene expression. These target genes are involved in promoting cellular proliferation and migration, such as the c-myc oncogene and the cell cycle regulator cyclin D1. Besides inactivation of APC, mutations in phosphorylation sites of the beta-catenin gene can also lead to stabilisation of the protein. In oesophageal adenocarcinomas, increased cytoplasmic and nuclear localisation of beta-catenin has been observed (173), implicating involvement of APC inactivation or beta-catenin mutations with subsequent activation of the signal transduction pathway.

Serine protease system

The serine protease system has been shown to play an important role in the invasive potential of a variety of tumours by breaking down the extracellular matrix. Urokinase plasminogen activator (uPA) is a serine protease. High levels of uPA were found in oesophageal adenocarcinomas (178,179), and correlated with higher pTNM categories, tumour stage, lymphatic invasion and survival (180). Therefore, uPA antigen content could identify oesophageal adenocarcinoma patients who will develop early tumour recurrences, thus providing a more accurate estimation of prognosis.

CD44 protein family

CD44 is a family of glycoproteins involved in cell-cell adhesion and cell-matrix interactions. As a result of alternative splicing of 10 exons (v1-10) more than 20 isoforms have been described. CD44-standard (CD44s) and its abnormal transcripts (CD44v) have been detected in oesophageal adenocarcinoma (181-183). Increased CD44s expression was seen in 50-66% of the oesophageal adenocarcinomas (Dr K.K. Krishnadath, personal communication, november 1997)(181,183). In Barrett's oesophagus CD44s (but not CD44v6) expression increases along with dysplasia and the proliferation rate and increased CD44v6 was seen in an early stage of malignant transformation (Dr K.K. Krishnadath, personal communication, november 1997)(183). A significant correlation between CD44s, v6 and v10 expression and clinicopathological characteristics has been reported (Dr K.K. Krishnadath, personal communication, november 1997)(181,183), but further studies on a larger patient cohort are required to validate the usefulness of CD44s and isoforms in clinical decision making.

Cathepsin B

The cysteine protease cathepsin B (CTSB) gene, which maps to 8p22, codes for a lysosomal enzyme that has been shown to be overexpressed or exhibit altered localisation in cancers (184). Overexpression or altered localisation of CTSB is thought to result in degradation of the basement membrane facilitating tumour invasion and metastasis. Hughes *et al.* found an amplicon at chromosome 8p22-23 resulting in CTSB gene amplification and overexpression (185). Moreover, abundant extracellular expression of CTSB protein was found in 29 of 40 (73%) oesophageal adenocarcinoma specimens (185). These data support an important role for CTSB gene amplification and CTSB protein overexpression in oesophageal adenocarcinomas.

SUMMARY AND CONCLUSIONS

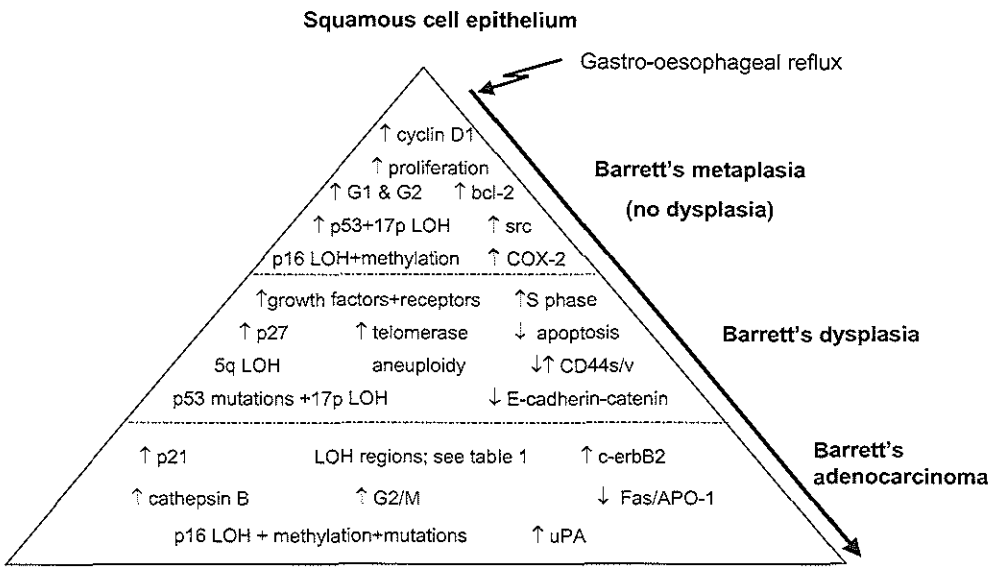
There is need for improved understanding of the molecular biology of Barrett's oesophagus and adenocarcinoma. Despite ongoing efforts to characterise the molecular changes in Barrett's oesophagus, its pathogenesis remains poorly understood. A wide variety of genetic events and mechanisms appear to play a role in the development and progression of Barrett's oesophagus-associated neoplastic lesions. Figure 5. summarises these molecular events. However, there is still no uniform molecular pathway of progression. In fact, a surprising degree of clonal heterogeneity in premalignant Barrett's epithelium has been found consistent with a complex pattern of evolution of neoplastic cell lineages rather than a simple linear pathway of progression.

Meaningful clinical intervention in patients with Barrett's oesophagus is still predicted on accurate histologic descriptions, but this information can be supplemented by biomarkers of cell proliferation and abnormalities in proto-oncogenes and tumour suppressor genes. Many of these markers do not have this potential: dysfunction of Ras-families of proto-oncogenes and cell cycle genes such as c-myc and cyclin D1 are not indicative for malignant transformation of Barrett's oesophagus. On the contrary,

development of Barrett's adenocarcinoma is associated with losses on chromosomes, *e.g.* losses of 4q, 5q, 16q and 18q are frequently observed. Results of these studies are promising, but need further attention. The same can be said about cell-cell adhesion molecules and growth factors and their receptors, such as EGF(R), c-erbB2, src and the prostaglandins. Abnormalities involving the p16 and p53 tumour suppressor genes and aneuploidy or increased 4N populations are among the most common somatic genetic lesions in the progression from Barrett's oesophagus to oesophageal. These biomarkers are potential candidates for objective molecular markers that can be used in combination with histologic staging to stratify patients 'risk of progressing to oesophageal adenocarcinoma.

In the next several years the subsequent genetic events critical for the initiation and progression of Barrett's adenocarcinoma will be further characterised and may clinically be useful as biomarkers for early cancer detection or prognostication. With the disturbing increase in incidence of Barrett's adenocarcinomas, further research into this area is vital.

FIGURE 5. *Summary of the genetic alterations involved in the progression of Barrett's metaplasia towards Barrett's adenocarcinoma. Alterations that occur in the early stages are (usually) also present in the more advanced histological stages. Within each histological category, the alterations are placed randomly and there is no hierarchical order.*



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CHAPTER 2

E-CADHERIN-CATENIN CELL-CELL ADHESION COMPLEX AND HUMAN CANCER

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PRÉCIS

Disturbance in the E-cadherin-catenin complex is one of the main events in the early and late steps of cancer development. Getting more insight into the early molecular interactions critical to the initiation and progression of tumours should aid the development of novel strategies for both prevention and treatment of cancer.

INTRODUCTION

It has long been known that cell-cell adhesion is generally reduced in human cancers. Reduced cell-cell adhesiveness removes contact inhibition of proliferation, thus allowing escape from growth control signals (1). Moreover, invasion and metastases, which are the most life-threatening properties of malignant tumours, are considered to be later, but critically important, carcinogenic steps. The invasion and metastatic steps consist of sequential steps involving host-tumour interactions. The suppression of cell-cell adhesiveness may trigger the release of cancer cells from the primary cancer nests and confer invasive properties on a tumour. In order for a metastatic nodule to form, cancer cells must detach from the primary site, invade through the surrounding host tissue, enter the circulation, lodge in a distant vascular bed, extravasate into the target organ, and proliferate (2). Therefore, reduced cell-cell adhesiveness is considered indispensable for both early and late carcinogenic steps. In recent years, there has been increasing interest in a large family of transmembrane glycoproteins, called cadherins, which are the prime mediators of calcium-dependent cell-cell adhesion in normal cells (3). There is increasing evidence that modulation of this complex by different mechanisms is an important step in the initiation and progression of human cancers.

Cadherin

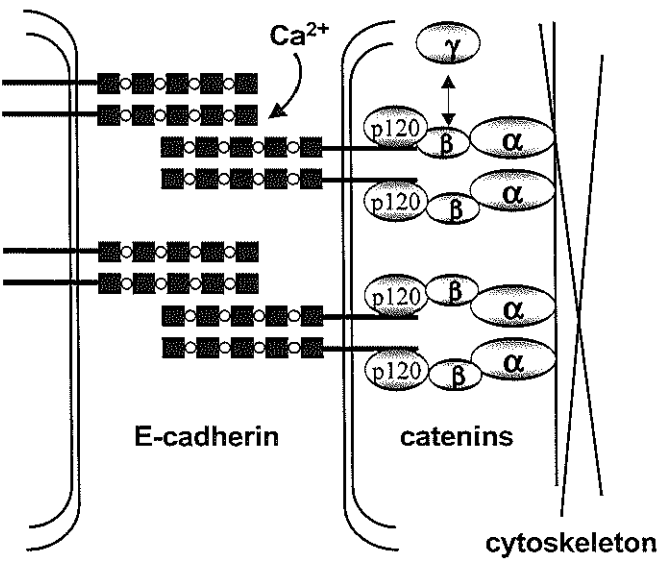
The cadherins have been divided up into more than 10 subclasses dependent on their tissue distribution, including E- (epithelia), N- (neuronal) and P- (placental) cadherin (4,5). E-cadherin (120 kDa; chromosome 16q), otherwise known as uvomorulin, L-CAM, cell-CAM 120/80 or Arc-1, is a classical cadherin and forms the key functional component of adherens junctions between epithelial cells (6). Normal E-cadherin expression and function is essential for the induction and maintenance of polarized and differentiated epithelia during embryonic development (3). The critical importance of E-cadherin in normal development and tissue function is demonstrated by the lethality of E-cadherin knockout mice at an early stage in embryogenesis (7). The binding of cadherins is homotypic in nature, i.e. they only bind to identical molecules on adjacent cell surfaces. The contribution of cadherins to intercellular binding is achieved through the formation of cell surface multi-molecular structures with a "zipper" confirmation (8).

Catenins

Cadherin-binding requires a complex series of interactions between cadherins and cytoplasmic molecules. E-cadherin is bound via series of undercoat proteins, the catenins, to the actin cytoskeleton (9) (Figure 1). This linkage between transmembranous cadherins and actin filaments of the cytoskeleton is necessary to form strong cell-cell adhesion (10). Deletion of the intracellular catenin-binding domain of E-cadherin or alterations in the functionally active catenins, results in the loss of the ability of E-cadherin to establish cell-cell adhesion, even if the extra-cellular binding domain is intact (10). The catenin family comprises α- (102kDa, chromosome 5q21-22) (11), β- (92kDa; chromosome 3p22) (12) and γ- (plakoglobin; 83 kDa ; chromosome 11q11) (13), with β- and γ-catenin sharing the

greatest homology. β- and γ-catenin bind directly to the cytoplasmic tail of E-cadherin in a mutually exclusive manner. α-catenin then links the bound β- or γ-catenin to the to the actin microfilament network of the cytoskeleton. Recently, another catenin-like molecule, p120^{ctn}, has been identified in association with E-cadherin at the cell-cell junctions although this complex does not appear to form a link with the actin cytoskeleton (14). p120 ^{ctn}, originally identified as one of the several substrates of the tyrosine kinase pp60src, also associates with β-catenin and E-cadherin (15). Unlike the other catenins, it has 4 isoforms, which appear to be expressed differentially in a variety of cell types (14). Recently, it was found that p120^{ctn} actually acts as an inhibitory regulator of cadherin function in colon carcinomas (16).

FIGURE 1. *Schematic representation of cadherin-mediated cell-cell adhesion in epithelial cells.. Intercellular binding is achieved through the formation of E-cadherin dimers that interdigitate to form a continuous linear "zipper" structure. The interaction is dependent on extracellular calcium levels. The cytoplasmic domain of E-cadherin is complexed with either β- or γ-catenin. α-Catenin links the complex to the actin cytoskeleton of the cell.*



THE E-CADHERIN-CATENIN COMPLEX IN TUMOUR DEVELOPMENT

An intact E-cadherin-catenin complex is required for maintenance of normal intercellular adhesion. Therefore, several groups have proposed that, in carcinomas,

E-cadherin functions as an invasion-suppressor molecule such that its loss permits or enhances the invasion of adjacent normal tissues. There are many data supporting this hypothesis. Immunohistochemical studies in human cancers have frequently shown that a proportion of invasive carcinomas and carcinomas *in situ* show aberrant levels of E-cadherin and/or catenin expression in comparison to their related normal tissue (17-19). In general, E-cadherin and catenin staining is strong in well differentiated cancers which maintain their cell adhesiveness and are less invasive, but is reduced in poorly differentiated tumours which have lost their cell-cell adhesion and show strong invasive behaviour. Changes in E-cadherin expression may therefore be an important step in the development and progression of a malignant tumour. *In vitro* studies have shown that in various human cancer cell lines with an epithelioid, differentiated morphology were generally non-invasive and expressed E-cadherin, whereas cell lines with a fibroblast-like morphology were invasive and had often lost E-cadherin expression (20). Reconstruction of cadherin binding in human carcinoma cell lines by transfection with E-cadherin cDNA resulted in a more differentiated phenotype and loss of invasiveness (20-22). Furthermore, non-transformed Madin-Darby Canine Kidney epithelial cells as well as well-differentiated colon carcinoma cell lines, acquire a dedifferentiated and invasive phenotype when intercellular adhesion is inhibited by anti-E-cadherin monoclonal antibodies (23,24). This clearly demonstrates that E-cadherin can suppress the overt features of advanced tumour progression. However, it has remained unsolved whether the loss of E-cadherin-mediated cell adhesion is a prerequisite for tumour progression or is a consequence of dedifferentiation during tumour progression *in vivo*. Using a transgenic mouse model of pancreatic β-cell tumourigenesis this question was recently addressed. Perl *et al.* demonstrated that the loss of E-cadherin-mediated cell-cell adhesion is causally involved in the transition from adenoma to invasive carcinoma (25).

Loss of cadherin-mediated adhesion may also act by promoting tumour cell detachment from the primary site resulting in dissemination of malignant cells to distant organs. Direct evidence implicating E-cadherin in the development of metastases is based on the association between high-metastasising carcinomas and low-E-cadherin immunoreactivity (26,27). An *in vitro* study has shown that E-cadherin-negative tumour cells are more likely than E-cadherin-positive cells to be dislodged from the primary tumour by low shear forces, such as these found in venules or lymphatic systems (28). The involvement of E-cadherin in metastasis was studied using an *in vivo* model of nude mice (29,30). Injections of E-cadherin-negative breast cancer cells in the circulation gave rise to multiple lung and osteolytic bone metastases in these mice. However, breast cancer cells that were transfected with E-cadherin cDNA showed a significant impaired capacity to form osteolytic metastases (29). It has also been shown in breast cancer patients that tumours with reduced E-cadherin expression have a higher frequency of lymph node and distant organ metastases than those with preserved E-cadherin expression (27).

E-cadherin as a growth suppressor

Recently it became clear that E-cadherin is involved in contact inhibition of cell growth by inducing cell cycle arrest (1,31). Sequential activation and inactivation of a family of cyclin-dependent kinases govern the cell cycle. P27 is one such cyclin-dependent kinase inhibitor, which results in cell cycle arrest. A recent report demonstrated that E-cadherin has the ability to inhibit cell proliferation by the upregulation of p27 (1). The mechanism by which E-cadherin regulates p27 is still unclear. Inhibiting the activity of mitogenic pathways such as the epidermal growth factor receptor (EGFR), which in turn regulate the level of p27 in cells is suggested. Therefore, E-cadherin, classically described as an invasion suppressor (21), is also a major growth/ proliferation suppressor. This attractive model for E-cadherin might explain earlier findings by Hermiston and Gordon. They showed that inactivation of E-cadherin in intestinal crypt cells leads to formation of adenomas, and that forced expression of E-cadherin suppresses proliferation (32,33).

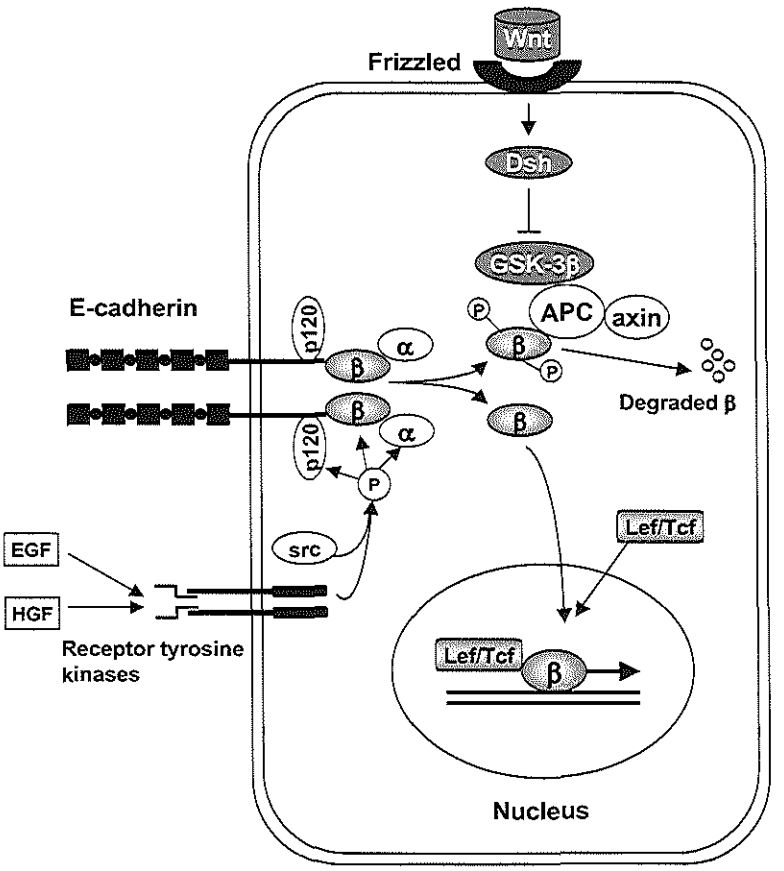
THE ROLE OF β-CATENIN IN SIGNAL TRANSDUCTION

An important function of β-catenin, namely its role in cell signalling, has been elucidated in the past years (Figure 2) (34-36). β-Catenin is the vertebrate homologue of the *Drosophila* segment polarity gene armadillo, an important element in the Wingless/Wnt (Wg/Wnt) signalling pathway. Wingless is a cell-cell signal in *Drosophila* that triggers many key developmental processes, Wnt being the vertebrate homologue (37). In the absence of a mitotic signal from outside the cell, β-catenin is sequestered in a complex with the adenomatous polyposis coli (APC) gene product, a serine threonine glycogen kinase (GSK-3β) and an adapter protein axin (or a homologue conductin) enabling phosphorylation and degradation of free β-catenin by the ubiquitin-proteasome system (38). Our understanding of the function and interactions between the proteins in the complex was rather a mystery until recently. Axin, the newly recognised component of the complex acts as a scaffold protein in the multiprotein complex (39). Formation of an axin regulatory complex is critical for GSK-3β activity and β-catenin phosphorylation and degradation, since GSK-3β does not directly bind to β-catenin but requires the presence of axin which binds to both proteins (40). This complex formation leads to the maintenance of low levels of free cytoplasmic β-catenin. Residual catenins hold cells together by binding to cadherins both at the adherens junctions and the actin cytoskeleton.

When a mitotic signal is delivered by the Wnt pathway, by association of the Wg/Wnt family of secreted glycoproteins and their membrane receptor frizzled, it leads to activation of the dishevelled (Dsh) protein, which is recruited to the cell membrane. The activated Dsh downregulates the protein complex, so that it can no longer phosphorylate β-catenin, which then is not degraded (41). How exactly Wnt signalling leads to the stabilization of β-catenin remains unclear, although it is

suggested that that a critical step could be the dissociation of GSK-3β from axin with the help of Dsh (42). With GSK-3β no longer bound to axin, it can not phosphorylate β-catenin, leading to an elevation in β-catenin levels. Another model proposed is that inhibition of GSK-3β activity upon Wnt signalling by Dsh leads to the dephosphorylation of axin, resulting in a reduced efficiency of binding to β-catenin. The release of β-catenin from the phosphorylation and degradation complex promotes β-catenin stabilisation and signalling (43,44). The resulting increase in free cytosolic β-catenin then enters the nucleus and directly binds the transcription factors Lef and Tcf, leading to the activation of gene expression (45-47). Recently, the target genes of these transcription factors have been identified. They are thought to be involved in inhibiting apoptosis and promoting cellular proliferation and migration, such as the c-myc oncogene and one of the cell cycle regulators cyclin D1 (48-51). γ-Catenin can also enter the nucleus and interact with these transcription factors, but γ-catenin has been shown to have transactivation capacities different to β-catenin thus leading to distinct signalling properties (52).
Although β-catenin performs distinct functions in E-cadherin-mediated cell-cell adhesion and in Wnt-signalling (53), there appears to be some cross talk between the adhesive and the signalling pathways. The expression of excess E-cadherin interferes with Wnt-signalling by competing for β-catenin binding (54). However, loss of cadherin binding does not lead to accumulation of β-catenin free to bind Tcf/Lef-1 and to modulate transcription (55). Interestingly, LEF-1/β-catenin is also able to bind the E-cadherin promoter possibly leading to downregulation of E-cadherin (56).

FIGURE 2. *Schematic representation of the catenins and the interaction with other transmembrane, cytosolic and nuclear proteins. The unbound cytoplasmic pool of β-catenin is sequestered in a protein complex and phosphorylated by glycogen synthetase kinase 3β (GSK-3β), enabling its degradation. Abnormalities in this degradation pathway, activation of the Wnt pathway, mutated adenomatous polyposis coli (APC) or mutated β-catenin, can lead to an increase in free β-catenin levels, which then translocates to the nucleus, binds transcription factors (Lef/Tcf), and stimulates transcription of target genes. The catenins also interact with receptor tyrosine kinases, such as the epidermal growth factor (EGF) and the hepatocyte growth factor (HGF) receptor, which alter their phosphorylation status and, as a consequence, modulates E-cadherin-catenin cell-cell adhesion. See the text for more details.*
Dsh, dishevelled.



Activation of the Wg/Wnt pathway in human cancers

It is now believed that transformation of adult mammalian cells into malignant tumours reflect an exaggeration of the Wg/Wnt pathway, at least in some tumours. The APC gene is mutated in many cancers. In fact, it is supposed to be the first genetic alteration in the multistep process in inherited (familial adenomatous polyposis coli) and sporadic colorectal cancers (57). Recently, it was found that APC regulates levels of free-β-catenin in the cell, as discussed above. Most of the mutations in APC result in truncated APC protein which can still complex with, but not degrade β-catenin (58). The result of APC mutation is therefore an increase in cellular free β-catenin, which may trigger a cascade of events resulting in the initiation of adenomas (59). APC mutations in colorectal cancers have been shown to result in an increase in β-catenin/Tcf-4 signalling, leading to overexpression of the c-myc proto-oncogene and promotion of neoplastic growth (48,60). However, somatic mutations or small deletions (but no germline mutations) in the β-catenin gene, targeting the serine and threonine residues necessary for phosphorylation by GSK-3β and breakdown by the APC/GSK complex, are also associated with colon carcinogenesis (60,61). These β-catenin mutations have a dominant effect, suppressing the APC dependent binding and degradation of β-catenin leading to accumulation of cytoplasmic free β-catenin, again with subsequent translocation of β-catenin to the nucleus.

In the past two years many studies revealed that activation of the Wg/Wnt pathway by mutated β-catenin is a rather frequent event in malignancies including hepatocellular- and thyroid carcinomas, desmoid tumours and pilomatricomas (62-65), but uncommon in melanomas, head and neck cancers, oesophageal and prostate carcinomas (66-69). This implicates β-catenin as an oncogene in cancer development giving a possible explanation for the uncontrolled proliferation observed in cancers. Perhaps this is why widespread nuclear and cytoplasmic staining of beta-catenin in colorectal cancers was an independent prognostic factor for short survival (70). Similarly, mutations in GSK-3β, axin or other (yet unknown) elements involved in the Wnt/Wg pathway may contribute to β-catenin signalling. Future studies need to focus on these other components of the complex and try to elucidate the Wg/Wnt pathway in human cancers.

MECHANISMS OF INACTIVATION OF THE E-CADHERIN-CATENIN COMPLEX IN CANCER

Human cancers possess both irreversible and reversible mechanisms for inactivation of the E-cadherin-catenin complex which lead to invasion and metastases. Loss of the E-cadherin locus on the long arm of chromosome 16 (16q22) has been reported to occur at rather high frequency in hepatocellular (50%), lobular breast (50%) and oesophageal carcinomas (66%) (71-73). There have been several reports on E-cadherin gene mutations in human cancers (74). In

poorly differentiated tumours like lobular breast cancer and the diffuse type gastric cancer, E-cadherin mutations play an important role in tumour development (75,76). However, mutations are rare in oesophageal, thyroid and colorectal carcinomas (73,77,78). Interestingly, E-cadherin mutations with loss of the remaining wild-type allele were also detected in the pre-invasive *in situ* component of lobular breast cancer and in intramucosal lesions of gastric signet ring cell carcinomas (79,80). This, together with the findings of Perl *et al.* (25), indicates that genetic alterations of the E-cadherin gene are involved in the early developmental stages of some histological types of human cancer, and that both E-cadherin alleles are inactivated. The importance of loss of function of E-cadherin in the onset of cancer has now been well established. There have been several studies reporting germ-line mutations in the E-cadherin gene in families with an inherited diffuse type of gastric cancer (81,82). However, the clinical implications for these findings are not clear yet. Only a minority of gastric cancers can be accounted for by E-cadherin mutations. Recently, it has been reported that early onset of lobular breast carcinomas may also be associated with E-cadherin germline mutations(82,83).

Thus far, mutations in the α-catenin gene has been described only for cell lines, not in tumours *in vivo*(84,85). Human cultured cancer cell lines with a genetically altered α-catenin only regained their cell-cell adhesiveness when transfected with wild-type α-catenin cDNA (86). Therefore, α-catenin meets the criteria of an invasion suppressor gene.

Genetic alterations in β-catenin abolishing cell-cell adhesiveness have been observed in two gastric cancer cell lines HSC 39 and 40A, both derived from the same signet ring cell carcinoma of the stomach, show a diffuse growth pattern (87,88). This mutation results in a truncated β-catenin lacking the region for interaction with α-catenin. Transfection of these cell lines with wild-type β-catenin restores cellular adhesiveness in these cell lines (88). Recently, a mutation in γ-catenin was described in a gastric cancer cell line, but no mutations have been reported in sporadic gastric cancers (55).

Down-regulation of E-cadherin expression can be induced by a low activity of the E-cadherin promoter due to chromatin rearrangement in the regulatory domain or due to DNA methylation (89,90). *In vivo* experiments showed that methylation in the E-cadherin promoter region correlated significantly with reduced E-cadherin expression in hepatocellular carcinomas and that methylation was also detected frequently in pre-cancerous conditions (91). Furthermore, an *in vitro* experiment showed that downregulation of E-cadherin by stimulating c-erbB2 transcription further reduces E-cadherin promoter activity, suggesting a role for c-erbB2 overexpression in tumour progression and metastases (92). However, an immunohistochemical study strongly argued against a role for c-erbB2 as a transcriptional regulator of E-cadherin expression in breast carcinomas *in vivo* (93). In addition, downregulation of β-catenin in cholangiocarcinomas was associated with c-erbB2 downregulation (94). Furthermore, EGFR and the tumour suppressor p53 may also play a role in the regulation of E-cadherin and α-catenin expression

and perturbation of the E-cadherin-catenin complex (95,96). Recently, it was found that *Helicobacter pylori* infection was associated with downregulation of E-cadherin in gastric mucosa and as such might play a role in the onset of neoplastic growth (97).
In some tumours, including pancreatic, thyroid, gastric, bronchopulmonary, oesophageal, colorectal and bladder cancer, the staining pattern of the E-cadherin-catenin complex is not always an absence or reduction in the expression but shows a redistribution from the cell membrane to the cytoplasm (19,98-103). The mechanism responsible for this redistribution in tumour cells remains elusive. Immunoprecipitation experiments have been performed to address this point with modifications in the interactions between E-cadherin and the catenins being observed (98,104). In colorectal tumours with heterogeneous cytoplasmic immunoreactivity for E-cadherin as well as the catenins, one of the catenins was not present in the complex or the cytoskeletal bound fraction was reduced in spite of the overall increase in expression of the proteins (Dr M. El-Bahrawy, personal communication) (99,105). These studies have shown that the expression of the proteins does not necessarily imply that they are functioning, as binding of the E-cadherin-catenin complex to the cytoskeleton is essential for its role in cell adhesion.
Failure of E-cadherin and catenins to localise to the membrane and/or bind to the cytoskeleton in spite of their abundant presence may be due to alterations in their phosphorylation status (100,101). A number of receptor and non-receptor tyrosine kinases and phosphatases, including the epidermal growth factor receptor (EGFR) and the c-erbB2 oncogene and the hepatocyte growth factor (HGF) receptor c-met interact with the catenins. This interaction alters the phosphorylation status of the catenins, and as a consequence cadherin-mediated adhesion (106-110). For example, EGFR has tyrosine kinase activity, which is activated through autophosphorylation upon its binding to epidermal growth factor (EGF). By this mechanism EGF induces immediate phosphorylation of β- and γ- catenin which is inhibited by herbimycine, a tyrosine kinase inhibitor (111). Overexpression of EGFR, c-erbB2 and c-met has been described in several cancers. This was associated with cellular redistribution of E-cadherin from the membrane to the cytoplasm along with suppression of its function (111,112). In addition, there is evidence to suggest that the association between E-cadherin and α-catenin can be prevented by tyrosine phosphorylated β-catenin (113).
Further evidence for the possible role of tyrosine phosphorylation as mechanism by which E-cadherin-catenin function is modulated comes from the finding in cells transfected with the v-src oncogene. Increased tyrosine phosphorylation of β-catenin and E-cadherin was observed and this posttranslational modification resulted in functional changes such as decreased cell-cell adhesion, increased migration and increased invasiveness, without affecting the overall expression of either of the catenins or the cadherins (114,115). The inhibition of tyrosine phosphorylation restores cadherin function to normal (114). Other studies showed that up-regulation of tyrosine phosphorylation of β-catenin and p120 occurs

frequently in surgical specimens of colorectal and lung cancer, and that phosphorylation of β-catenin correlated well with poor survival of patients after surgery (116,117). These results suggest that tyrosine phosphorylation of the catenins might be a significant mechanism that modulates their function and in turn that of E-cadherin-catenin, and this may have important prognostic value.

E-CADHERIN-CATENIN EXPRESSION IN CANCER AND THE POSSIBLE CLINICAL RELEVANCE

Immunohistochemical studies of many different types of human carcinomas (including skin, head and neck, lung, breast, thyroid, oesophageal, gastric, pancreatic, hepatocellular, colon, renal, bladder, prostate, endometrial and ovarian carcinomas) have shown that a proportion of carcinomas show reduced levels of E-cadherin expression in comparison to their related normal tissues. Indeed, E-cadherin loss is most pronounced in those types of carcinoma that have strikingly infiltrative growth patterns associated with little or no intercellular cohesion, such as invasive lobular breast cancer and diffuse type gastric adenocarcinoma. Therefore, it is not surprising that abnormal expression of the E-cadherin-catenin complex correlates with pathological characteristics of the tumour, such as grade of differentiation, invasion of the tumour, venous permeation, peritoneum seeding, lymph node, liver and bone metastases and tumour stage (26,118-124). Interestingly, aberrant expression of E-cadherin, α-, β-, γ-catenin and p120 correlated with clinical parameters, such as disease relapse, disease free survival and overall survival in cancer patients (121,125-131). Moreover, aberrant expression of E-cadherin and/or the catenins was shown to be an independent prognostic marker for shorter survival, although its predictive value was usually less strong than the standard parameters like tumour grade, tumour stage and lymph node metastases (128,132-136). Of particular interest is the finding that E-cadherin was an independent predictor of occult lymph node metastasis and micrometastases in nodes classified as N0 by routine histopathological methods (137). This is in accordance with studies that show an additive value for E-cadherin-catenin expression in patients with no signs of lymph node or distant metastases (N0 and M0) (131,138). Immunohistochemical detection of E-cadherin and the catenins could therefore not only be useful in predicting the disease free or overall survival but also in identifying patients with clinically negative lymph nodes who are at risk for occult metastases and who may benefit from more extensive lymph node dissection.

A number of studies did not show a relationship between E-cadherin-catenin expression and clinico-pathological parameters (139,140). Several explanations for this discrepancy can be given: the histological type and number of cancers analysed, selection of the tumours (stage, tumour grade), demographics of the study population (141), differences in the surgical approaches (extent of lymph node dissection) (118,142), as well as differences in staining evaluation.

Some studies report that the combination of E-cadherin and one of the catenins has a better prognostic value than evaluation of the individual components (27,143). It is important to note that alterations in any component may lead to disrupted function of the complex. Since catenins play a critical role in the regulation of cadherin-mediated adhesion, this indicates that E-cadherin immunoreactivity does not always imply the presence of a functionally normal cadherin-catenin complex. Thus, to predict tumour invasion and metastasis in carcinomas, it is useful to investigate not just the expression of E-cadherin but also the expression of the catenins. In addition, these results re-emphasise the importance of understanding the regulatory pathways of cell adhesion in order to interpret correctly the immunohistochemical data on adhesion molecule expression in tumours. Interestingly, the lack of correlation between cadherin and catenin immunoreactivity is also consistent with the promiscuous and yet selective association of catenins not only with E-cadherin, but also with other transmembrane (e.g. EGFR), cytosolic (e.g. APC) and nuclear protein (Tcf). Undoubtedly, large clinico-pathological studies evaluating all members of the adhesion complex within a well defined population and complete follow-up are needed to validate the use of E-cadherin and catenins as predictors of tumour cell behaviour.

Alterations in E-cadherin and catenin expression have also been found in pre-invasive lesions of the colon (adenoma), oesophagus (Barrett's dysplasia), stomach (gastric dysplasia) and breast (ductal carcinoma in situ) (18,19,144,145). In addition, elevated levels of a soluble form of E-cadherin (sE-cadherin; 80Kda) can be detected in the serum from patients with cancers, possibly induced by tumour-associated proteolytic degradation. In bladder cancer patients a correlation was found between the levels of sE-cadherin and grade, stage of the tumour and with tumour recurrence on cystoscopy (146,147). Furthermore, determination of sE-cadherin levels may serve as a tumour marker with a rather high sensitivity when compared to CA19-9 and CEA in gastric cancer patients (148). In ovarian carcinoma patients the levels of E-cadherin showed no correlation with the response to chemotherapy or 5-yr survival. Therefore, it was concluded that determination of pre-operative levels of sE-cadherin does not offer useful clinical information for the management of patients with ovarian cancer (149). Undoubtedly, more evidence from larger studies is needed to address the possible usefulness of sE-cadherin as a disease marker in cancer patients.

A combination of decreased E-cadherin expression with altered expression of other proteins involved in cancer invasion and metastasis has been described. For example, a combination of decreased E-cadherin expression with upregulation of urokinase-type-plasminogen activator (a protease involved in cancer invasion and metastasis) was shown to be an independent predictor of prognosis in gastric cancer patients (150). Simultaneous reduced expression of E-cadherin and CD44v6 in breast cancer and low E-cadherin expression in combination with high CD44s in renal cancer was correlated with poorer survival (129,151). Combination of low E-cadherin immunoreactivity and high type IV collagenase expression was an

independent predictor for disease recurrence and overall survival, but not stage, nodal metastases and histological type in pancreatic cancer (152). Finally, Otto et al. found that abnormal expression of both E-cadherin and gp78 (the receptor for a tumour-derived autocrine motility factor) in patients with bladder carcinoma results in poor disease outcome, independent of tumour stage and grade (118).
Recent studies suggest that modulation of E-cadherin and catenins may be more complex than previously thought. For example, it has been demonstrated that in 40% of adenocarcinomas E-cadherin levels are elevated in their intravascular tumour components in comparison to their extravascular compartments (153). Moreover, expression of E-cadherin or the catenins has been found to be higher in the lymph node metastases in comparison to the primary tumour (122,136,145) and, surprisingly, there was a greater tendency for liver metastasis in tumours in which the integrity of E-cadherin-mediated cell adhesion is intact (154). It strongly suggests that the levels of cadherin-catenins are not necessarily fixed, but in an appropriate microenvironment may be subject to transient manipulation. An explanation could be that entrance of a carcinoma into an intravascular compartment is associated with an upregulation of E-cadherin expression and that subsequent exit into extravascular tissues is associated with down-regulation (155). Second, upregulation of E-cadherin expression at implantation sites is maybe necessary for tumour cells in order to cluster and grow out as metastases. These concepts are supported by *in vitro* data and the fact that the staining pattern of E-cadherin in human tumours is often heterogeneous and unstable in some tumours (156,157).

E-CADHERIN-CATENIN: A TARGET FOR ANTI-CANCER THERAPY?

Since the function and expression of the E-cadherin-catenin complex is often reduced in cancer cells, it is suggested that restoration of the E-cadherin-catenin will lead to differentiation and anti-invasive properties. Several drugs have been described to alter the expression of E-cadherin, some of which are already used in the treatment of cancer patients. At least *in vitro*, insulin-like growth factor-I, tamoxifen, taxol, retinoic acid and progestagens were shown to upregulate the functions of E-cadherin/catenin complex including inhibition of invasion (158-162). The mechanisms by which these drugs induce these changes include upregulation of E-cadherin, α- and β-catenin mRNA expression, dephosphorylation of β-catenin, increased stability of the β-catenin protein, and localisation of β-catenin at the cell-cell junctions (160,162-164). A few more drugs can be added to the list of components which restore E-cadherin-catenin dependent cell-cell adhesion, of which aspirin is probably the most intriguing (165). Epidemiological, animal model, and clinical studies all suggest that nonsteroidal anti-inflammatory drugs are potent preventive agents for colon cancer. Mahmoud *et al.* showed that aspirin decreased the rate of tumour formation in MIN mice, an animal with a germline mutation in APC which leads to increases in cytoplasmic β-

catenin with subsequent cell signalling. Aspirin produced a decrease in intra-cellular β-catenin levels, suggesting that modulation of this protein is associated with tumour prevention (165).

TABLE 1. *The E-cadherin-catenin complex: findings and clinical implications*

Findings	*References*
Expression of E-cadherin-catenin correlates with histopathological findings: tumour grade, invasion and wall infiltration, venous permeation, lymph node, liver and bone metastases, tumour stage	(26,118-124)
Expression of E-cadherin-catenin can predict survival after surgery: overall 5-yr survival, 5-yr disease free survival, disease relapse	(121,125-131)
Expression of E-cadherin can predict occult lymph node metastases/micrometastases	(137) (131,138)
Malignant degeneration: normal epithelium → dysplasia → carcinoma sequence	(18,19,144,145).
Soluble E-cadherin in sera of cancer patients could serve as a tumour marker	
follow up of cancer patients, detecting recurrence	(148) (146,147)
response to (adjuvant) therapy ?	
Identifying families with hereditary diffuse type of gastric cancer (and lobular breast cancer?)	(166,167)
Anti-cancer therapy and cancer prevention: restoring abnormal expression of E-cadherin-catenin by drugs	(158-162) (165)

CONCLUSIONS

Inactivation of the E-cadherin-catenin cell–cell adhesion complex is mediated by genetic and epigenetic events mechanisms which occur in both the early and late stages of carcinogenesis. Previous and current studies suggest that these molecules can be useful in the assessment of the malignant potential of pre-invasive lesions and the development of prognostic markers in cancer. Table 1 summarises these findings and the possible clinical implications. In addition to epithelial cell-cell adhesion, E-cadherin-catenin is involved in a much further extent in cancer cell biology. Surprising findings regarding the interaction between APC and β-catenin,

and its role in cell signalling, have clearly shown that this complex has a key role in malignant cell transformation. Moreover, elucidation of the mechanisms underlying the changes in E-cadherin and catenin function may lead to the development of novel therapeutic approaches based on their biochemical and genetic manipulation.

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PART II

EPIDEMIOLOGICAL AND CLINICAL ASPECTS

CHAPTER 3

INCREASED INCIDENCE OF ADENOCARCINOMAS OF THE GASTRO-OESOPHAGEAL JUNCTION IN DUTCH MALES SINCE THE 90'S

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Submitted

PRÉCIS

The incidence of adenocarcinomas of the gastro-oesophageal junction increases in the South-eastern of the Netherlands and largely occurs in middle aged males.

INTRODUCTION

Cancers of the oesophagus and stomach are fairly common malignancies. Worldwide, cancer of the oesophagus is the eighth and cancer of the stomach the second most common form of cancer (1). Since before the Second World War the incidence and mortality rates of stomach cancer have been declining in industrialised countries (2). In contrast, the incidence rates of adenocarcinomas of the lower third of the oesophagus and/or of the gastric cardia have increased significantly over the last two decades in the United States (3), Australia (4) and some European countries (5,6). Longstanding gastro-oesophageal reflux leading to Barrett's oesophagus is the strongest risk factor for the development of oesophageal adenocarcinoma and (probably) cardia carcinoma (7,8). Whether the rise in incidence rates for adenocarcinomas of the oesophagus and cardia truly reflects an increase in gastro-oesophageal reflux and premalignant Barrett's oesophagus is presently unclear.
Several pitfalls may bias the observed trends in incidence rates for oesophageal and gastric carcinomas. The upward trend in incidence of adenocarcinomas of the distal oesophagus may well result from an increase in the number of histological verified cases, due to increased use of flexible endoscopy. A more precise discrimination of adenocarcinomas from other histological subtypes or a shift in the classification of tumours arising at the gastro-oesophageal junction (9), could also affect incidence rates over time. In this study, we addressed these issues by studying the time trends in mortality and incidence rates of oesophageal and gastric carcinomas in the Netherlands according to subsite and histology in the Southeast of the Netherlands between 1978-1996, where a regional population-based cancer registry, that collects data from the hospitals, has been in operation since the 50's.

METHODS

Study population

Data were collected from the population-based Eindhoven Cancer Registry (IKZ), which covers approximately 2 million inhabitants in the Southeast of the Netherlands since 1988. Only data from the eastern part of the area (900,000 inhabitants, registered since 1955) are used for this analysis. Registration is based on notification of newly diagnosed cases by the departments of pathology and radiotherapy and data from the medical records in all community hospitals in the region as well as from specialised departments and hospitals outside the region. In the study period, access to specialised care was excellent, although the number of hospitals declined from 12 to 7 by mergers. Data are collected during regular visits to these institutions, generally within 6 months since diagnosis by trained data abstracters. The registry does not record death certificate only cases, because this would require informed consent from both patients and doctors in the Netherlands.

Selection of tumours

All primary tumours of the oesophagus and stomach (International Classification of Oncology (ICD-O-9) codes 150 and 151, respectively) were included in the analysis. Non-epithelial neoplasms (sarcomas, lymphomas) were excluded from analysis. Between 1978-1996, a total of 507 oesophageal cancers and 3007 gastric carcinomas were recorded. The location of the tumours in the oesophagus was categorised into four groups based on the last ICD-O digit of each classification: upper- (codes 150.0 and 150.3), middle- (150.4), lower (150.5) oesophagus and other (overlapping and not otherwise specified (NOS); 150.8/9). The cancer site in the stomach was categorised into four groups: cardia (151.0), mid-stomach (fundus, body or curvatures; 151.3/4/5/6 and 151.8), antrum/pylorus (151.1/2) and others (unspecified; 151.9). Endoscopic, radiological and surgical data are taken into consideration for subsite assignment. Oesophageal tumours were classified into the following morphology categories: squamous cell carcinoma (codes 8050-8082 and 8047), adenocarcinoma (codes 8480-8490, 8140-8473 and 8500-8550) and all other morphology (NOS, no histological confirmation and other histological types; codes 8000-8004, 8010-8040, 8041, 8043 and 8560-8580). Gastric carcinomas were classified into adenocarcinomas and other morphology, including unspecified cases and cases with no histological confirmation.

Between 1978 and 1996, incidence rates were computed per 100,000 person-years for each gender. Age adjustment was performed by direct standardisation to the world standard population (WSR). Incidence rates are presented as three-year moving means to ensure stability of rates. Incidence rates for adenocarcinomas of the oesophagus and cardia were also combined for analysis of time trend.

Mortality rates

Regional data on the number of people with cancer of the stomach and oesophagus as the underlying cause of death from 1978-1996 were obtained from the Causes of Death Registry of Statistics Netherlands (CBS). Mortality rates per 100.000 people were calculated by gender, standardised to the WSR. Neither subsite nor subtype could be discriminated for oesophageal and gastric cancers.

RESULTS

Oesophageal cancer

During the study period, 352 males and 155 females were diagnosed as having carcinoma of the oesophagus. Table 1 summarises the gender, age and subsite details on the histological total numbers of oesophageal cancers. The predominant histological type was squamous cell carcinoma, making up two-thirds of the whole group. One fourth of all oesophageal carcinomas were adenocarcinomas. The group with other morphology included tumours with no histological confirmation in 12 cases (2%) and 39 cases (8%) which were classified as not otherwise specified or undifferentiated carcinomas. Oesophageal carcinomas were mainly localised in the distal third of the oesophagus. The subsite could not be determined in 13% of the cases (overlapping or unspecified), and 8 out of the 9 being $\geq$ 75 years of age.

TABLE 1. *Distribution of histological subtypes of oesophageal carcinomas diagnosed from 1978 to 1996, according to gender, age and subsite.*

	adenocarcinoma	*squamous cell carcinoma*	*other*[a]	*total*
Total cases (%)	**130 (26)**	**326 (64)**	**51 (10)**	**507 (100)**
gender (%)				
M	90 (26)	233 (66)	29 (8)	352
F	40 (26)	93 (60)	22 (15)	155
M/F ratio	2.3	2.5	1.3	2.3
age (yrs)				
median	69	67	76	68
range	27-94	28-97	48-94	27-93
age category (%)				
≤44 yrs	4 (24)	13 (76)	0	17
45-59 yrs	25 (22)	83 (72)	7(6)	115
60-74 yrs	62 (26)	157 (66)	19 (8)	238
≥75 yrs	39 (28)	73 (53)	25 (19)	137
subsite (%)				
upper 1/3	5 (9)	47 (81)	6 (10)	58
middle 1/3	16 (13)	93 (76)	13 (11)	122
lower 1/3	96 (37)	140 (54)	23 (9)	259
other[b]	13(19)	46 (68)	9 (13)	68

[a] *Not otherwise specified (n=32), no histological confirmation (n=12) and other morphology (n=7).* [b] *Tumours overlapping two or more subsites (n=28) and subsite not otherwise specified (n=40)*

The age-adjusted incidence and mortality rates for oesophageal cancer in males increased over the entire 19-year study-period. In males, the age-adjusted incidence rate doubled from 2.4 to 4.8 per 100,000 person-years and the mortality rate increased from 2.7 to 5.6 (Figure 1A). The increase was more pronounced in the older age groups (≥ 60 years). In females age-adjusted incidence and mortality rates increased from 0.5 to 2.3 and from 1.2 to 2.0, respectively. The rising trend in incidence rates was seen in all age groups whereas the increase in mortality is more pronounced for the age groups 45-59 and 60-74 years. Stable mortality rates were seen for females ≥ 75 years.

The rising trend for adenocarcinomas in males increased between 1978 and 1996 and was most pronounced between 45-74 years. At the end of the study period, the incidence rate of oesophageal adenocarcinoma became similar to the incidence rate of squamous cell carcinoma: 2.2 per 100,000 person-years *versus* 2.3, respectively (Figure 2A). In females, the incidence rates for squamous cell carcinomas appeared to increase faster than the adenocarcinomas (Figure 2B). The increase in squamous cell carcinomas was mainly seen in women aged between 45 and 75 years. For both sexes the combined incidence rates for unspecified oesophageal carcinomas and carcinomas of other histology were stable. An increase occurred in oesophageal

carcinomas arising from the distal third of the oesophagus in males and the middle and distal third in females (Figure 3).

FIGURE 1. *Age-adjusted incidence of and mortality rates from oesophageal (left) and gastric (right) cancers in the period 1978-1996.*

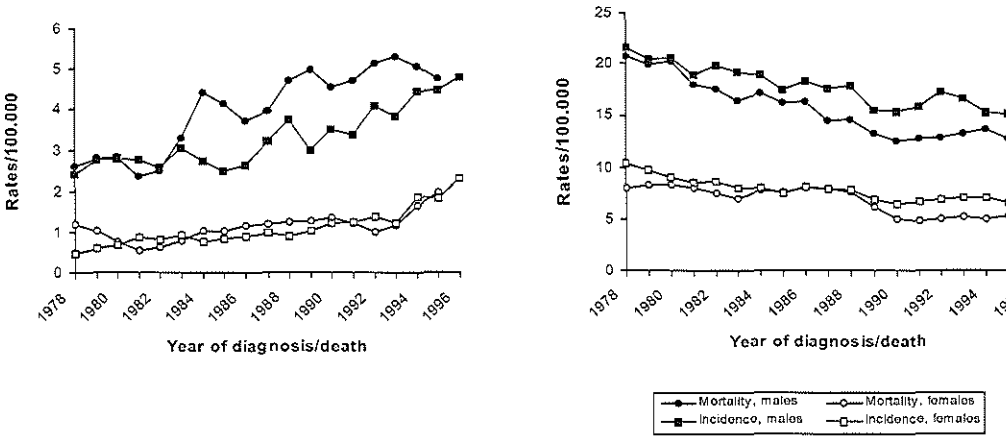
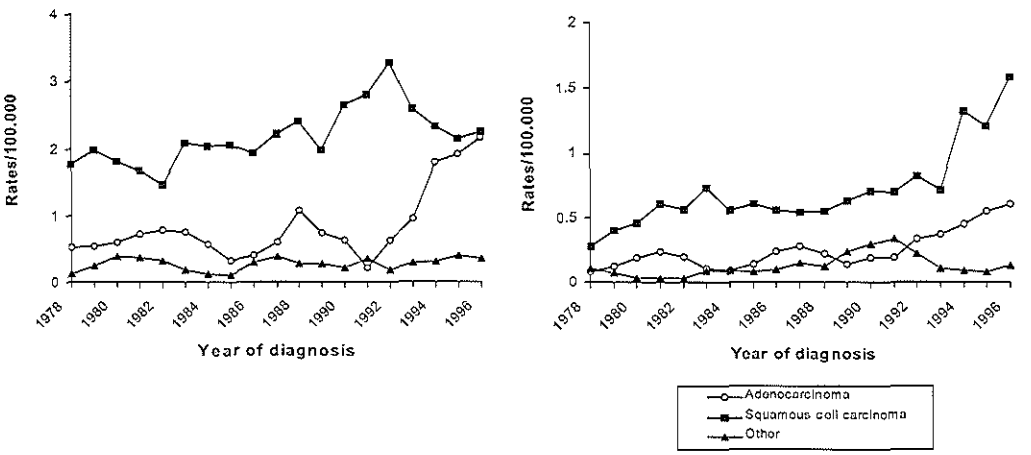


FIGURE 2. *Age-adjusted incidence rates of oesophageal cancer according to morphology in males (left) and females (right).*



Gastric cancer

During the study period, 1885 males and 1122 females were diagnosed as having carcinoma of the stomach. Table 2 shows the sex, age and subtype details on the subsites of all gastric cancers. Most cancers arise from the midstomach, but the subsite was not known in 398 cases (13%). Almost all diagnosed cases of gastric cancers were histologically proven (99%), whereas in 210 cases the morphology was unspecified. The group of cardia cancers mainly consisted of males at a younger age than those with tumours at other subsites. Table 3 shows that male/female ratios for cardia cancer (and oesophageal adenocarcinomas) decreased with increasing age.

TABLE 2. *Gender, age and subtype distribution of subsites of all gastric carcinomas diagnosed from 1978 to 1996, according to gender, age and subtype.*

	cardia	*midstomach*	*antrum/pyloric*	*other*[a]	*total*
Total cases (%)	**565 (19)**	**1275 (42)**	**796 (26)**	**398 (13)**	**3007**
gender (%)					
M	428 (23)	802 (42)	415 (22)	240 (13)	1885
F	137 (12)	473 (42)	354 (32)	158 (14)	1122
M/F ratio	3.1	1.7	1.1	1.5	1.6
age (yrs)					
median	68	71	74	73	72
range	25-93	21-97	24-99	21-99	21-99
age category (%)					
≤44 yrs	27 (27)	32 (31)	34 (33)	9 (9)	102
45-59 yrs	112 (24)	212 (45)	81 (17)	65 (14)	470
60-74 yrs	255 (21)	535 (44)	283 (23)	144 (12)	1217
≥75 yrs	171 (14)	496 (41)	371 (30)	180 (15)	1218
Subtype (%)					
adeno	524 (19)	1196 (43)	712 (26)	324 (12)	2756
[b]other	41 (16)	79 (32)	57 (23)	74 (29)	251

[a] Tumours not otherwise specified [b] Tumours without histological confirmation (n=37), other morphology (n=13) or not otherwise specified (n=201)

TABLE 3. *Male/Female ratios for oesophageal and gastric carcinomas diagnosed from 1978 to 1996, according to subtype and subsite, respectively.*

	M/F ratio				
Age category	*oesophageal cancer*			*gastric cancer*	
	squamous cell	*adeno*	*cardia*	*midstomach*	*antrum/pyloric*
≤44	3.3	4	27	1.3	1
45-59	2.5	5.3	10.2	2.7	2.5
60-74	3.4	2.9	3.3	1.9	1.5
≥75	1.4	1.0	1.5	1.3	0.9
total	2.5	2.3	3.1	1.7	1.1

Age-adjusted incidence declined from 21.6 to 15.9 per 100,000 person-years in males and from 10.4 to 6.2 in females (Figure 1B). Stable rates were seen for the age group 30-44 years. The age-adjusted mortality rates decreased over the study period from 21 to 11 per 100,000 person-years in males and from 8.2 to 4.2 in females. Rather stable mortality rates were seen in males aged 30-44 years and females aged 45-59 years.

Incidence of tumours with subsite unspecified decreased dramatically from 1978 to 1989 (Figure 4), which was accompanied by an upward trend in the incidence rates for midstomach and antrum/pylorus carcinomas. Since 1987 the incidence rates for midstomach and antrum/pylorus carcinomas declined, but increased for cardia carcinomas especially among men aged 30-44 years (from 3.1 to 4.6 per 100,000 person-years, respectively). In females, a similar picture was seen, but the incidence of cardia carcinomas remained stable in the last 10 years.

FIGURE 3. *Age-adjusted incidence rates of oesophageal cancer according to subsite in males (left) and females (right).*

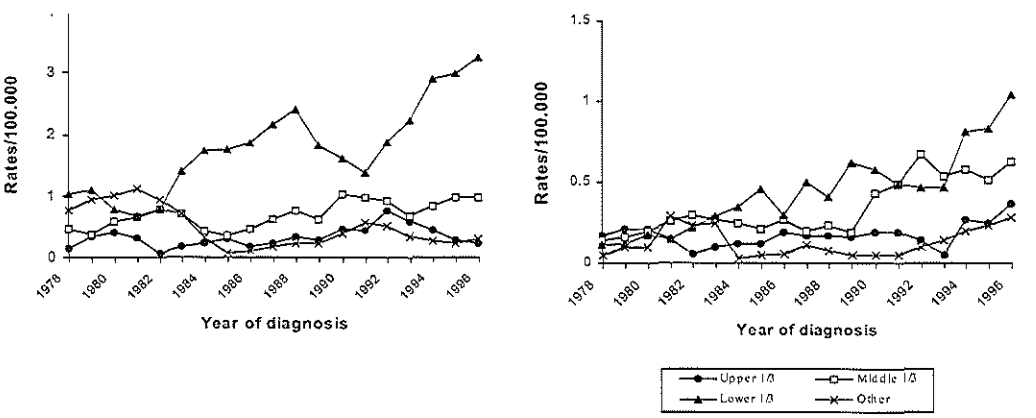


FIGURE 4. *Age-adjusted incidence rates of gastric cancer according to subsite in males (left) and females (right).*

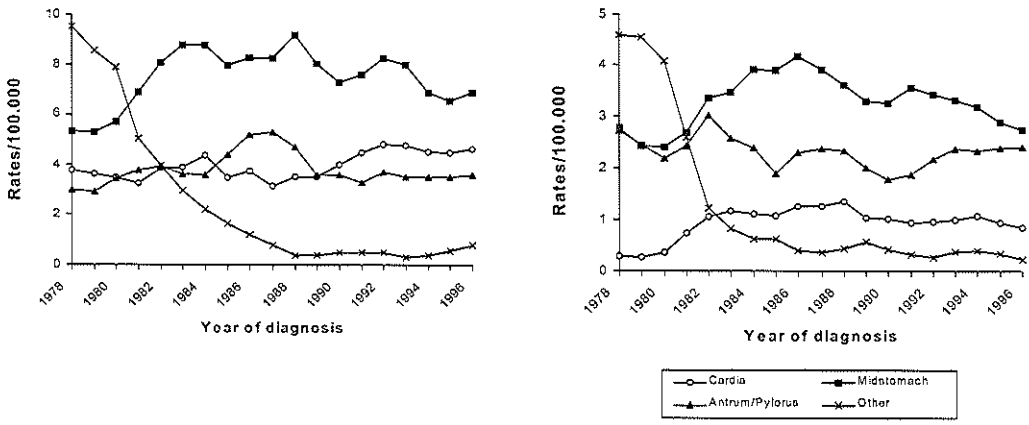
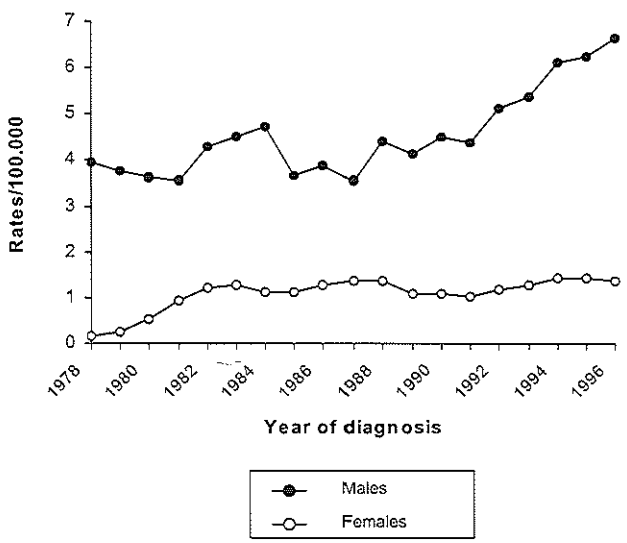


FIGURE 5. *Age-adjusted incidence rates of adenocarcinomas of the oesophagus and gastric cardia combined.*



Time trends in the occurrence of adenocarcinomas of the oesophagus and gastric cardia combined

For both sexes an increase in incidence is observed over the study period (Figure 5). In males, rather stable rates were seen during the 80's, whereas an increase in rates was seen during the nineties. Incidence rates for females were rather stable.

DISCUSSION

The temporal trends in the occurrence of oesophageal and gastric carcinoma in the Dutch population between 1978 and 1996 exhibited a diverse pattern. The occurrence of oesophageal squamous cell carcinoma increased in females and to a lesser extent in males. A marked increase of oesophageal adenocarcinoma in males occurred during the 90's. The incidence of adenocarcinoma of the oesophagus became approximately equal to that of squamous cell carcinomas. These changes were not reported by Botterweck *et al.* They studied incidence rates for adenocarcinomas of the gastro-oesophageal junction as a group, i.e. distal oesophagus and cardia combined, in the Netherlands until 1992 (6).

Furthermore, we observed that carcinoma of the gastric cardia increased somewhat in males in the last decade, whereas rather stable rates were seen for females. Distal gastric cancer (midstomach and antrum/pylorus) rates clearly decreased for both sexes, as is seen in other countries throughout the industrialised world (10,11). In males the decline in incidence seems to be slower than the decline in mortality. This may largely reflect early diagnosis through the general "open-access" use of gastroscopy within the Netherlands. The decline in mortality could also reflect better surgical treatment and lower peri-operative mortality rates. Indeed, survival of gastric cancer has improved slightly in some European countries (12). In contrast, sizeable increases are recorded in the incidence of and mortality from oesophageal cancer in the former communist countries of Central Europe, in countries of Northern Europe (including the United Kingdom) and among black males and females in the United States (5,13). However, in the Netherlands the incidence is relatively low, with only 1.7% and 1.0% of all malignancies in males and females, respectively (14).

Several biases hamper the interpretation of the trends in subsite- and histology-specific incidence rates. Changing completeness of reporting of diagnosed cancer cases seemed hardly a problem in this study, because access to medical care was adequate and reporting to the regional Cancer Registry in the Netherlands is known to be nearly complete. However, the incidence rates for oesophageal cancer are lower than the mortality rates within the same geographical region. Some oesophageal cancer cases may not be registered by the Eindhoven Cancer Registry. Subjects with a clinical diagnosis only of oesophageal or gastric cancer without pathological verification, and who are seen in the outpatient clinic only, may not be notified to the Registry. But under-reporting of diagnosed cases is unlikely to vary according to sex or morphological subtype.

Could increased use of endoscopy and better imaging explain the increase in adenocarcinomas of the oesophagus or gastric cardia? The high percentage histologically verified tumours did not change significantly over time: 97.6% for oesophageal cancers and 98.8% for gastric cancers and access to specialised care was very good during the study period. If this factor would play a role, a shift towards a better classification/stage of the tumours should be visible. Unfortunately, our data did not allow us to perform an analysis of stage at diagnosis. But others found that stage at diagnosis and survival have remained unchanged, despite likely improvements in early detection through better access to endoscopy and better supportive care after surgical treatment (15,16). Furthermore, a more frequent use of endoscopy does not explain the increasing incidence, since the increase of oesophageal and cardia adenocarcinomas is more marked in males than in females.

Because the incidence rates for unspecified tumours and tumours of other histology were stable during the study period, and the diagnosis of adenocarcinoma or squamous cell carcinoma can be easily established by routine light microscopy examination, it is unlikely that a more accurate histopathological classification has inflated the upward trend of adenocarcinomas at the gastro-oesophageal junction.

Oesophageal cancer with unknown subsite decreased during the first years of the study period, but rates were rather stable when an increase in tumours in the middle and lower third of the oesophagus was seen. In the first half of the study period, the occurrence of gastric cancer with unknown subsite decreased dramatically from 34% to 4%, as reported by others (9,17). The male to female ratios among tumours of unknown subsite was close to those of known subsites, collectively. The unknown subsite group was likely to be composed in similar proportions of tumours of all subsites. Assuming that 20% of the gastric carcinomas are situated at the cardia, then the observed increase in gastric cardia carcinomas between 1978 and 1996 should also likely to be 20%. Recently, Ekstrom *et al.* showed that accuracy in registering of cardia tumours by the National Swedish Cancer Registry is low and that the true cardia carcinoma incidence in the Swedish population could be up to 45% higher or 15% lower (18). Since data recording in the Eindhoven Cancer Registry is performed within the hospitals by trained data abstractors, misclassification is of cardia cancers is not a major problem in our study.

A gradual transfer to the oesophagus of cases previously reported as being of proximal gastric (cardiac) origin might also have biased our incidence rates. Since 1970 carcinomas of the gastric cardia received a separate code (code 151.1), distinguishing it from stomach cancer at other sites. It was recommended in the ICD-9 (that applied since 1978) that tumours arising at the gastro-oesophageal junction should be coded to the stomach (19). Gradually, the entity has been widely recognised by the medical community. Because of the proposed association of adenocarcinoma with Barrett's oesophagus (oesophageal mucosa lined with columnar epithelium), a great number of such tumours are more likely to have recently been classified as arising from the oesophagus (20). However, such a

spillover effect is unlikely, because a decline of incidence rates for gastric cardia cancer has not occurred.

In practise, it is often difficult to decide whether the tumour has originated at the gastric cardia or in the distal oesophagus. Dolan *et al.* reported that misclassification of oesophageal adenocarcinomas as carcinomas of the gastric cardia is common error in the data of the British Merseyside and Cheshire Cancer Registry (5). Adenocarcinomas of the oesophagus and gastric cardia are very similar in growth pattern, tumour grade and presence of nodal metastases (5,21). The remarkable similarity in survival between carcinoma of the oesophagus and cardia also suggest that the division of these carcinomas into oesophageal and gastric carcinomas is inappropriate (5,21). So, there can be little doubt that the incidence of adenocarcinomas at the gastro-oesophageal junction has been rising in the 90's, at least in males (and to a lesser extent in females) in the Southeast of the Netherlands.

What changes in the prevalence of risk factors could have occurred during the 70's and 80's? The rise in incidence rates for adenocarcinomas at the gastro-oesophageal junction could reflect an increase in Barrett's oesophagus. However, the prevalence of Barrett's oesophagus paralleled(22) or even exceeded the increased use of upper endoscopy since the 70's (23). Increased awareness of the endoscopic features of Barrett's oesophagus could have resulted in more increase in endoscopic diagnosis (24). If an increased presence of Barrett's oesophagus underlies the increasing trend of adenocarcinoma of the oesophagus and gastric cardia, then risk factors for Barrett's oesophagus must have been on the increase. Current opinion is that Barrett's oesophagus arises from persistent (duodeno) gastro-oesophageal reflux (25), which is more likely in obese individuals (26). Obesity is increasing in prevalence in the Netherlands and other European countries (27,28), but not (yet) to the extent as reported in the Unites States (29). Furthermore, a significant relationship was found between oesophageal adenocarcinoma and either obesity or total fat intake (30,31).

Case-control studies of patients with oesophageal or gastric cardia adenocarcinoma have also suggested cigarette smoking and possibly heavy liquor consumption as risk factors. The upward trend for squamous cell carcinomas, particularly in females (between 45-74 years old), also occurred in Great Britain (5), but not in the United States and New Zealand (3,32). The extent to which alcohol and tobacco can explain both the level and the trend in the rates is unclear. But the incidence of and mortality from oral and lung cancer in the Netherlands among females, also strongly related to alcohol and tobacco, have likewise been found to be increasing in the past decades (33).

It is suggested that infection with *H. Pylori* might actually protect against gastro-oesophageal reflux, Barrett's oesophagus and adenocarcinoma of the gastro-oesophageal junction. *H. Pylori*-positive subjects have impaired gastric acid production and some even develop chronic atrophic gastritis, which lead to a persistent decrease in acid production. This is supported by several studies that show a lower prevalence of *H. Pylori* among subjects with gastro-oesophageal reflux

disease compared to controls with normal findings (34). Also infection with cagA+ *H.Pylori* strains (which is the strain strongly associated with severe gastritis) was associated with a reduced risk for Barrett's oesophagus and oesophageal and cardia adenocarcinomas (35,36). This might explain the observed time trends of a decrease in the prevalence of *H. Pylori* infection and *H. Pylori*-associated disorders (such as peptic ulcer disease and adenocarcinoma of the (distal) stomach) and the increase in the incidence of gastro-oesophageal reflux disease, Barrett's oesophagus and adenocarcinomas of the gastro-oesophageal junction (24,37)

In conclusion, both major histological types of oesophageal carcinoma were on the increase: in males, mainly due to the rise of (Barrett's related) adenocarcinomas of the oesophagus since the early 90's, and in females due to an increase of squamous cell carcinomas since 1978. The sharp decrease in unspecified tumours and difficulties in classification until the mid 80's might have biased the observed trends to some extent.

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CHAPTER 4

ADENOCARCINOMAS OF THE DISTAL OESOPHAGUS AND GASTRIC CARDIA ARE ONE CLINICAL ENTITY

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PRÉCIS

Adenocarcinomas of the distal oesophagus and gastric cardia should be regarded as one clinical entity. Uniform staging criteria for both malignancies are recommended.

INTRODUCTION

According to the pathological tumour node metastasis (pTNM) criteria established by the Union Internacional Contre la Cancrum (UICC) and the American Joint Committee on Cancer (AJCC), carcinoma of the gastric cardia is classified as gastric cancer and carcinoma of the distal 8cm of the oesophagus including the intra-abdominal oesophagus is classified as oesophageal cancer (1,2). However, several studies suggest common risk factors and a similar phenotype for adenocarcinomas arising from the distal oesophagus and gastric cardia (3-8). Therefore, the distiction in classification seems rather artificial.
The present study questioned whether these tumours should be regarded and staged as two separate entities. Pathology, TNM stage and survival were studied in 252 patients who underwent resection for adenocarcinoma of the distal oesophagus or gastric cardia.

PATIENTS AND METHODS

From 1 January 1987 to 1 January 1997, 499 patients with an adenocarcinoma of the distal oesophagus or gastric cardia were evaluated at this hospital. After preoperative analysis 391 patients were operated on with curative intent. In 59 (15%) of these, resection was not possible because of metastatic spread or local irresectability. Patients who received pre-operative radiation and/or chemotherapy (n=43), and those who underwent oesophageal resection and total gastrectomy reconstructed by colonic interposition (n=25) or a transthoracic approach (n=12), were excluded from the study.
The remaining 252 patients underwent transhiatal resection of tumour and the continuity of the gastrointestinal tract was restored by a gastric tube with cervical anastomosis. In all patients a standard dissection of the perigastric, left gastric and the coeliac nodes was performed. Macroscopic tumour clearance was aimed at in all cases but no extended lymph node dissection was done.

Pathology

The pathology records of all patients were reviewed. A tumour was considered to arise from the distal oesophagus when the epicentre of the mass was located in the tubular oesophagus extending from the tracheal bifurcation to the gastro-oesophageal junction including the intra-abdominal oesophagus, according to the TNM classification (International Classification of Diseases for Oncology C15.5). The tumour was considered to be cardiac when the epicentre was at the gastric cardia, defined as the area at and immediately below the gastro-oesophageal junction, extending approximately 2cm downwards. It was preferable to rely on the muscular wall rather than the mucosal Z-line to define the transition between oesophagus and stomach, because many tumours destroyed the cardiac mucosa. Tumours arising from the fundus or corpus of the stomach and infiltrating the gastric cardia or distal oesophagus were excluded. Gross specimens were processed according to a standard laboratory protocol. Multiple 4-µm sections of the tumour

and surrounding mucosa were taken and stained with haematoxylin-eosin. Barrett's metaplasia was diagnosed when specific evidence of intestinal metaplasia was present. Lymph nodes were identified in the fixed specimens by the pathologist and subsequently evaluated for metastases.

Staging

Adenocarcinomas of the oesophagus and gastric cardia were classified according to the pTNM criteria for carcinoma of the oesophagus or stomach, established by the UICC in 1992 (1).

Statistical analysis

Differences in patient and tumour characteristics and TNM-classifications were assessed with the χ^2-test. Follow-up was until January 1 1997 or until death if earlier. Overall survival rates were calculated according to the Kaplan-Meier method and included perioperative deaths. For calculation of intercurrent death-corrected survival, patients who died of causes unrelated to carcinoma were considered as withdrawn from the study at the moment of death. Differences in survival rates were assessed with the log-rank test. The Cox proportional hazard model was used to evaluate various factors simultaneously. Statistical significance was set at the 5 percent level.

RESULTS

Some 252 patients who underwent transhiatal resection and stomach tube reconstruction for adenocarcinoma of the oesophagus or gastric cardia were included in the study. The in-hospital mortality rate was 4% (n=9), causes of death were anastomotic leak (n=3), pneumonia and respiratory failure (n=3), thrombosis of basilar artery (n=1), tracheo-oesophageal fistula (n=1) and stomach tube-aortic fistula (n=1).

In 111 patients the tumour originated from the distal oesophagus and in 141 patients the tumour originated from the gastric cardia. In both groups the median age of the patients was 66 (33-82) years. Male : female ratios were 3.1 : 1 for patients with oesophageal tumours and 7.8 : 1 for those with gastric cardia tumours (P=0.007).

Macroscopic appearances

The median diameter of oesophageal and gastric cardia tumours was 4 cm. Macroscopically, 53 oesophageal adenocarcinomas (48 percent) were limited to the distal oesophagus and 58 tumours (52 percent) involved the cardia with a median length of 1cm. Thirty gastric cardia tumours (21 percent) were limited to the cardia and 111 tumours (79 percent) showed some infiltration of the distal oesophagus (median length 1.5cm). A mean of 13 lymph nodes was dissected out of each specimen by the pathologist (mean 3.4 nodes positive for tumour microscopically).

Microscopy

Table 1 shows the microscopic characteristics of adenocarcinomas of the oesophagus and gastric cardia. Tumour-free resection margins were achieved in 67-74 per cent of the patients. Most carcinomas were moderately (G_2) or poorly

(G_3) differentiated; signet ring cells were detected in 20 per cent of the oesophageal carcinomas and 19 per cent of gastric cardia carcinomas. There was microscopic evidence of Barrett's epithelium in 54 per cent of the oesophageal adenocarcinomas and in 13 per cent of the gastric cardia carcinomas.

TABLE 1. *Microscopic characteristics according to location of primary tumour*

Microscopic characteristics	*Oesophagus (n=111)*	*Gastric cardia (n=141)*	*P-value*
Residual tumour classification (%)			0.2
R_0 (tumour free margins)	74 (67)	105 (74)	
R_{1-2} (tumour-positive)	37 (33)	36 (26)	
Circumferential	32	30	
Distal plane of resection	1	3	
Proximal plane	3	3	
Both	1	0	
Grade of Differentiation (%)			0.6
G_1 (well)	5 (4)	5 (3)	
G_2 (moderate)	63 (57)	70 (50)	
G_3 (poor)	43 (39)	66 (47)	
Signet ring cells (%)			0.9
Yes	22 (20)	27 (19)	
No	89 (80)	114 (81)	
Barrett's epithelium (%)			<0.001
Yes	60 (54)	18 (13)	
No	51 (46)	123 (87)	

Values in parentheses are percentages

Tumour stage

Gastric cardia carcinomas were more likely to be found at an advanced T stage (table 2). The frequency of metastatic locoregional lymph nodes was similar in the two groups. Twenty-one patients (19 per cent) with adenocarcinoma of the oesophagus were classified as having metastatic disease: metastases in distant lymph nodes were detected in 19 patients, whereas two patients had visceral metastases. In 15 of 19 patients these nodes were located around the coeliac axis (N2 for gastric carcinomas). Only three patients with gastric cardia carcinomas had positive distant nodes and two patients had visceral.

TABLE 2. *Tumour node metastasis subclassification according to location of primary tumour*

	Oesophagus (n=114)	*Gastric cardia (n=150)*	*P-value*
Tumour			
T_{is}/T_1	18 (16%)	9 (6%)	0.03
T_2	16 (14%)	28 (20%)	
$T_{3/4}$	77 (70%)	104 (74%)	
Nodes			
N_0	52 (47%)	53 (38%)	0.13
$N_{1,2}$	59 (53%)	88 (62%)	
Metastases			
M_0	92 (83%)	132 (94%)	0.007
M_1	19 (17%)	9 (6%)	

Survival

Follow-up was complete for all 252 patients. Median follow up was 19 (range 1-118) months. Median follow-up for patients who survived was 31 months (range 3-118). The overall 3- and 5-year survival rates (n=252) were 36 and 24 per cent respectively, with a median survival of 22 months. As only 16 patients died without suspected or proven recurrence of disease, overall survival rate was similar to the intercurrent death-corrected 5-year survival rate (26 per cent). Therefore, only overall survival is considered further.

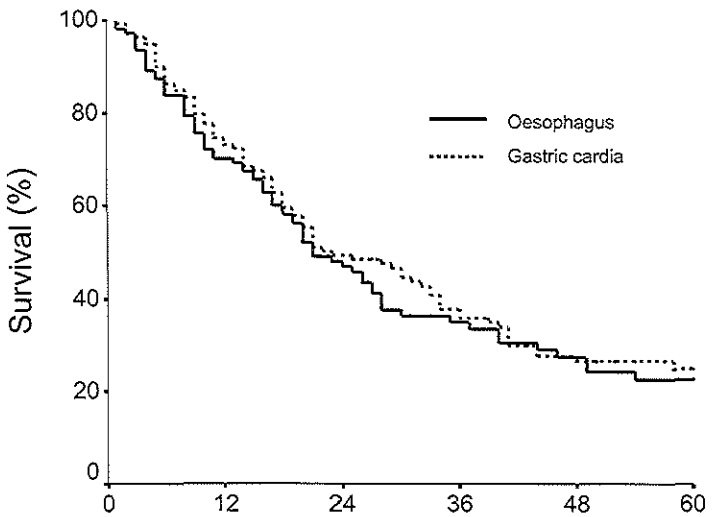
Survival in relation to TNM subclassifications for the whole group was as follows: patients with T_{is}/T_1 tumours had a 5-year survival rate of 70 per cent, compared with 37 per cent for T_2 and 14 per cent for those with $T_{3/4}$ carcinomas. Patients with negative lymph nodes (N_0; n=102) had a 5-year survival rate of 42 per cent, whereas those with positive lymph nodes (N_1 or N_2; n=150) had a survival rate of 11 per cent (P<0.0001). In the gastric cardia group the 5-year survival rate for patients with N_1-lymph nodes was not statistically different from that for patients with N_2 lymph nodes: 11 and 14 per cent, respectively (P=0.4). The 5-year survival rate for patients without metastasis (M_0) was 27 per cent compared with zero for patients with metastasis (M_1) (P<0.0001).

Analysis for survival according to histopathological grading showed that there was a survival advantage for patients with well and moderately differentiated carcinomas ($G_{1,2}$: 31 per cent) over patients with poorly differentiated carcinomas (G_3: 18 per cent) (P=0.004). The 5-year survival rate of patients with positive resection margins (R_{1-2}) was 8 per cent and that of patients with tumour-free margins was 33 per cent (P<0.0001). When adenocarcinomas were classified as Barrett-related (n=78) and non-Barrett-related (n=174) carcinomas, no significant difference in survival was observed between the groups, with rates of 38 and 27 per cent respectively (P=0.2). The 3- and 5-year survival rates for patients with oesophageal adenocarcinoma were 40 and 26 per cent respectively, and were similar to those for patients with an

adenocarcinoma of the gastric cardia (38 and 27 per cent) (P=0.9; figure 1). Survival was also assessed after stratification of the patients in the following groups: $T_{is-2}N_0M_0$, $T_{3-4}N_0M_0$, $T_{is-2}N_1M_0$, $T_{3-4}N_1M_0$, M_0 and M_1. No significant differences in 5-year survival rates between adenocarcinomas of the oesophagus and gastric cardia were observed within these groups.

Multivariate analysis showed that age, T, N and M-category, radicality of the resection and grade of differentiation were independent variables predicting survival. Location of the primary tumour was not an independent prognostic factor (table 3).

FIGURE 1. *Kaplan Meier survival curves according to location of the tumour.*



No. at risk						
Total	252	175	100	62	42	25
Oesophagus	111	76	43	25	18	12
Gastric cardia	141	99	57	37	24	13

TABLE 3. *Multivariate analysis regarding survival of various factors (Cox's regression)*

Factor	*Number of patients*	*Relative death rate (RDR)*	*P-value*	*95% Confidence interval RDR*
Age group				
50-60yrs	47	1.1	0.6	0.6-2.1
61-70yrs	109	1.8	0.3	1.0-3.0
>71yrs	63	2.3	0.004	1.3-4.1
T-category				
T_2	44	2.1	0.1	0.9-5.3
$T_{3,4}$	181	2.6	0.03	1.1-6.1
N category				
$N_{1,2}$	147	2.0	<0.001	1.4-2.9
M category				
M_1	28	1.6	0.03	1.0-2.6
Grade of differentiation				
G_3	109	1.4	0.04	1.0-1.9
Radicality				
$R_{1,2}$	73	1.8	<0.001	1.3-2.6
Location of tumour				
Gastric cardia	141	0.8	0.6	0.6-1.3

Reference categories used are: age group <50yrs (n=33); T_1 (n=27); N_0 (n=105); M_0 (n=224); $G_{1,2}$ (n=143); R_0 (n=179) and location of the tumour in the oesophagus (n=111).

DISCUSSION

The incidence of adenocarcinomas of the oesophagus and gastric cardia has shown large increases in many populations examined (9,10). This trend is in contrast to a decrease in the incidence of distal gastric adenocarcinomas and a relative stability of oesophageal squamous cell carcinomas. However, some studies have found only small rises in incidence or stable rates of gastric cardia cancers and non-parallel rates of oesophageal adenocarcinomas (11,12).

The present study showed that patients with gastric cardia carcinomas share characteristics in terms of age, sex distribution (predominance of males) and histological features with patients with adenocarcinomas of the distal oesophagus, as has been reported by others (6,13). When gastric cardia carcinomas were compared with non-cardiac gastric carcinomas significant differences were found. (14,15) This suggest common risk factors and a similar phenotype for adenocarcinomas arising from the distal oesophagus and gastric cardia, and that gastric cardia tumours are more closely related to oesophageal adenocarcinomas than to distal gastric carcinomas.

It has been postulated that adenocarcinomas of the distal oesophagus arise from the Barrett metaplasia-dysplasia-carcinoma sequence, and that this might be true for all adenocarcinomas arising in the region of the lower oesophagus as well as the

gastric cardia (7,16-18). However, the risk of developing an adenocarcinoma in intestinal metaplasia of the gastric cardia needs to be determined in larger prospective studies (19,20). In general, the length of Barrett's epithelium in patients with an adenocarcinoma of the cardia is shorter than in patients with oesophageal adenocarcinoma (21,22). The low incidence of Barrett's epithelium found in the present group of cardiac carcinomas may be explained by the fact that these shorter lengths may be easily overgrown by invasive tumour (23). These findings identical to those of Steup *et al.* (24) but in contrast with reported incidences of Barrett's metaplasia in adenocarcinomas of the gastro-oesophageal junction and gastric cardia of approximately 40 per cent (22,25). A possible explanation involves the differences in definition of the gastric cardia. Furthermore, the specimens were examined for the presence of Barrett's epithelium by several pathologists over the years, based on haematoxylin and eosin staining only. Interobserver variability in diagnosing Barrett's epithelium and failure to use mucin staining, which increases the sensitivity for detection of intestinal metaplasia, could be responsible for the lower prevalence of Barrett's epithelium in the present group.

A significant better survival for patients with adenocarcinomas associated with Barrett's epithelium *versus* non-Barrett-related carcinomas has been reported (26,27). Significant differences in survival rates were not observed in this study but a tendency towards a better survival rate was noted for the Barrett-related carcinomas. The median diameter of the tumour was 3 cm for Barrett related carcinomas and 4.5cm for the non-Barrett-related carcinomas. This may indicate that the so-called non-Barrett-related carcinomas are simply late tumours that have overgrown the Barrett's oesophagus and therefore show a tendency towards a worse prognosis.

Location of the tumour was not an independent prognostic parameter for survival, and 3- and 5-year overall survival rates were similar for adenocarcinomas of the oesophagus and gastric cardia. Similar results have been reported by other groups (22, 24, 28,29,30). However, a more favourable T and N stage was seen in patients with oesophageal carcinoma. This could be explained by the fact that carcinomas of the oesophagus give rise to symptoms of dysphagia earlier and are therefore detected at an earlier stage. Moreover, the higher prevalence of early stage (T_{is}/T_1) tumours in oesophageal cancer might reflect the fact that patients with a known Barrett's oesophagus were under endoscopic surveillance at this or the referring hospital. Ruol *et al.* reported prevalence rates for early cancer (T_1) of the oesophagus and cardia of 27 and 4 per cent respectively, and also found no difference in overall survival between the groups (31). Apparently, at the time of diagnosis cardiac tumours are at a more advanced stage of disease but this has no impact on survival.

At this institution carcinomas of the gastric cardia and the distal oesophagus are mostly treated as one clinical entity, by subtotal oesophagectomy and proximal gastrectomy. Total gastrectomy with oesophagojejunostomy for 'true' cardiac cancer not infiltrating the oesophagus, as favoured by others (32-34), is not used because of the higher risk of positive resection margins. The majority of the

patients with a positive plane of resection showed involvement of the circumferential plane and only five patients (three patients with gastric cardia carinomas) had involvement of the distal resection margin in this series. Closer analysis of the seven patients with involvement of the proximal resection plane in the neck revealed that these were all poorly differentiated carcinomas with submucosal satellite lesions infiltrating the middle and upper oesophagus.
Adenocarcinomas with their epicentre in the distal oesophagus are regarded as oesophageal carcinoma and regional lymph nodes for these carcinomas are the mediastinal and perigastric nodes, excluding the coeliac nodes. Adenocarcinomas with their epicentre at or just distal of the gastro-oesophageal junction are regarded as gastric carcinomas; regional lymph nodes are the perigastric nodes along the lesser and greater curvature (N_1 less than 3 cm and N_2 more than 3cm from the edge of the primary tumour) and the nodes along the left gastric, common hepatic, splenic and coeliac arteries (N_2). In the individual patient assignment of the primary tumour to one of the two localisations is artificial, so the analysis of lymph nodes is highly biased. Steup *et al.* reported that, when carcinomas of the gastro-oesophageal junction were staged as oesophageal carcinomas compared with staging as gastric cancer, no major difference was seen between the two staging modalities either in overall survival or in survival by stage (24). Another staging system currently proposed for oesophageal carcinomas is the modified Skinner classification based on the wall, node, metastases (WNM) concept similar to the modified Dukes' system for colon cancer (35,36). Carcinomas of the cardia are also included in this classification because the surgical approach to such lesions is similar; a comparison of staging criteria for oesophageal and gastric cancer showed no difference in the staging results (28). In the present study 15 of 21 patients with adenocarcinoma of the oesophagus had positive lymph nodes around the coeliac axis and were staged as M_1, whereas these would be N_2 nodes in cases of gastric cardia cancer. Therefore, these data support a classification in which involved lymph nodes resected by standard lymphadenectomy are all considered as locoregional lymph nodes for adenocarcinomas of the distal oesophagus as well as the gastric cardia, and both carcinomas are regarded as one entity.

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PART III

ESTABLISHMENT AND CHARACTERISATION OF CELL LINES FROM ADENOCARCINOMAS OF THE GASTRO-OESOPHAGEAL JUNCTION

CHAPTER 5

ESTABLISHMENT OF CELL LINES FROM ADENOCARCINOMAS OF THE OESOPHAGUS AND GASTRIC CARDIA GROWING *IN VIVO* AND *IN VITRO*

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PRÉCIS

We established succesfully 12 gastro-oesophageal junction adenocarcinoma cell lines growing in vivo (n=9) and in vitro (n=3). All cell lines resemble histologically, immunocytochemically and genetically the tumours from which they originated.

INTRODUCTION

Despite the rising incidence of adenocarcinomas of the gastro-oesophageal junction (GOJ), little is known about the cellular and molecular mechanisms underlying the origin of these tumours. This is partly caused by the lack of sufficient *in vivo* and *in vitro* model systems. Tumour xenografts are useful models to study the *in vivo* behaviour of tumours. Furthermore, human tumour xenografts are a source of tumour cells without admixture of normal human cells. This facilitates the analysis of tumour cell specific molecular alterations e.g. loss of heterozygosity (LOH). Till now, only a limited number of GOJ adenocarcinoma xenografts have been reported in the literature (1,2). Also only few *in vitro* human GOJ adenocarcinoma cell lines have been established (3,4).
In this study we report the generation of 28 xenografts and 3 cell lines growing *in vitro* obtained from primary GOJ adenocarcinomas and lymph node metastases. From these xenografts 9 permanent *in vivo* growing tumour lines were obtained.

MATERIALS AND METHODS

Xenografting and *in vitro* culture

The surgical resection specimens were placed on ice shortly after removal. Small tissue fragments were taken from the most vital parts of the primary tumour and lymph node metastases and were used for subcutaneous xenografting or tissue culture. Xenografting was performed by subcutaneous implantation of 2-6 small tumour fragments in female nude NMRI mice (6-8 weeks of age). When growing tumours reached a size of about 5-mm diameter (between 2-4 months) the tumours were removed and small parts were used for retransplantation. For tissue culture, a piece of tissue from the primary tumour or metastasis was rinsed in sterile Hanks Balanced Salt Solution supplemented with penicillin and streptomycin and cut in small pieces with sterile scissors and forceps. The tissue fragments were transferred to 5-10 petri-dishes to which RPMI -1640 (Bio-Whittakker,Verviers, Belgium) as culture fluid was added, supplemented with 10% fetal calf serum and antibiotics. No growth factors or other additives were used. After a week of culture, growth of cells could be observed along the edges of some fragments. Initially, also fibroblasts started growing, but after a number of passages they stopped growing, whereas the tumour cells become adapted to the growth medium and start to divide more rapidly. Cultures were passaged by trypsinization and after a number of passages *in vitro* the cell lines were used for transplantation to nude mice to verify their tumourigenic nature. Cultured cells were trypsinized and after washing with tissue culture medium and phosphate buffered saline (PBS) suspended in PBS in a concentration of $50x10^6$ cells/ml. From this suspension $5x10^6$ cells (0.1ml) were injected subcutaneously in a nude mouse.

Histology and immunocytochemistry

Primary tumours, metastases and xenografts were routinely fixed in 10% buffered formalin and embedded in paraffin. 5-µm sections were routinely stained with

hematoxylin and eosin. The presence of sialomucin was investigated by alcian blue staining at pH=2 by routine procedure.
Cells were also cultured on glass slides, washed with PBS and fixed in cold aceton for immunocytochemical detection of the epithelial marker keratin. To visualise the presence of specific antigens the Peroxidase-Anti Peroxidase (PAP) staining method was followed. After preincubation with 10% normal rabbit serum the slides were incubated overnight with a specific monoclonal antibody cocktail (clone AE1 and AE3 from Biogenex, Ramon, CA, USA) in a dilution of 200x. This antibody cocktail detects high molecular weight (basic) as well as low molecular weight (acidic) cytokeratins. After rinsing with PBS the slides were incubated with a rabbit anti-mouse immunoglobulin serum (Dako, Glostrup, Denmark) in a dilution of 25x. Subsequently a mouse anti-peroxidase monoclonal to which peroxidase was coupled was added (Sigma, Montesanto, St-Louis, MI, USA) in a dilution of 200x. The cells were stained with diaminobenzoic acid (DAB) to which H_2O_2 was added and sealed. As a positive control for cytokeratin staining the colon carcinoma cell line HT-29 was used.

DNA isolation, microsatellite analysis and chromosome number

Polymorphic microsatellite marker analysis was used to verify the derivation of the xenografts and cell lines and to confirm their tumour cell nature by loss of heterozygosity. DNA was isolated from cultured cells, frozen xenografts and frozen patient normal and tumour tissue by standard proteinase-K digestion and phenol/chloroform extraction. Eight highly polymorphic microsatellite markers on chromosomes 14 and 16 were PCR amplified as described (5). Markers D14S67, D14S78, D14S1010, D14S1037, D16S265, D16S398, D16S503 and D16S512 were amplified in 15µl reaction volume containing 1.5 mM $MgCL_2$, 0.02 mM dATP, 0.2 mM dGTP, dTTP and dCTP each, 0.8 µCi α-^{32}P-dATP, 20 pmol of each primer and 0.2 U *Taq* polymerase. PCR was carried out under standard conditions. PCR products were size separated by electrophoresis in a 6% polyacrylamide gel. After drying, the gels were exposed overnight to X-ray film.
The chromosome number of the *in vitro* growing cell lines was determined by a standard procedure. In short, the cell cultures were treated overnight with colcemid and the harvested cell suspensions underwent a hypotonic shock in KCl. Then the cells were fixed in methanol/acetic acid and the metaphase spreads were stained with DAPI. Chromosome number was determined by counting 10-20 well spread metaphases.

RESULTS

Tumours

Based on the macroscopic and microscopic examination of the resection specimens the tumours around the GOJ were classified as Barrett-related (n=16) or non-Barrett-related adenocarcinomas both from gastric and oesophageal origin. The presence or absence of Barrett's transformed mucosa adjacent to the tumours discriminated Barrett-related from non-Barrett-related adenocarcinomas respectively. Of 16 Barrett's adenocarcinomas 7 were classified as poorly differentiated, 7 as moderately

differentiated and 2 as well differentiated. Of the 54 non-Barrett-related adenocarcinomas 22 were undifferentiated, 30 moderately differentiated and 2 well differentiated (Table 1).

TABLE 1. *Xenografting adenocarcinomas of the gastro-oesophageal junction. Xenograft take compared with histological grade of the primary tumours or metastases. The data are expressed as the number of xenografts/ number of primary tumours or metastases.*

Type		*Grade of Differentiation*			*Total take*
		Undifferentiated	*Moderately*	*Well*	
Barrett	*n=16*	1/7	2/7	0/2	3 (19%)
Non-Barrett	*n=54*	7/22	10/30	2/2	19 (35%)
Metastases	*n=17*	2/9	4/8	0/0	6 (35%)

Xenografting

70 primary adenocarcinomas of the GOJ and 17 lymph node metastases were xenografted to nude mice. This resulted in initial growth of 3 tumours from 16 xenografts from Barrett-related adenocarcinomas and 19 tumours from 54 transplantations of non-Barrett-related adenocarcinomas. After transplantation of the 17 lymph node metastases (2 originating from Barrett-related tumours and 15 from non-Barrett-related carcinomas) 6 xenografts were obtained, all originating from gastric cardia carcinomas. So the total success rate of growing xenografts was 32%. Repeated transplantations of xenografts resulted in the establishment of 6 long-term xenografts from primary tumours and 3 from metastases. One of these *in vivo* lines originates from a primary Barrett-related adenocarcinoma. The lines were given the notation OAC (Oesophageal AdenoCarcinoma) of respectively the Primary tumour or of the Metastasis followed by the tumour number and the Xenograft. The following lines were obtained from primary adenocarcinomas OACP33X, OACP47X, OACP56X, OACP58X, OACP67X and OACP76X and from metastases OACM2.1X, OACM30X and OACM53X. There was no strict correlation between the degree of differentiation and successful xenografting. Seven of the 9 lines originated from moderately differentiated and 2 from poorly differentiated tumours. The 3 cell lines established from metastases were derived from moderately differentiated tumours.

In vitro culture

Thirty four of the 70 primary GOJ adenocarcinomas and 9 of the 17 metastases were also cultured *in vitro*. Many of them became infected and a number were overgrown by fibroblasts. Ultimately 3 long term *in vitro* growing cell lines were established (7%).

Two cell lines, i.e. OACP4C and OACM4.1C, derived from the same patient grow adherent in monolayer and are pleiomorphic, with epithelioid and fibroblastoid cells with cytoplasmic protrusions (Figure 1A). In the cultures several multinucleated giant cells (Figure 1B) are observed. The third cell line, OACM5.1C, is derived from a lymph node metastasis from a patient suffering from a Barrett's adenocarcinoma. This cell line grows loosely attached to the culture flask and in multicellular floating spheroids. The cells are round or have a fibroblastoid morphology (Figure 1C). These 3 *in vitro* cell lines appeared to be tumorigenic in nude mice and developed subcutaneous tumours within 6-8 weeks.

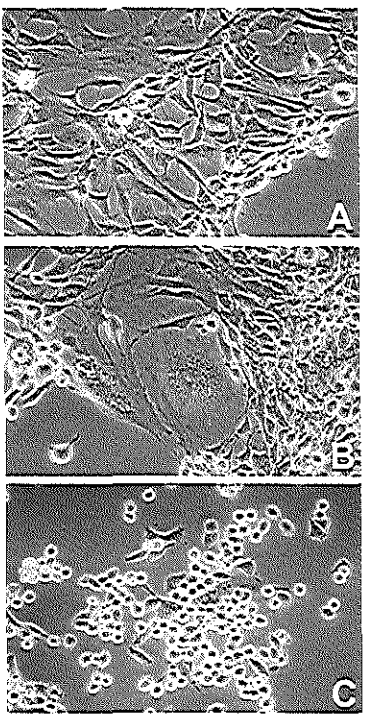


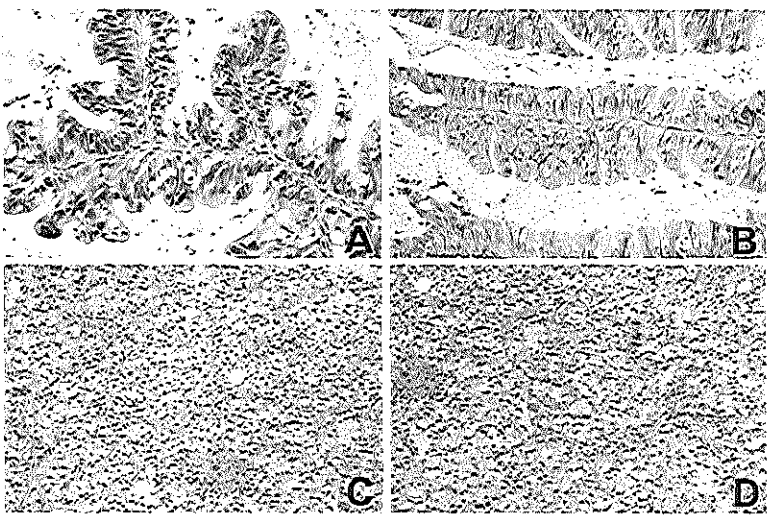
FIGURE 1 A-C. *Phase-contrast images of OACP4C (A). Note pleiomorphic nature and multinucleate giant cells (B) and of OACM5.1C forming multicellular spheroids (C).*

Histology and immunocytochemistry

The histology of all xenografts, including those formed by transplantation of cells from the 3 *in vitro* cell lines, completely resembled the histology of the primary tumour and

metastatic derivatives resected from the patients. Figures 2A+B shows a primary differentiated tumour and the corresponding xenograft and Figures 2C+D the comparison of an undifferentiated primary tumour with the xenograft from the *in vitro* cell line. With the alcian blue staining all xenografts appeared to harbour sialomucin producing cells, as did the primary tumours. The established xenografts and *in vitro* cell lines were derived from invasive tumours. However, no invasion into the surrounding tissue of the subcutaneous xenografts was observed. All xenografts presented as encapsulated subcutaneous tumours. Variable cytoplasmic expression of cytokeratin was demonstrated in all 3 *in vitro* cell lines confirming their epithelial nature (not shown).

FIGURE 2 A-D. *Histology of differentiated xenograft OACP58X (A) compared to that of the primary tumour (B). Comparison of undifferentiated xenograft of the in vitro cell line OACM4.1C (C) with corresponding primary tumour (D).*



Microsatellite analysis and chromosome number

To confirm the derivation of the xenografts and *in vitro* cell lines their DNA was compared with the patients tumour and normal DNA. With 8 highly polymorphic microsatellite markers all alleles in the xenografts and *in vitro* cell lines were present in the corresponding primary tumours and normal tissues (Figures 3A+B). In addition, LOH was found with at least one of these markers in the xenografts and 3 cell lines. The LOH patterns were in all cases comparable to the LOH patterns found in the matched primary tumours or metastases (Figures 3A+B).

All 3 *in vitro* cell lines appeared to be aneuploid. OACP4C had 53-57 and OACM4.1C 52-65 chromosomes. OACM5.1C was hypodiploid with 39-41 chromosomes. Further karyotypic characterisation will be published as part of a separate report (Rosenberg *et al.*, manuscript in preparation).

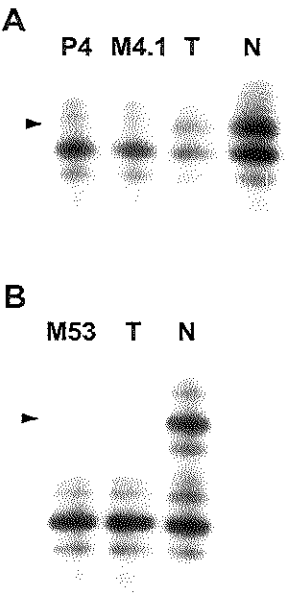


FIGURE 3 A-B. *A. Microsatellite analysis with polymorphic marker D14S1010 on in vitro primary tumour cell line OACP4C (P4) and metastasis cell line OACM4.1C (M4.1), primary tumour (T) and normal DNA (N) from the same patient. The patient is heterozygous for this marker and a complete loss of the upper allele (arrowhead) is observed in the cell lines and a partial loss in the primary tumour. The upper allele in the primary tumour is most likely derived from normal human cells present in the tumour specimen. B. microsatellite analysis with polymorphic marker D14S1010 on metastasis xenograft (M53X, first passage), primary tumour (T) and normal DNA (N) from the same patient. The patient is heterozygous for this marker. A complete loss is observed in the xenograft and a partial loss in the primary tumour (arrowhead).*

DISCUSSION

In general, the success rate of xenografts from GOJ adenocarcinomas on nude mice is very reasonable, although long term establishment of these xenografts was limited compared to that of other tumours (6). The tumour growth does not correlate with the tumour grade of the tissue transplanted. Most lines were derived from moderately and not from poorly differentiated tumours. It is likely that the subcutaneous microenvironment or impaired vascularisation may explain the many negative cases. Alternatively, the sensitivity of tumour cells to the natural killer cells of nude mice may hamper their outgrowth. Orthotopic transplantation of the established cell lines to the cardia did not improve the outgrowth (unpublished results) despite the malignant nature (7) and the genetic instability of these tumours (8). Eighty-seven primary transplantations resulted in the initial growth of 28 xenografts (32%). After repeated re-transplantations only 9 (10%) xenografts could be established as permanent lines. There was no correlation with pathological criteria and long term xenograft growth. LOH analyses revealed that already in the first passage xenografts, obtained 2-4 months after primary transplantation, no contaminating normal human cells are present in the tumours (Figure 3B).

Since the xenografts are not contaminated with normal human stromal cells they are of value for comparative genomic hybridization (9). DNA from xenografts was also used for mapping of deletions on the long arm of chromosome 4 (2) and in studying E-cadherin and β-catenin mutations in GOJ adenocarcinomas (10,11).
The number of well-described permanent *in vitro* GEJ adenocarcinoma cell lines is limited. Altorki *et al.* have reported the growth of three permanent cell lines (SK-GT-1, 4 and 5) from which one was derived from a Barrett's carcinoma(3). Rockett *et al.* generated a series of tumour cell lines called JROECL 19, 33, 47 and 50 (4). The cell line JROECL 33 originated also from a Barrett's adenocarcinoma. However, 2 lines, JROECL 47 and 50, proved to be subclones of the colon carcinoma cell line HCT 116 (12). Palanca-Wessels *et al.* established 4 permanent cell lines from Barrett's metaplastic epithelium all of which showed already polyploidy, loss of heterozygosity of chromosome 9p and in 3 of 4 cultures loss of 17p and p53 mutations(13). More recently, a number of less well characterised lines Seg-1, Flo-1 and Bic-1 were preliminary reported in a study on the differential expression of heat-shock proteins in Barrett's metaplasia and oesophageal adenocarcinomas (14).
Our *in vitro* cell lines express cytokeratin indicating their epithelial nature. All xenografts, also from the *in vitro* cell lines and from undifferentiated tumours, harboured sialomucin-containing cells demonstrating their derivation from adenocarcinomas. It is obvious that the histology of the xenografts corresponds to that of the primary tumour. Also when the cells of the permanent *in vitro* cell lines were transplanted to nude mice they gave rise to tumours which are histologically similar to the tumours from which they originated. However, all primary tumours were invasive whereas none of the subcutaneous xenografts showed invasiveness. This is comparable to e.g. colorectal tumour cell lines which show no invasion after heterotopic transplantation but invasion is observed at the orthotopic site (15).
The microsatellite analysis of the primary tumour and the corresponding xenograft or cell line proves their common origin. All three *in vitro* cell lines are aneuploid in agreement with reports that most GOJ have aberrant ploidy levels (16). The chromosome numbers of cell lines OACP4C and OACM4.1C are in the same range and this is consistent with numerous reports on comparable ploidy of paired primary tumours and metastases. Cell line OACM5.1C has a hypodiploid karyotype. Although uncommon, hypodiploidy has been described in GOJ adenocarcinoma (17).
In conclusion, the results show that GOJ adenocarcinoma cell lines growing *in vivo* and *in vitro* can be successfully established and resemble histologically, immunocytochemically and genetically the tumours from which they originated. These xenografts and cell lines are a valuable source of homogeneous tumour tissue for further investigations.

ACKNOWLEDGEMENTS

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CHAPTER 6

HUMAN OESOPHAGEAL ADENOCARCINOMA CELL LINES JROECL 47 AND JROECL 50 ARE ADMIXTURES OF THE HUMAN COLON CARCINOMA CELL LINE HCT 116

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PRÉCIS

We demonstrate by HLA-typing, mutation analysis and microsatellite analysis that oesophageal adenocarcinoma cell lines JROECL 47 and 50 are in fact admixtures of the human colon carcinoma cell line HCT 116.

INTRODUCTION

Recently, four human oesophageal and gastric cardia adenocarcinoma cell lines were established (1). These cell lines were included in two studies on E-cadherin and β-catenin gene mutations in adenocarcinomas of the oesophagus (2,3). Cell lines JROECL 47 and JROECL 50, derived from one tumour, harbour E-cadherin and β-catenin gene mutations. These mutations could not be detected in the primary tumour from which the cell lines were established. Recently, identical E-cadherin and β-catenin gene mutations have been described in the human colon tumour cell line HCT 116, established in 1981 (4-6). These results prompted us to investigate the derivation of the cell lines JROECL 47 and JROECL 50 by HLA typing, mutation analyses, microsatellite allelotyping and microsatellite instability (MSI) analysis.

MATERIALS AND METHODS

Cell lines, primary tumour, xenografts and DNA isolation

Cell lines JROECL 47 (passage 16) and JROECL 50 (passage 10) were obtained from the European Collection of Cell Cultures (ECACC). From cell lines JROECL 47 and JROECL 50 also the early passages, before submission of these cell lines to the ECACC, (passages 2 and 4, respectively) were investigated. These early passages were a gift from Dr A.G. Morris, University of Warwick, Coventry, United Kingdom. Sections from the original paraffin tissue blocks of the patient's oesophageal tumour, from which the cell lines JROECL 47 and 50 were presumable derived, were gifted by Dr. S.J. Darnton, Birmingham Heartlands Hospital, Birmingham, United Kingdom. Colon cancer cell line HCT 116 was a generous gift from Dr. P. van der Saag, Hubrecht Laboratory, Utrecht, the Netherlands. Cells were cultured under standard conditions in RPMI 1640 supplemented with 10% FCS.

To study the histological characteristics, 5 x 10^6 trypsinized tumour cells from cell lines JROECL 47 and 50 (passages 16 and 10, respectively) and HCT 116 were injected subcutaneously in female NMRI nude mice. Xenografts were removed and routinely processed for histological examination. The animal experiments were licensed and done in accordance with approved protocols by the Erasmus university Medical Centre Rotterdam, The Netherlands.

DNA was isolated by standard proteinase K digestion and phenol extraction from the cultured cell lines and from the tissue block of the original oesophageal tumour, from which cell lines JROECL 47 and JROECL 50 were presumably established.

HLA typing

HLA-DRB1 typing was performed on cell lines JROECL 47 and 50, cell line HCT116 and the tissue blocks of the original tumour, as described (7). The polymorphic exon 2 was amplified and subsequently sequenced on an ABI373 automated sequencer (Perkin Elmer, Foster City, USA). HLA-DRB allele

assignment was established by comparing the sequences obtained to the HLA-allele database similar to HLA-DPB allele assignment (8).

Mutation analyses

Cell line HCT 116 has heterozygous mutations in the E-cadherin gene (codon 120; exon 3), the β-catenin gene (codon 45; exon 3) and the K-ras gene (codon 13; exon 1) (4,5,9). PCR-SSCP was performed to detect these mutations, as described (2,9,10). Samples with aberrant migrating bands were reamplified, cloned and sequenced.

Microsatellite analyses

Nine polymorphic dinucleotide repeat markers: D8S136, D8S133, D9S161, D9S156, D16S265, D14S292, D14S977, D17S786 and CHRNB1 were investigated by radioactive PCR as described previously (11).

Because HCT 116 is reported to have the microsatellite instable (MSI) phenotype (12), MSI markers BAT26, BAT40 and BAT-RII were also investigated (13).

RESULTS AND DISCUSSION

To date, only very few *in vitro* growing human oesophageal adenocarcinoma cell lines are known. The availability of these cell lines is of great value to study the biology and the genetic alterations in these poorly-understood cancers, which show a dramatic increase in incidence over the past decades (14). Recently, 4 such cell lines were established (1). Here we report that 2 of these cell lines, JROECL 47 and JROECL 50 are in fact admixtures of the human colon cancer cell line HCT 116.

In all experiments identical results were obtained for the early and late passages of cell lines JROECL 47 and JROECL 50. In cell culture JROECL 47, JROECL 50 and HCT 116 have the same morphology with spindle shaped cells and similar growth rates. Xenografting of these 3 cell lines resulted in undifferentiated solid tumours, without glandular differentiation (results not shown). HLA typing revealed that cell lines JROECL 47, JROECL 50 and HCT 116 all have the same HLA-DR allele DRB1*03011/1102, which is different from the original primary oesophageal tumour from which the cell lines JROECL 47 and 50 were presumably established: DRB1*08032/04011. An example of the difference between the cell lines and the original primary tumour is shown by a characteristic sequence of exon 2 of the primary tumour and the cell lines (Figure 1). The frequency of the patient primary tumour allele combination DRB1*08032/04011 in the population is less than 0.0041 compared to the frequency of 0.0098 of the allele combination DRB1*03011/1102 of the cell lines (15).

Furthermore, PCR-SSCP analyses of exon 3 of the E-cadherin gene, exon 3 of the β-catenin gene and codon 12/13 of the K-*ras* gene showed an identical, aberrant mobility pattern in all 3 cell lines (Figure 2).

FIGURE 1. *HLA-typing of the original primary tumour and the cell lines (JROECL 47 and 50 and HCT 116). The polymorphic positions of HLA-DRB1 exon 2 are shown in vertical orientation. Dots indicate identity to the nucleotide of the DRB1*08032 allele. Numbers are the polymorphic positions of nucleotides in exon 2 shown in vertical orientation (position 28, 30,....258). The polymorphic positions of the original primary tumour (DRB1*08032/04011) have been compared with the cell lines. The alleles present in the primary tumour do not exist in the cell lines.*

```
                      1111111 1122222222 222
                2333333345 7990046777 7900111122 355
                8012567867 7472909013 4978012801 078
                ---------- ---------- ---------- ---
Primary tumour  TCTCCGGGTT TTAATAAGCC CAAGCAGCCT CGT DRB1*08032
                CGGTAACACC ..C...GAT. .CGCG.A.GC ... DRB1*04011

Cell lines      ......TCC. AC.GATGAT. .CGCG.AG.G ATG DRB1*03011
                ......TCC. .....TGATA G....GA.GC .TG DRB1*1102
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FIGURE 2. *PCR-SSCP analysis of the E-cadherin gene exon 3 (A), β-catenin gene exon 3 (B) and K-Ras gene codon 12/13 (C). Lanes 1 and 5, normal, non-mutated control DNA from one individual; lane 2, JROECL 47; lane 3, JROECL 50 and lane 4, HCT 116. Note the same aberrant migration patterns in all 3 cell lines (arrowheads), as compared to normal control DNA.*

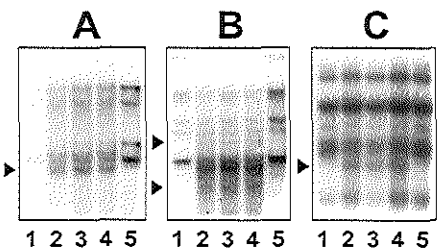
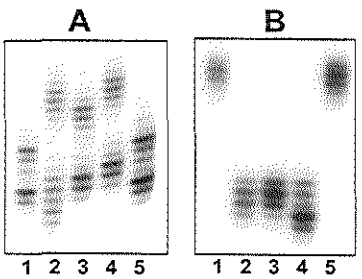


FIGURE 3. *Allelotyping with polymorphic marker CHRNB1 (A) and microsatellite analysis with BAT26 (B). Lanes 1 and 5, normal control DNA from one individual; lane 2, JROECL 47; lane 3, JROECL 50 and lane 4, HCT 116. Note the different allele sizes in the three cell lines, indicating different origins (A). Note the alteration in mononucleotide repeat size in the cell line DNA's compared to normal, control DNA, indicating MSI (B).*



Upon sequencing of the samples with aberrant migration patterns, the reported mutations in all three genes were confirmed (results not shown) (4,5,9). Allelotyping, however, showed different allele sizes between the 3 cell lines with 7/9 polymorphic markers, indicating a different origin of the cell lines (Figure 3A). With 2 markers the allele patterns were identical between the cell lines. But all three MSI markers demonstrated pronounced microsatellite instability with different allele sizes in the three cell lines. Figure 3B represents an example of MSI in the three cell lines as demonstrated by BAT26. Indeed, HCT 116 has been reported to have an extremely microsatellite instable phenotype (16). Obviously, separate cultures of HCT 116 resulted in different microsatellite alterations. Therefore, microsatellite analysis is not appropriate for allelotyping MSI cell lines.
Our assumption that cell lines JROECL 47 and 50 are admixtures of HCT 116 was confirmed by the ECACC with DNA fingerprinting (personal communication). Therefore, we conclude that cell lines JROECL 47 and JROECL 50 are not human oesophageal adenocarcinoma cell lines, but are admixtures of the human colon adenocarcinoma cell line HCT 116. Furthermore, allelotyping of cell lines by microsatellite analysis can be hampered by MSI.

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Part IV

E-Cadherin-Catenin Cell-Cell Adhesion Complex

CHAPTER 7

E-CADHERIN GENE MUTATIONS ARE RARE IN ADENOCARCINOMAS OF THE OESOPHAGUS

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PRÉCIS

In two tumour cell lines, JROECL 47 and 50, E-cadherin mutations were detected. In the remaining 55 tumour samples, we did not detect mutations, despite frequent loss of heterozygosity of the E-cadherin locus at 16q22.1.

INTRODUCTION

Cadherins are a family of Ca^{2+}-dependent cell-cell adhesion molecules. E-cadherin is a transmembrane protein with five tandemly repeated extracellular domains and a cytoplasmic domain that connects to the actin cytoskeleton through a complex with α-, β- and γ-catenins. E-cadherin is expressed on the cell surface in most epithelial tissues and is important for establishing cell polarity, maintaining epithelial integrity and cellular differentiation (1). The potential for E-cadherin to serve as an invasion or metastatic suppressor in epithelial tumourigenesis has been elucidated from *in vitro* studies. E-cadherin-negative epithelial cells grow invasive with a mesenchymal phenotype. After transfection with E-cadherin cDNA, epithelial structure is restored (2,3).
Consistent with this observation is a reduced or absent E-cadherin expression in various epithelial cancers showing invasive growth (4-6). Decreased expression of E-cadherin in Barrett's oesophagus, adenocarcinomas of the oesophagus and gastro-oesophageal junction was found to be related with progression of Barrett's oesophagus to adenocarcinoma, tumour stage and lymph node metastases (7,8). Furthermore, E-cadherin expression was an independent variable predicting survival in patients after resection for adenocarcinomas of the oesophagus (9).
Loss of E-cadherin expression resulted in the transition from well differentiated adenoma to invasive carcinoma in mouse pancreatic β-cell carcinogenesis (10). Inactivating mutations in the E-cadherin gene have been described for various tumours such as lobular breast cancer (*in situ*) and diffuse infiltrating gastric cancer concomitant with loss of heterozygosity at the E-cadherin locus in E-cadherin negative tumours (11-14). Moreover, two recent studies showed that germline mutations in the E-cadherin gene are associated with early onset familial gastric cancer (15,16). Whether genetic alterations in the E-cadherin gene play a role in the pathogenesis of adenocarcinomas of the oesophagus and gastro-oesophageal junction is not known. Therefore, we screened adenocarcinomas of the oesophagus and gastro-oesophageal junction for E-cadherin gene mutations and loss of heterozygosity.

MATERIALS AND METHODS

Tumour specimens

Fresh samples of adenocarcinomas of the distal oesophagus or gastro-oesophageal junction were obtained from 45 resection specimens. For analysis the tumour samples were microdissected to enrich for cancer cells (>75%). Nineteen tumours showed microscopic evidence of surrounding intestinal metaplasia indicative for Barrett's carcinomas. Samples of tumour and normal gastric epithelium or squamous epithelium of the oesophagus were snap frozen and stored in liquid nitrogen. Four lymph nodes infiltrated by tumour were frozen as well.

Cell lines and xenografts

In vitro cell lines from three adenocarcinomas JROECL19, JROECL33, JROECL50 and one adenosquamous carcinoma JROECL47 established by Rockett *et al.* were obtained from European Collection of Cell Cultures (ECACC) (17). *In vivo* xenografts were obtained after transplantation of tumour tissue to female nude mice, 4-6 weeks of age. From 3 lymph node metastases M2.1X1, M9X1, M4.1X2 and 1 primary tumour P23X1, xenografts were obtained.

DNA preparation

DNA from cell lines was isolated according to standard procedures. Genomic DNA from xenografts and tumour samples and normal tissue was isolated from consecutive 5-μm cryostat tissue sections by overnight proteinase K incubation at 55 ^{0}C followed by phenol extraction and ethanol precipitation. DNA pellets were dissolved in TE (10mM Tris.HCl, pH 7.8; 1mM EDTA). The tumour tissue samples contained at least 75% tumour cells.

Analysis of the E-cadherin gene by PCR-SSCP

The entire open reading frame of the E-cadherin gene was screened for mutations using 15 exon-spanning primer pairs (12). Genomic DNA was used at 50-100ng per 15μl reaction mixture containing 1.5mM $MgCl_2$, 0.02mM dATP, 0.2 mM dGTP, dTTP and dCTP each, 0.8μCi α-^{32}PdATP (Amersham, Buckinghamshire, UK), 20 pmol of each primer and 0.2U Taq polymerase (Promega, Madison WI, USA). Each PCR was overlaid with mineral oil. PCR was performed for 35 cycles (denaturing at 95 °C for 30 s, annealing at the appropriate temperature for 45 s. and extension at 72 °C for 1 min.). A final extension step was carried out at 72 °C for 10 min. PCR products were diluted 1:4 with a loading buffer (95% formamide, 10mM EDTA (pH 8.0), 0.025% bromophenol blue and 0.025% xylene cyanol), denatured at 95 °C for 4 min. and snap-cooled on ice. Appropriate aliquots of the radiolabelled PCR products were separated on a non-denaturing polyacrylamide gel (6% polyacrylamide) containing 10% glycerol and run at 7W overnight at room temperature in 1 x TBE running buffer. Gels were fixed in acetic acid (10%), dried on blotting paper (Schleicher & Schuell, Dassel, Germany) on a vacuum gel dryer and exposed to X-ray film overnight at -70 °C, using intensifying screens. DNA with aberrantly migrating PCR/SSCP fragments was reamplified and purified over QIAquick spin columns (Qiagen, Hilden, Germany), cloned into a PGEM-T easy vector (Promega, Madison WI, U.S.A.), and sequenced with α-^{35}SdATP according to the dideoxy chain termination method. Electrophoresis of the sequenced samples was carried out on a 8% denaturing polyacrylamide gel. After fixation and drying, gels were exposed to X-ray film for 1-3 days at room temperature.

LOH determination

In 51 tumours loss of heterozygosity was determined with microsatellite markers that map on 16q22.1 where the E-cadherin gene is located. Markers tested were: D16S503, D16S265, D16S398 and D16S512. Markers were tested on 100ng of tumour and normal DNA in a PCR reaction as described previously (18). LOH was

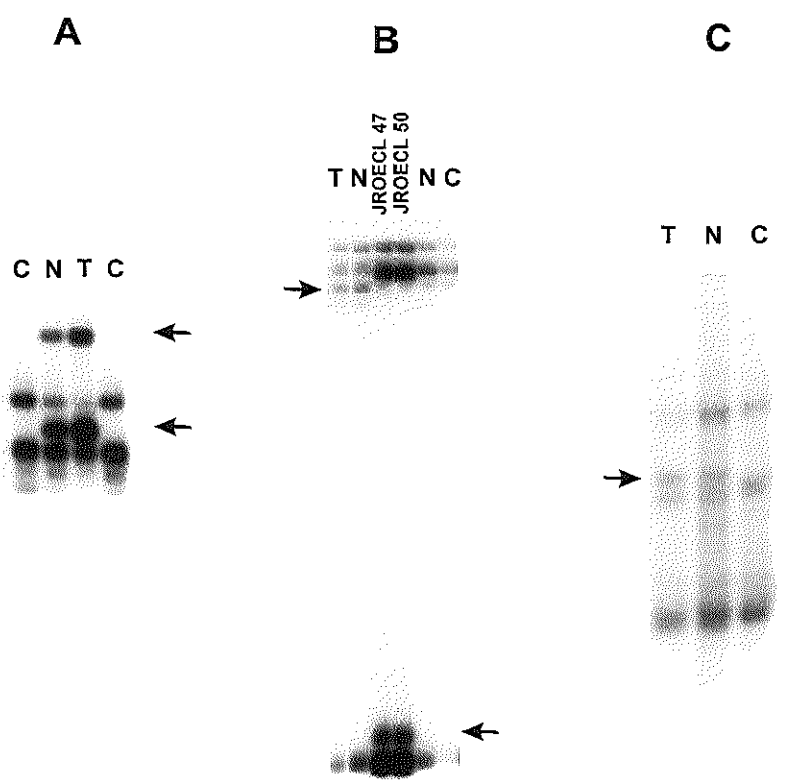
established by visual comparison of the intensity of allelic bands obtained from tumour samples with those from normal DNAs.

RESULTS

PCR-SSCP

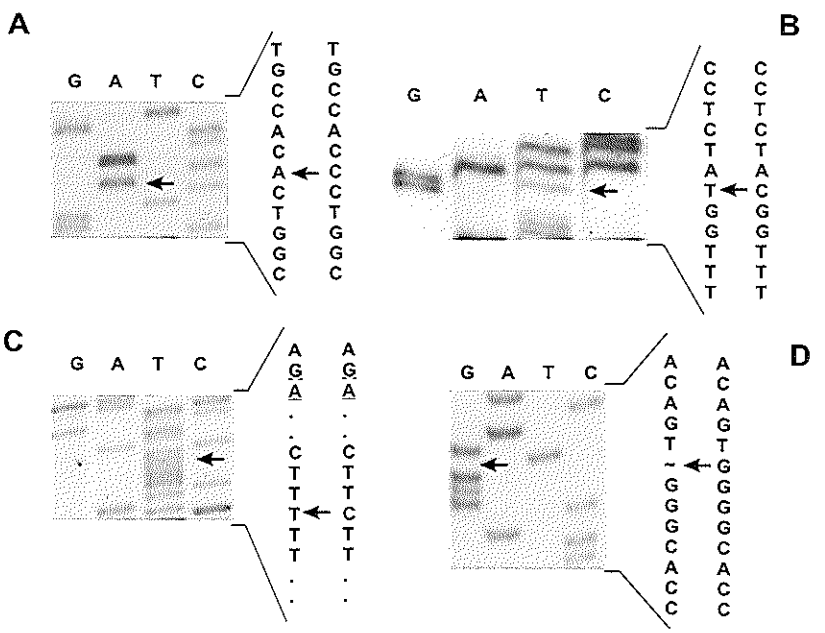
All 16 exons and exon-intron boundaries of the E-cadherin gene were analysed for genetic alterations in four cell lines, four xenografts and 49 tumour samples (45 primary tumours and four corresponding locoregional lymph node metastases). In total 37 aberrant bands were detected throughout 9 different amplicons. Cell line, xenograft and tumour DNA with aberrant SSCP patterns were compared with the amplification products of patient matched non-tumorous DNA. In most tumours the aberrant SSCP pattern was also present in the corresponding normal DNA (Figure 1). A tumour-restricted mobility shift was found in cell lines JROECL47 and JROECL50 in amplicon 3 (Figure 1B).

FIGURE 1. *PCR-SSCP analysis of E-cadherin amplicons 2 (A), 3 (B) and 4/5 (C) from three oesophageal adenocarcinoma specimens and two cell lines JROECL 47 and JROECL 50. Mobilty shifts are identified by arrows. C=control DNA; T= tumour DNA; N= corresponding non-tumorous DNA.*



Upon sequencing an one base-pair deletion in codon 120 (exon 3) was detected (Figure 2D and Table 1). Both cell lines were derived from the same primary tumour (Dr S.J. Darnton, personal communication), in which this mutation could not be detected. Sequence analysis of all the other SSCP band shifts revealed eight known polymorphisms and three new genetic alterations (Figures 2A, B, C and Ttable 2). Furthermore, upon sequencing of amplicon 16 a discrepancy with the published sequence of intron 15 of the E-cadherin gene deposited in EMBL/GenBank database libraries (accession no. Z13009) was detected (5' intron15-...cttgag-3' exon 16).

FIGURE 2. *Sequence analysis for the three tumours and cell line JROECL 47 with abnormally shifted PCR-SSCP bands as shown in Figure 1. Mutated sequence (left) and wild type sequence (right) are shown at the right of each figure. Base pair alterations are indicated by arrows. Splice recognition site is shown by the underlined characters in panel C. For exon 2 (A) and exon 3 (B) we identified missense mutations, also present in the normal control DNA of these patients. For amplicon 4/5 a base pair change was observed in intron 4 (C). One frameshift mutation caused by a 1-bp deletion in was detected for exon 3 (D) in the two cell lines JROECL 47 and JROECL 50.*



LOH determination

In Figure 3 examples are presented of the analysis of the 4 microsatellite loci in different tumour DNAs which showed allelic loss. Of the 48 informative cases, 31 (65%) showed LOH of at least one marker (Figure 4).

FIGURE 3. *Loss of heterozygosity in tumour #3 (D16S503), #10 (D16S265), #12 (D16S398) and #22 (D16S512). Loss of one allele is visible in the lane containing tumour DNA (T) compared with the lane containing normal DNA (N).*

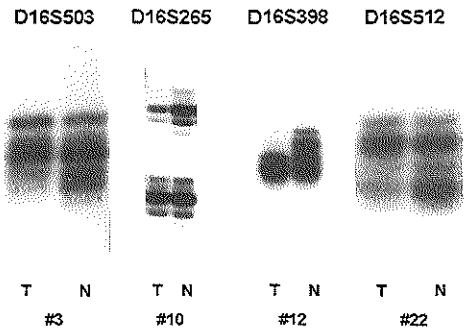


TABLE 1. *Tumour restricted mutation in cell lines JROECL 47 and JROECL 50 (2 cell lines derived from a primary tumour of one patient)*

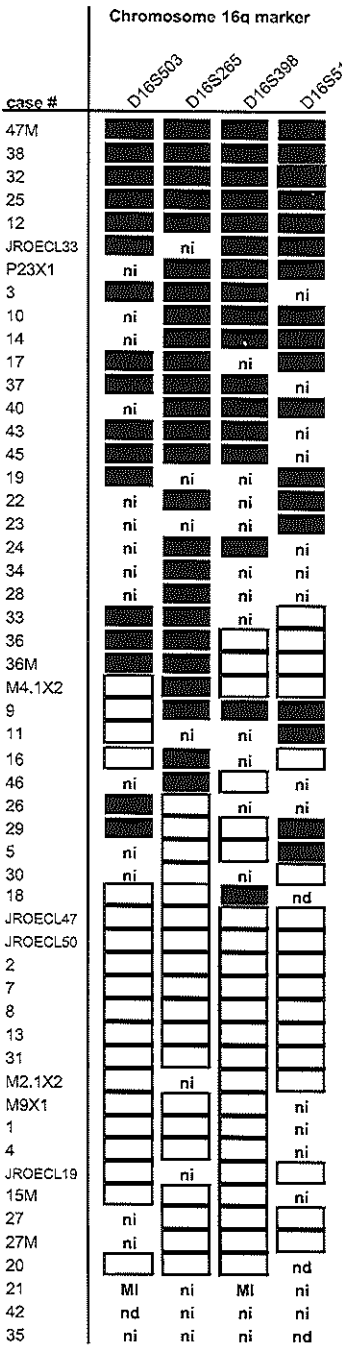
Amplicon	*Site*	*Nucleotide change*
3	Cdn 120	Deletion G: frameshift leading to stopcodon at cdn 214 in exon 5

TABLE 2. *Polymorphisms identified in the E-cadherin gene*

Aplicon	*Site*	*Nucleotide change**	*Observed frequency*	*Literature***
1	Non-coding (5' UTR)	TCC→TGC	2/57	1
2	Cdn 30	CCT→ACT Pro→Thr	1/57	unknown
3	Cdn 90	CGG → TGG Arg → Trp	1/57	unknown
4/5	Intron 4	gtagagaaag→..ac	2/57	1
	Intron 4	C→T 18 nt upstream exon 5	1/57	unknown
11	Cdn 560	ACG→ACC Thr→Thr	2/57	1,2
12	Cdn 632	CAC→CAT His→His	1/57	1,2,3
13	Cdn 692	GCT→GCC Ala→Ala	14/57	1,2,4
	Intron 12	T→C	12/57	1,4
14	Cdn 751	AAC→AAT Asn→Lys	1/57	1,2
16	Cdn 878	GGC→GGT Gly→Gly	1/57	3

** Splice recognition site is shown by the underlined characters ** Polymorphisms also reported by [1]Berx et al., 1996; [2]Guilford et al., 1998; [3]Risinger et al., 1994; [4]Soares et al., 1997.*

FIGURE 4 *Patterns of allelic loss in oesophageal adenocarcinomas at the E-cadherin locus on chromosome 16q22.1. Closed rectangle, loss of heterozygosity; white rectangle, heterozygosity retained; ni, non-informative; MI, microsatellite instability; nd, no data obtainable. Note that 36M and 27M are lymph node metastases from primary tumours 36 and 27, respectively.*



DISCUSSION

Reduced expression of E-cadherin is frequently seen in the majority of adenocarcinomas of the oesophagus and gastro-oesophageal junction with or without Barrett's epithelium (7,8,19). However, E-cadherin immunoreactivity does not provide information about the function of the protein and gene integrity. For example, mutations in the E-cadherin gene leading to loss of adhesive potential but with normal cellular localisation of the protein have been described (20,21). In this study we have analysed if genetic alterations in the E-cadherin gene are involved in the reduced expression of E-cadherin in adenocarcinomas of the oesophagus and gastro-oesophageal junction. In an earlier study, eight out of 49 tumours were analysed for expression of E-cadherin by means of immunohistochemistry. Seven out of eight of these adenocarcinomas showed reduced expression for E-cadherin (9).

In our series of 57 adenocarcinomas of the oesophagus only one tumour restricted DNA alteration in the E-cadherin gene was detected. In two cell lines JROECL 47 and JROECL 50, derived from the same tumour, a one base pair deletion was found leading to a premature stopcodon resulting in a truncated protein lacking part of the extracellular binding region as well as the transmembrane and intracellular domain. So far, this mutation has not been reported in the literature. Both cell lines still have a non-mutated E-cadherin allele as demonstrated by the heterozygous exon 3 SSCP pattern indicating two different alleles. We were unable to demonstrate this mutation in the primary tumour. Obviously, the mutation was present in a minor subpopulation of the primary tumour or has developed during establishment of both cell lines.

PCR-SSCP analysis is a robust mutation detection procedure (22,23), indicated by the finding of 8 known polymorphisms and 3 not yet described DNA alterations. However, we can not rule out the possibility that mutations remained undetected by this procedure. All 3 new DNA alterations are also present in the patients' normal DNA. Two of these alterations lead to amino acid substitutions in the precursor sequence of the E-cadherin gene. Recently, germline mutations leading to truncated E-cadherin were identified in familial gastric cancer in New Zealand and Europe (15,16). Because no loss of the normal E-cadherin allele in the tumours occurred we consider these DNA alterations as polymorphisms. Furthermore, we have no evidence of a familial predisposition to oesophageal cancer in any of our patients.

Allelic loss at the E-cadherin gene locus 16q22.1 has been reported in 30-50% of breast, prostate and hepatocellular cancer (24). Our data show LOH of this locus in two-thirds of the oesophageal adenocarcinomas. But the high percentage of 16q22.1 LOH without concomitant mutation of the remaining E-cadherin gene might point to another tumour suppressor gene on 16q involved in the genesis or progression of oesophageal adenocarcinomas. We can not rule out the possibility that homozygous deletions are present in these carcinomas. Especially in DNA derived from resection specimens homozygous deletions can escape detection due

to the contamination with non-tumorous DNA. However, all exons could be amplified with DNA derived from the cell lines and xenografts with exon spanning primers, which makes it unlikely that homozygous deletions have occurred.
Alternatively, if the high frequency of LOH at 16q22.1 truly reflects loss of one E-cadherine allele, this could point in the direction of gene dosage effects as formerly proposed on the basis of experimental studies (3). However, a previous study by Ilyas *et al.* could not establish a correlation between allelic loss and immunohistochemical E-cadherin expression in colorectal cancers (25).
Besides loss of one E-cadherin allele, other mechanisms leading to downregulation of E-cadherin in oesophageal adenocarcinomas could also be involved. Hypermethylation of the 5' CpG islands within the proximal promoter region of the E-cadherin gene has been found responsible for (temporary) downregulation of E-cadherin in several cancers (26-28). However, not only E-cadherin but also other members of the junctional complex have been shown to play a role in tumourigenesis. In several tumour cell lines showing impaired cell-cell adhesion and reduced expression of E-cadherin, mutations in α-catenin and β-catenin were identified (29,30). A cytoplasmic and nuclear staining pattern of β-catenin has been observed in adenocarcinomas of the oesophagus in cases in which E-cadherin was reduced (7,9). These tumours could harbour mutated β-catenin, as was recently described for colon carcinomas and hepatocellular carcinomas (31,32). Whether or not mutations in the catenins play a role in the pathogenesis of oesophageal cancer is presently unknown.
In conclusion, our results show that E-cadherin gene mutations are not involved in the subsequent progression of Barrett's epithelium to dysplasia and to adenocarcinoma of the oesophagus. Whether LOH at the E-cadherin locus contributes to the heterogenous expression of E-cadherin remains to be determined.

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CHAPTER 8

GENETIC ALTERATIONS INVOLVING EXON 3 OF THE β-CATENIN GENE DO NOT PLAY A ROLE IN ADENOCARCINOMAS OF THE OESOPHAGUS

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PRÉCIS

We detected no mutations in exon 3 of the β-catenin by PCR-SSCP analysis nor did we find large interstitial deletions involving exon 3. β-Catenin immunostaining in 54 tumours showed focal nuclear staining in 7 tumours and strong homogenous nuclear staining in 3 tumours; in the latter, no mutations in the mutation cluster region of the APC gene were detected.

INTRODUCTION

The E-cadherin-catenin complex is a key mediator of cell-cell adhesion and has been shown to function as suppressor of invasiveness (1). Not only E-cadherin itself, but also the interaction between cadherins and catenins appears to be crucial for its adhesive function. Therefore, disturbed cell-cell adhesion in tumours with normal E-cadherin expression may also be caused by impaired function of one of the catenins (2).

In addition to its function in cell-cell adhesion, β-catenin plays an important role in signal transduction; it is involved in Wingless-Wnt pathways that regulate cellular differentiation and proliferation (3,4). In the absence of growth or differentiation signals, cytoplasmic β-catenin is kept low because the protein is sequestered in complexes, which include the adenomatous polyposis coli (APC) protein, a serine threonine glycogen kinase (GSK-3β) and conductin or axin, enabling degradation by the proteasome. The binding of β-catenin by APC requires phosphorylation of β-catenin by GSK-3β on three serine and one threonine residue, all of which are encoded by exon 3 of the β-catenin gene. Mutations in these serine/threonine sites in exon 3 of β-catenin result in stabilisation of β-catenin (3,5). The increased concentration of this protein in the cytoplasm favours its binding to the T-cell factor/lymphoid enhancer binding factor (Tcf/LEF) family of DNA binding proteins and it subsequently translocates to the nucleus where it induces transcription of specific genes which stimulate tumour formation, such as cyclin-D1 and c-myc (6,7). This mechanism has been proposed as an important step in colorectal carcinogenesis. In colorectal cancers *APC* is most commonly mutated. Most mutations in *APC* lead to a truncated protein which can still complex with, but not degrade β-catenin. The result of *APC* mutation is therefore an increase in cellular free β-catenin. In approximately half of the colorectal cancers with an intact *APC*, mutations in exon 3 of the β-catenin gene were found, which is consistent with their effect on β-catenin stability and Tcf activation (8,9). Mutations, including large interstitial deletions involving exon 3 of the β-catenin gene, have also been found in several other tumors, including ovarian and endometrial carcinomas, medulloblastomas, hepatoblastomas and hepatocellular carcinomas and prostate cancers (10-14). Recently, mutations have been identified at high frequency in pilomatricomas and anaplastic thyroid carcinomas (15,16).

Immunohistochemical studies on β-catenin immunoreactivity in oesophageal adenocarcinomas have indicated loss of membranous staining in combination with cytoplasmic or nuclear staining (17). This has been taken to indicate β-catenin activation or APC inactivation leading to cytoplasmic accumulation of β-catenin and Tcf/LEF activation. In view of the complementation of β-catenin and *APC* mutations in colorectal cancers and because *APC* mutations are rare in oesophageal adenocarcinomas, we deemed it of interest to focus on β-catenin as a possible target for gene mutations. Here, we report the screening of genetic

alterations in the β-catenin gene involving exon 3 in adenocarcinomas of the oesophagus.

MATERIALS AND METHODS

Tissues

The study population included 59 primary tumors and 8 regional lymph node metastases obtained from patients who underwent surgery for adenocarcinoma of the distal oesophagus or the gastro-oesophageal junction at the University Hospital Dijkzigt, Rotterdam, the Netherlands. Tumours were classified according to the pTNM criteria for carcinoma of the oesophagus, established by the Union Internationale Contre le Cancer (UICC) in 1992. The vast majority of the tumours invaded the muscularis propria and/or adventitia (pT3-category, 80%) and had loco-regional lymph node metastases (pN1-category, 71%). There were 3 stage I, 14 stage IIA, 2 stage IIB, 22 stage III and 18 stage IV tumours.

Tumour samples were snap-frozen and stored in liquid nitrogen. Xenografts were obtained from 5 human oesophageal adenocarcinomas and 4 lymph node metastases by subcutaneous transplantation of tumour tissue on female nude NMRI mice, 4-6 weeks of age. Generally, the xenotransplants were harvested after 2-3 months. We also analysed exon 3 of the β-catenin gene in 2 human oesophageal cancer cell lines (JROECL 19 and 33) established by Rocket *et al.* and obtained from the European Collection of Cell Cultures (ECACC) (18). Cell lines were cultured in RPMI enriched with 10% foetal calf serum. DNA was isolated according to standard methods with proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation. Colon cancer cell line HCT 116 and 3 desmoid tumour samples with known mutations in exon 3 of the β-catenin gene (kindly provided by Dr. S.Tejpar, KULeuven, Leuven, Belgium) were used as positive controls for validation of the mutation detection procedure.

Mutation analysis: exon 3 of the β-catenin gene

PCR-Single stranded conformation polymorphism (SSCP) analysis for exon 3 was performed using primer pairs 1F (catttccaatctactaatgct) and 1R (ctgcattctgactttcagtaa) spanning exon 3 (315 bp). Because the sensitivity of the SSCP analysis varies with the size of the DNA fragment being analysed, we also amplified all tumour samples with primers 2F (atttgatggagttggacatggc) and 2R (ccagctacttgttcttgagtgaagg) within exon 3 which gives a shorter PCR product (226 bp) (10). The 15-µl PCR reaction mixture contained 50-100ng of DNA, 1.5 mM $MgCl_2$, 0.02 mM dATP, 0.2 mM dGTP, dTTP and dCTP each, 0.8µCi of [^{32}P]dATP (Amersham, Buckinghamshire, UK), 20 pmol of each primer and 0.2U *Taq* polymerase (Promega, Madison WI, USA). The PCR was performed for 35 cycles of 95 °C for 30s, 55 °C for 45s, and 72 °C for 1min. The PCR products were diluted 1:4 with loading buffer (95% formamide, 10mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol), heated at 95°C for 5 min, cooled and

electrophoresed in 6% polyacrylamide gels containing 10% glycerol at 7 W overnight at room temperature in 1x TBE running buffer.

Mutational analysis: interstitial deletions involving exon 3

We also performed PCR amplifications on all specimens with the primers (3F (ttgaagtataccatacaactg) and 3R (gcagcatcaaactgtgtagat), corresponding to parts of β-catenin exons 2 and 4, respectively, to screen for interstitial deletions involving exon 3 (8,12). The same PCR conditions were used as described above, except for using a different polymerase (Takara Ex Taq; Takara Biomedicals, Shiga, Japan). The PCR products of 1037 bp were electrophoresed in a 1% agarose gel and visualised by staining with ethidium bromide.

Sequencing

PCR products were purified over QIAquik spin columns (Qiagen, Hilden, Germany) and cloned into a pGEM-T vector (Promega, Madison, WI). Plasmid clones were sequenced with ^{35}SdATP and T7 and SP6 primers according to the dideoxy chain termination method.

Immunohistochemistry

In 54 tumours immunostaining for β-catenin was performed on 5-μm formalin-fixed, paraffin embedded tissue-sections with the avidin-biotin-peroxidase method. Sections were deparaffinised, immersed in 0.01 M sodium citrate buffer (pH 6.0) and incubated at 100°C in the microwave oven for 15 minutes. The mouse anti-human β-catenin monoclonal antibody (Transduction laboratories, Lexington, KY, USA) was applied overnight at a dilution of 1:200. When intense nuclear staining was present, the specimen was microdissected and DNA was isolated from these areas with nuclear staining.

Replication error phenotype (RER)

RER status was assessed in all tumour samples. The mononucleotide repeats BAT26, BAT40 and transforming growth factor-β type II receptor (BAT RII) were amplified under the conditions mentioned above, using published primer sets to determine microsatellite instability (19). Carcinomas demonstrating a shift in BAT26, BAT40 or BAT RII compared to normal DNA from the same tumour were classified as demonstrating microsatellite instability.

Analysis of APC mutations

In 3 tumours with nuclear localisation of β-catenin the mutation cluster region of *APC* was analysed as described by Mulkens *et al.* (20). DNA was extracted from frozen sections and two overlapping fragments that cover the mutation cluster region were amplified using the PCR. Mutations were screened for by temperature gradient electrophoresis and in aberrant cases mutations were identified by DNA sequencing.

TABLE 1. *Summary of β-catenin mutations found in human benign and malignant tumors and cell lines according to site.*

site	*number of tumours with β-catenin mutations/ number of tumours analysed (%)*	*reference*
Colorectum	4/24 adenonomas (17)	(9)
	2/16 adenocarcinomas (13)	
	8/33 adenocarcinoma cell lines (24)	
	7/58 adenocarcinomas (12)	(8)
	2/92 adenocarcinomas (2)	(21)
	1/57 adenocarcinoma cell lines (2)[1]	
	1/72 adenocarcinomas (1)	(22)
	8/130 adenomas (6)	
Skin	7/26 melanoma cell lines (27)	(23)
	1/50 melanomas (2)	(24)
	12/16 pilomatricomas (75)	(16)
Oesophagus	0/15 squamous cell carcinomas	(25)
Stomach	0/21 diffuse- and intestinal type carcinomas (0)	(26)
Breast	0/11 lobular and ductal carcinomas (0)	(26)
Prostate	5/104 carcinomas (5)	(14)
	0/7 cell lines and xenografted carcinomas (0)	
Uterus	5/35 endometrial carcinomas (14)	(27)
Ovarium	7/25 ovarian carcinomas (28)	(28)
CNS	3/67 medulloblastomas (4)	(11)
Thyroid	19/31 anaplastic carcinomas (61)	(15)
Liver	12/75 hepatocellular carcinomas (18)	(12)
	25/52 hepatoblastomas (48)	(13)
	3/3 hepatoblastoma cell lines (100)	
	2/2 hepatocellular carcinomas (100)	
Bone and soft tissue	2/62 malignant tumours (3)[2]	(29)
	0/11 benign tumours (0)[3]	
	22/42 sporadic desmoid tumours (41)	(30)
Upper digestive and respiratory tract	0/22 squamous cell carcinoma pharynx (0)	(31)
	0/30 squamous cell carcinoma larynx (0)	
	0/31 squamous cell carcinoma floor mouth (0)	

[1] *Cell lines analysed: 7 colon, 5 brain , 2 prostate, 9 lung, 6 ovarium, 6 leukemia, 7 renal, 7 breast and 8 melanoma cell lines.*

[2] *Tumours analysed: 21 malignant fibrous histiocytomas, 8 osteosarcomas, 7 synovial sarcomas, 7 liposarcomas, 7 malignant schwannomas, 3 rhabdomyosarcomas, 2 epithelioid sarcomas, 2 neurofibrosarcomas, 2 chondrosarcomas, 1 fibrosarcoma, 1 leimyosarcoma, 1 alveolar soft-part sarcoma.*

[3] *Tumours analysed: 3 giant cell tumours, 3 lipomas, 3 schwannomas, 1 desmoplastic fibroma, 1 hamartoma.*

CNS, central nervous system

RESULTS AND DISCUSSION

Studies indicate that elevated levels of free cytoplasmic β-catenin are involved in the genesis of a variety of carcinomas. Increased β-catenin levels can occur through mutations in the serine/threonine phosphorylation sites necessary for breakdown of β-catenin encoded by exon 3 of the β-catenin gene. So far, a large panel of benign and malignant human tumours have been analysed for β-catenin mutations involving exon 3 (Table 1). Mutations were frequently observed in skin, thyroid, liver and soft tissue tumors including pilomatricomas, anaplastic thyroid carcinomas, hepatoblastomas and desmoid tumours, respectively. Elevated levels of free β-catenin could also be achieved by inactivating mutations in *APC*, or perhaps through disruption of E-cadherin. Reduced membranous and/or increased cytoplasmic expression of E-cadherin in combination with nuclear localisation of β-catenin has been detected in oesophageal adenocarcinomas suggesting that the absence of E-cadherin at the cell membrane might be one factor liberating these catenins to participate in intracellular signalling (17). We found no E-cadherin gene mutations in oesophageal adenocarcinomas (32). Although loss of the 5q locus (harbouring *APC*) occurs in early stages of genesis of Barrett's adenocarcinoma (33), mutations in *APC* are rare (34). Therefore, we hypothesised that β-catenin gene mutations might play a role in the progression of Barrett's epithelium to dysplasia and to invasive adenocarcinoma of the oesophagus.

A total of 69 oesophageal tumour samples, consisting of 54 primary tumours, 4 lymph node metastases, 2 well established cell lines and 9 xenografts, were screened for genetic alterations involving exon 3 of the β-catenin gene. None of the samples exhibited mobility shifts by SSCP analysis. However, SSCP analysis is limited in detecting DNA alterations. The sensitivity in detecting single base pair mutations lies between between 60 and 90% (35). Therefore, we used four tumour samples with known base-pair alterations as positive controls. The colorectal cancer cell line HCT 116 which has been shown to harbour a 3-bp deletion (cdn 45) in exon 3 of the β-catenin gene (9), and three desmoid tumors with different single base substitutions in codons 41 and 45 (Dr F. Nollet, personal communication). All positive controls showed aberrant bands with primers 2F/2R and only 2 out of 4 when the primers 1F/1R were used to amplify exon 3 (Figure 1).

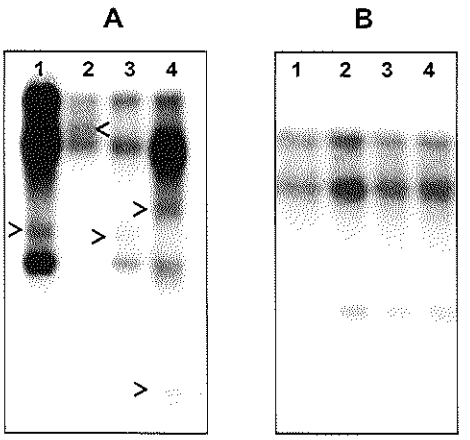


FIGURE 1. *PCR-SSCP analysis of β-catenin exon 3 with primers 2F/2R in tumour samples and normal control DNA. Panel A: lanes 1-3 are DNA's isolated from three desmoid tumors and lane 4 is DNA from colon cancer cell line HCT 116. Panel B: lanes 1-4 are from DNA extracted from oesophageal adenocarcinomas. Note that no SSCP aberration is seen in the oesophageal adenocarcinoma samples. Arrowheads, aberrantly migrating bands.*

This is consistent with the property of SSCP to be more sensitive for shorter DNA fragments (36). The efficiency of mutation detection can be elevated by changing the gel conditions (percentage and crosslinking of acrylamide, additives such as glycerol) or running conditions (room temperature or 4°C). The use of more than one set of gel conditions may have resulted in a greater detection of mutations, but others report only minimal improvement in detection (36). We used the same gel and running conditions as described by Orita *et al.*, who found this to be the most sensitive condition (37). Furthermore, the same PCR-SSCP method has been used recently and we detected three not yet described DNA alterations and eight known polymorphism's in the E-cadherin gene (32). However, we still can not rule out the possibility that mutations remained undetected by the SSCP analysis. But since we analysed a substantial number of tumour samples we think it is legitimate to conclude that mutations in exon 3 of β-catenin do not play a major role in adenocarcinomas of the oesophagus.

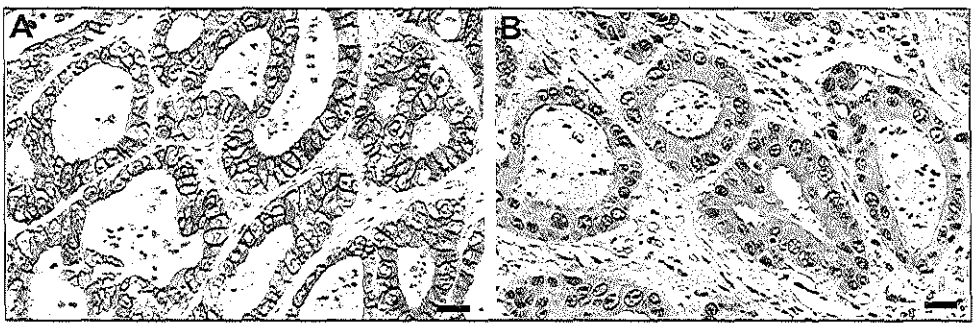
It has been suggested that mutations in β-catenin may be associated with an underlying defect in mismatch repair (9,21). Therefore, 69 oesophageal tumours were investigated for microsatellite instability but only 3 (4%) demonstrated the RER-phenotype. This confirms previous observations that widespread microsatellite instability occurs infrequently in adenocarcinomas of the oesophagus and that β-catenin mutations occur in a minority of RER-positive tumours (22,38).

In melanoma cell lines and in colorectal and hepatocellular carcinomas larger deletions involving exon 3 have been described previously (8,23,39). We screened 69 oesophageal tumour samples for larger deletions with primers spanning exon 3 and the adjacent introns. However, PCR products with reduced size were not identified.

β-Catenin mutations occur focally in prostate cancer and therefore detection is subjected to sampling errors (14). Garcia-Rostan *et al.* also demonstrated that β-

catenin mutations may be focal and that anaplastic thyroid carcinomas exhibit clonal heterogeneity (15). Therefore we performed β-catenin immunostaining on 54 tumours. Three tumours showed homogeneous nuclear staining in >90% of the tumour cells and 7 tumours had focal areas with intense nuclear staining, indicative of β-catenin deregulation (figure 2).

FIGURE 2. *Immunohistochemistry for β-catenin in oesophageal adenocarcinomas. Panel A shows a tumour with normal membranous localisation of β-catenin. Panel B demonstrates a tumour with prominent nuclear and cytoplasmic immunostaining. Scale bar is 25μm.*



These samples were microdissected and subsequently analysed by SSCP for β-catenin mutations with primer pairs 2F and 2R. However, in none of the samples mobility shifts indicative for mutations were found. Considering the possibility that some mutations are not detected by SSCP analysis, we sequenced exon 3 of β-catenin in the 3 samples with homogenous nuclear β-catenin staining, but no aberrations were detected. In these tumors APC inactivation could have been responsible for the increased cytoplasmic and nuclear β-catenin staining. Since 68-77 % of the reported somatic mutations are found in the mutation cluster region (20), we analysed this region of *APC*, but we did not identify any somatic mutations. This is in agreement with the results from Powell and co-workers who detected one *APC* mutation in the mutation cluster region in 18 oesophageal adenocarcinomas analysed (34). This demonstrates that *APC* mutations are rare in oesophageal adenocarcinomas.

Upregulation of the expression or nuclear concentration of β-catenin could result from alterations not only in the *APC* or β-catenin genes but also in other genes that function in this pathway. As yet, no genetic alterations in *GSK-3β*, its homologue *GSK-3α*, or γ-catenin have been reported in carcinomas with wild type *APC* and wild type β-catenin (9,11). Activation of the *Wnt1* oncogene promotes

the accumulation of β- and its homologue γ-catenin (40). Although Wnt expression has not been investigated in oesophageal cancer, it does promote mammary tumour formation in mice (41). Whether these components of the wingless/wnt-signalling are deregulated in adenocarcinomas of the oesophagus, is subject for further investigation.

In conclusion, we have shown that mutations involving exon 3 of the β-catenin gene do not play a role in the pathogenesis of adenocarcinomas of the oesophagus, which is in contrast with the prevalence of β-catenin mutations in other tumour types. The strong nuclear staining pattern of β-catenin in these tumours is unlikely to be explained by *APC* inactivation, indicating that other components of the wnt-signalling pathway must be involved.

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CHAPTER 9

REDUCED P120-CATENIN EXPRESSION CORRELATES WITH POOR SURVIVAL IN PATIENTS WITH ADENOCARCINOMAS OF THE GASTRO-OESOPHAGEAL JUNCTION

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Submitted

PRÉCIS

Reduced membranous expression of the cell-cell adhesion molecule p120-catenin is seen in approximately 70% of adenocarcinomas of the gastro-oesophageal junction, and correlates with poor 5-year survival rate.

INTRODUCTION

Cadherins are a family of transmembrane proteins implicated in calcium-dependent cell-cell adhesion. Normal cadherin function is necessary for embryological development, as well as morphogenesis of several tissues (1). One member of this family, E(epithelial)-cadherin is a molecule that plays a pivotal role in the establishment of cell-cell interactions in epithelial cells: disruption of E-cadherin-mediated cell-cell adhesion can lead to dissociation of epithelia and de-differentiation in fibroblast-like morphology (2-4). The activity of E-cadherin requires its interaction with the actin filaments of the cytoskeleton. This linkage is mediated by the catenins, a series of undercoat proteins. Both β- and γ-catenin bind directly to the cytoplasmic domain of E-cadherin and α-catenin links the complex to the actin microfilament network. Downregulation or defective interactions between any of the components of the cadherin-catenin complex appear to be associated with malignant transformation. A number of clinicopathological studies demonstrated that loss of E-cadherin-catenin expression is commonly associated with high tumour grade, advanced tumour stage, lymph node metastases and poor survival in a variety of malignancies, including prostate-, bladder-, breast-, colorectal-, and pancreatic cancers (5-10). In adenocarcinomas of the gastro-oesophageal junction (GOJ) in particular, others and we showed that abnormal expression of E-cadherin, α-, β- and γ-catenin is correlated with an aggressive phenotype (11-14). Moreover, aberrant expression of E-cadherin and β-catenin were independent predictors of poor survival (13).
Recently, another catenin-like molecule, p120-catenin ($p120^{ctn}$), has been identified as being associated with E-cadherin at the cell junctions (15-18). This protein is homologous to β- and γ-catenin and binds directly to the cytoplasmic domain of E-cadherin (19). The exact function of $p120^{ctn}$ has not been elucidated. There is some evidence that alterations in the level or distribution of $p120^{ctn}$ is a common event in inflammatory bowel disease and some human epithelial malignancies (6,20-23). Recently, it was postulated that $p120^{ctn}$ may act as an inhibitory regulator of cadherin function in colon carcinomas (24). The role of $p120^{ctn}$ in adenocarcinomas of the GOJ has not been studied. Therefore, we studied $p120^{ctn}$ expression and its cellular localisation in adenocarcinomas of the oesophagus and cardia. In addition, we evaluated the relationship between the expression and clinicopathological features in order to examine the application of $p120^{ctn}$ as a prognostic marker in adenocarcinomas of the GOJ.

MATERIALS AND METHODS

Patients and tumour specimen

From 1 January 1988 to 1 August 1999, 449 patients with an adenocarcinoma of the GOJ (*i.e.* distal oesophagus or gastric cardia) were evaluated at the University Hospital Dijkzigt. After pre-operative analysis, 343 patients (76%) were operated on with curative intent. In 52 (15%) of these, resection was not possible due to metastatic spread or local irresectability. Ninety-six consecutive cases of adenocarcinomas of the GOJ (84 males and 14 females, median age 64; range 37-80 years) who all underwent a transhiatal resection of the primary tumour were studied. The continuity of the gastro-intestinal tract was restored with a gastric tube with cervical anastomosis. In all patients a standard dissection of the perigastric, left gastric and coeliac nodes was performed. Macroscopic tumour clearance was aimed at in all cases but no extended lymph node dissection was done. None of the patients received neoadjuvant or post operative chemo-radiation therapy. Since adenocarcinomas at the GEJ (including distal oesophageal and gastric cardia carcinomas) can be considered as one clinical entity (25,26), TNM staging was performed according to the TNM classification for carcinoma of the oesophagus, established by the International Union against Cancer 1992 (27). Patients were followed at regular intervals after operation and data concerning disease recurrence, subsequent treatment and death were prospectively stored in a comprehensive database.

Of these 96 patients, formalin-fixed, paraffin-embedded tissue blocks were obtained from the resection specimens and 1-2 paraffin blocks representative for the whole tumour was selected. The selection was made on basis of presence of highest tumour grade within the oesophagectomy specimen assuming that these growth patterns within the tumour would predict patient's outcome. In addition, lymph nodes and premalignant Barrett's epithelium (present within the same slide as the tumour) were available from 13 and 20 patients respectively, and were also examined for p120^{ctn} expression.

Immunoperoxidase staining of paraffin-embedded tissues

Formalin-fixed, paraffin-embedded tissue blocks were cut into 4-μm thick sections, mounted on AAS-coated glass slides and stained using a standard avidin biotin immunoperoxidase technique. Sections were dewaxed in xylene and transferred to alcohol. Incubating the sections in 0.5% hydrogen peroxide in methanol for 20 minutes blocked endogenous peroxidase. To enhance the immunoreactivity, rehydrated sections were treated with an antigen retrieval solution in a microwave oven. The slides were submerged in 0.01M citrate buffer at pH 6.0 and were heated in a 700W microwave on full power for 3 x 5 min cycles, pausing to ensure that there was no fluid loss due to evaporation. After cooling, the slides were placed in a Sequenza immunostaining system (Shandon, UK) and pre-incubated with 10% normal goat serum (DAKO, Glostrup, Denmark) in PBS/BSA 5%. Then the slides were incubated overnight at 4°C with the primary antibody p120^{ctn} at 1:1000 dilution (Transduction Laboratories, Lexington, Kentucky, USA; packaged at

0.25mg/ml). Subsequently, the slides were washed in PBS and incubated with biotinylated goat anti-mouse IgG (Biogenex, San Ramon, USA) for 30min. After three washes in PBS, the slides were incubated with the streptavidin-peroxidase complex (Biogenex) for a further 30 minutes. Then the slides were developed with activated 3.3'-diaminobenzidine hydrochloride (Fluka, Neu-Ulm, Germany) with 0.08% H_2O_2 solution for 7 min. and the reaction was stopped in water. The slides were then counterstained with Mayer's Haemotoxylin and dehydrated in alcohol before mounting. Negative controls were duplicate sections similarly stained in which the primary antibody was omitted and replaced by normal mouse immunoglobulins. Positive controls using normal gastric epithelium were also run with each batch, in addition to using adjacent non-involved normal oesophageal or gastric mucosa as an internal positive control.

Interpretation of immunostaining

Two independent observers (BPLW and ANV) without prior knowledge of the tumour stage and patient profile carried out evaluation of staining. The sections were examined under light microscopy. The intensity of staining was always assessed relative to adjacent non-malignant epithelium within the same slide, which was used as an internal positive control. Staining was assessed in a semiquantative manner based on the percentage of cells with membranous staining. Initially, tumours were divided into 4 groups: >90% of the tumour cells were positive for $p120^{ctn}$ (*i.e.* membranous staining at virtually all intercellular borders), between 90 and 50%, 50-10% and <10% of the cells showed membranous staining. In addition, cytoplasmic staining was scored as either present at normal intensity (similar to adjacent normal mucosa) or increased. In case of tumour heterogeneity those areas within the tumour that showed the lowest membrane staining and/or highest cytoplasmic staining were assessed and analysed further. For purposes of statistical analysis all cases were grouped either as with normal membranous expression (>90%) or with reduced antigen expression of $p120^{ctn}$ (<90%). The cut-off point of 90% was used as an arbitrary measure, similar to previous published studies (13,28).

Statistical analysis

Statistical analysis was performed using the Statistical Software package SPSS 7.5 for Windows (SPSS Inc., Chicago, Illinois, USA). Correlations between p120ctn expression and clinicopathological indices were assessed with the χ^2 test.

Patients' follow-up was until August 1, 1999 or until death if before. Overall survival rates were calculated according to the Kaplan-Meier method. Differences in survival rates were calculated using the log-rank test. To identify independent prognostic factors, Cox's proportional hazard model (forward stepwise analysis) was used to enter variables in the model if these were statistically significant, while controlling for the other variables in the model. Backward stepwise elimination was done to verify that the same parameters remained in the final models. Statistical significance was set at the 5% level.

RESULTS

Immunoperoxidase staining of normal mucosa and GOJ adenocarcinomas

Normal squamous epithelium of the oesophagus and normal gastric epithelium showed a strong membranous expression of p120^{ctn} localised at the intercellular borders (Figure 1A). Some cytoplasmic staining was noted in the parabasal layers of the squamous epithelium and in the deeper parts of the gastric glands.

In our series, comparing the assessments of two observers for interobserver variability, less than 10% of cases changed category (*i.e.* normal to reduced expression, or vice versa). In adenocarcinomas, normal membranous expression of p120^{ctn} (>90% of the tumour cells) was seen in 31/96 (32%) and reduced expression in 65/96 (68%) of the tumours (Figure 1C/D). Absent of p120^{ctn} membranous expression (*i.e.* <10% of the tumour cells) was observed in 11 cases (Figure 1E). An increase in cytoplasmic staining of p120^{ctn} as compared to adjacent normal oesophageal or gastric epithelium within the same section was noted in 38 tumours (40%). The loss of membranous expression of p120^{ctn} correlated with increased cytoplasmic p120^{ctn} staining (P=0.05). Remarkably, reduced membrane staining and/or increased cytoplasmic expression of p120^{ctn} were mainly seen in singly infiltrating cells or clusters of cells. Staining patterns were often heterogeneous, with abnormal expression affecting some parts of the tumour but not others (Figure 1F). Interestingly, focal nuclear immunoreactivity for p120^{ctn} was seen in 5 tumours (Figure 1B). There was no difference in p120^{ctn} expression between oesophageal and gastric cardia adenocarcinomas.

Expression of p120^{ctn} in Barrett's metaplasia and lymph node metastases

In 20 patients, the expression of p120^{ctn} in Barrett's metaplasia adjacent to the tumour was evaluated. Loss of normal surface p120^{ctn} expression was seen in 4/20 cases (20%) and in 1 case this was accompanied by an increase in cytoplasmic staining (Figure 1G/H). In these 4 samples, p120^{ctn} staining was also reduced in the corresponding primary tumour. In the majority of the Barrett's specimens with normal membranous expression, a reduced expression of p120 was observed in the primary tumour.

Reduced p120^{ctn} expression was seen in 11/13 (85%) lymph node metastases. Interestingly, 4 primary tumours showed a higher percentage of positive cells with p120^{ctn} expression when compared to the metastases. In 6 tumours the p120^{ctn} expression was similar and in 3 tumours expression of p120^{ctn} was lower than in the corresponding metastases.

Correlation between staining and clinicopathological parameters

Reduced p120^{ctn} expression was only significantly associated with advanced tumour grade, but not with other pathological parameters (Table 1).

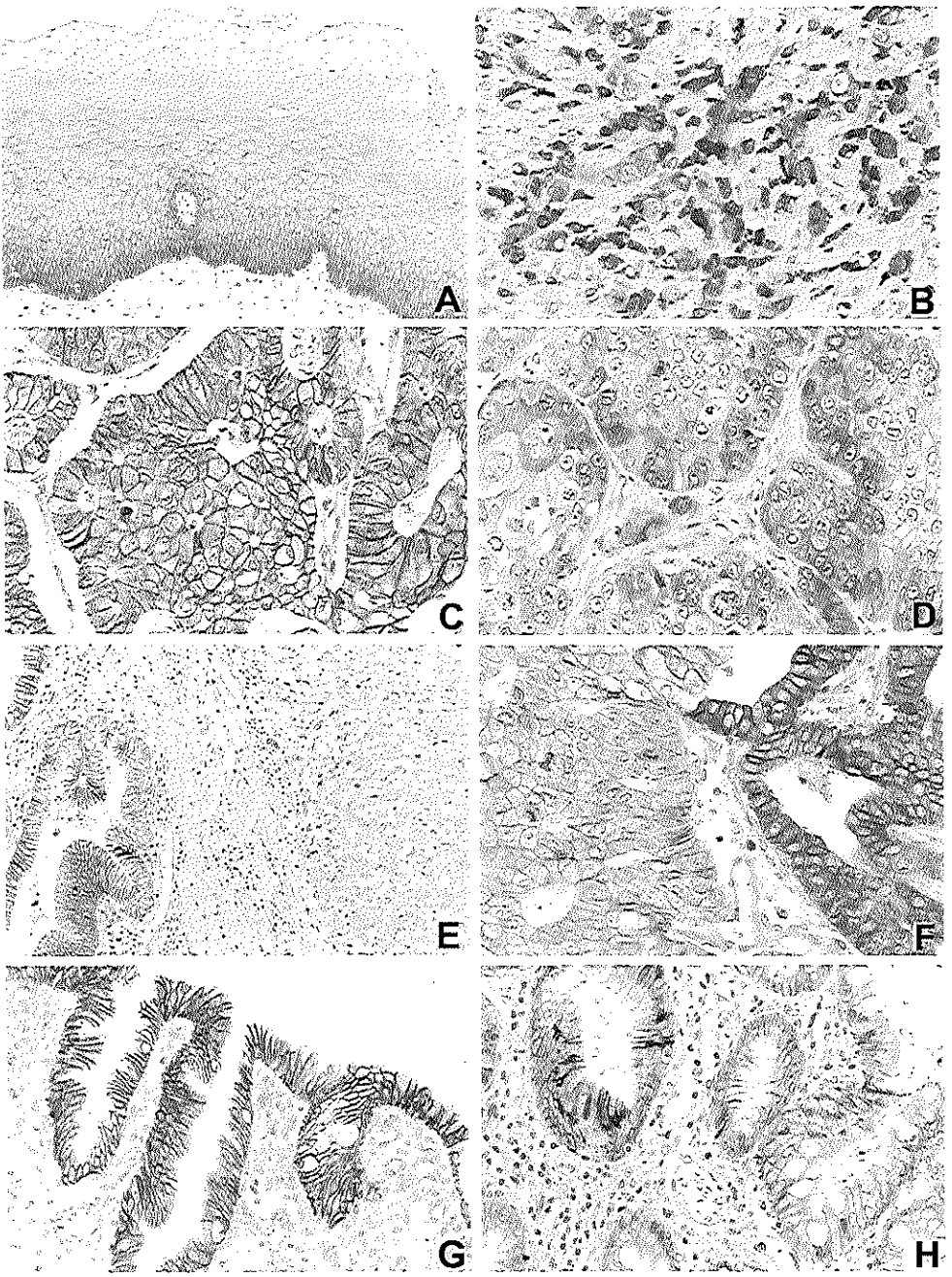
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TABLE 1. *Correlation between tumour characteristics and p120^{ctn} expression*

	p120^{ctn} expression			*P-value*
Variable	*Normal (>90%) No. (%)*	*Reduced (<90%) No. (%)*	*Total No. (%)*	
Tumour grade[a]				<0.0001
G1	3 (10)	3 (5)	6 (6)	
G2	21 (68)	13 (20)	34 (36)	
G3	7 (22)	49 (75)	56 (58)	
pT classification[b]				0.9
T1	3 (10)	6 (9)	9 (9)	
T2	7 (23)	14 (22)	21 (22)	
T3	21 (67)	45 (69)	66 (69)	
pN classification[c]				0.3
N0	14 (45)	22 (34)	36 (37)	
N1	17 (54)	43 (66)	60 (63)	
pM classification[d]				0.3
M0	23 (74)	42 (65)	65 (68)	
M1	8 (26)	23 (35)	31 (32)	
Tumour stage[e]				0.6
I	2 (6)	5 (8)	7 (7)	
IIA+B	14 (45)	20 (31)	34 (36)	
III	7 (23)	17 (26)	24 (25)	
IV	8 (26)	23 (36)	31 (32)	
Residual tumour classification[f]	26 (48)	44 (68)	70 (73)	0.09
R0	5 (16)	21 (32)	26 (27)	
R1				

[a] Grade of differentiation, G1: well, G2 moderate, G3 poorly differentiated; [b] Oesophageal wall infiltration; T1: tumour limited to submucosa, T2: tumour infiltrates muscularis propria, but not adventitia, T3: tumour infiltrates adventitia; [c] Locoreginal lymph nodes; N0: no regional lymph node metastasis, N1: regional lymph node metastasis present; [d] Distant lymph node metastasis; N0: no distant metastasis, N1: distant lymph node metastasis; [e] Oesophageal Cancer stage; I: T1N0M0, IIA: T2-3N0M0, IIB: T1-2N1M0, III: T3N1M0, T4anyNM0 ,IV: T4anyNanyM; [f] Residual tumour classification; R0: tumour free resection margins, R1: microscopic tumour positive resection margins.

FIGURE 1. *P120^{ctn} immunoreactivity in normal squamous epithelium of the oesophagus (A). Nuclear immunoreactivity for p120^{ctn} (B). Preserved p120^{ctn} membranous expression in tumour sample (C), reduced membranous and upregulation of cytoplasmic staining (D), negative staining of the tumour (right side) as compared to normal gastric epithelium (left side) (E) and a heterogeneous staining pattern seen in some tumours, demonstrating increased cytoplasmic staining with some preservation of membranous staining, while adjacent tumour cells show mainly membranous staining without up-regulation of cytoplasmic staining (F). Preserved membranous expression of p120^{ctn} (G) and reduced membranous expression (H) in Barrett's epithelium. Original magnification X 200 (A, E) and X 400 (B, C, D, F, G, H).*

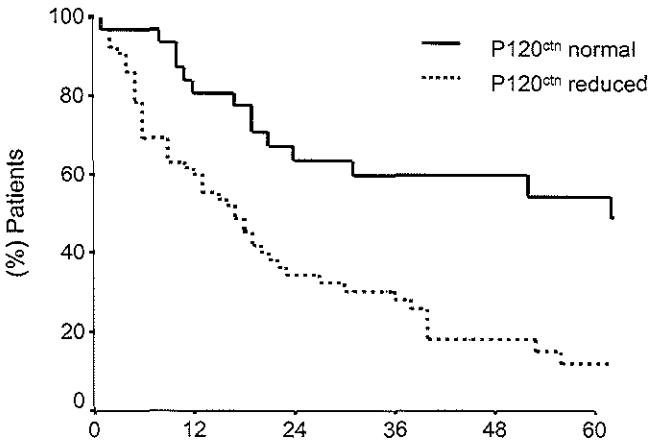
Follow-up was complete for all 96 patients. Univariate analysis revealed that tumour grade, pT, pN and pM classification, radicality of the operation and $p120^{ctn}$ expression were significantly associated with survival (Table 2). The 5-yr survival rate for patients with GOJ adenocarcinomas who showed normal membranous expression of $p120^{ctn}$ was 54% (median 52 months) compared to 12% (median 17 months) for patients with reduced expression (Figure 2). Multivariate analysis using the Cox regression model showed that abnormal expression of $p120^{ctn}$ was an independent variable in predicting overall survival. Grade of differentiation was the most powerful predictor of overall survival (Table 2).

TABLE 2. *Five-year survival probabilities subsequently stratified for tumour grade, pT, pN, pM classifications, tumour stage, radicality of the operation and $p120^{ctn}$ expression.*

		Univariate Analysis			*Multivariate Analysis*		
	No. of Patients	*5-yr[a]*	*SD[b]*	*P-value*	*RR[c]*	*CI[d]*	*P-value*
Overall	96	26%	--	--	--	--	--
Tumour grade					2.0	1.2 - 3.4	0.01
G1-2	40	47%	0.09	0.0003			
G3	56	11%	0.05				
pT classification					1.6	1.1 - 2.4	0.02
T1	9	50%	0.22	0.07			
T2	21	28%	0.12				
T3	66	22%	0.06				
pN classification					NS[e]	--	--
N0	37	41%	0.09	0.005			
N1	59	18%	0.06				
pM classification					NS	--	--
M0	65	33	0.06	0.006			
M1	31	8	0.07				
Tumour stage				0.005	NS	--	--
I,IIa	35	38	0.09				
Iib, III, IV	61	19	0.06				
Radicality				0.02	NS	--	--
R0	70	29	0.06				
R1	26	16	0.08				
$p120^{ctn}$ expression				0.0002	2.1	1.1 - 4.2	0.003
Normal	31	54	0.09				
Reduced	65	12	0.06				

[a] 5-yr Survival Rate; [b] Standard Deviation; [c] Relative Risk; [d] 95% Confidence Interval; [e] Not Significant

FIGURE 2. *Kaplan-Meier survival curve for 96 patients with tumours of the GOJ with normal or reduced expression of p120^{ctn}. Reduced expression of p120^{ctn} was correlated with poor survival (P=0.0002)*



No. at risk						
total	96	64	34	24	17	14
P120^{ctn} normal	31	25	17	12	11	10
P120^{ctn} reduced	65	38	17	12	6	4

DISCUSSION

In the United States (US) and in some European countries the incidence rates of adenocarcinomas of GOJ have increased over the past two decades (29-32). In the US, the rate of increase has surpassed that of any other cancer (33). The aetiology of adenocarcinomas of the GOJ is associated with gastro-oesophageal reflux and Barrett's oesophagus (34-36). Despite an increase in the number of endoscopies, screening for malignant transformation of Barrett's epithelium, improved surgical techniques and lower peri-operative mortality, the prognosis of patients with adenocarcinoma of the GOJ remains poor. Several biological and molecular parameters have been considered as potential prognostic markers for adenocarcinomas of the distal oesophagus and gastric cardia, but up to now tumour grading, and staging still remain the most important prognostic variables (25,37-39). However, current methods of disease staging are not always sensitive enough and fail to detect small numbers of tumour cells in lymph nodes (40). Therefore, the

development of additional indicators to refine the staging system for GOJ cancers and to identify patients who will not be cured by surgery is required.
Immunohistochemical detection of E-cadherin and the catenins can be useful in predicting disease-free or overall survival but also identify patients with clinically negative lymph nodes who are at risk for occult metastases and who may benefit from more extensive lymph node resection (41). In this study we examined the expression, distribution and prognostic value of $p120^{ctn}$, a recently identified member of the E-cadherin-catenin complex. We show that reduced membranous expression of $p120^{ctn}$ occurs in 68% of the carcinomas and true loss of expression (<10% of tumour cells show positive staining) in 11 cases (11%). There is very little information available concerning $p120^{ctn}$ expression in malignant disease. Abnormal $p120^{ctn}$ expression was seen in 40-80% of the patients with colorectal-, bladder-, breast-, and gastric carcinomas (6,21,22,28). Only in bladder carcinomas, $p120^{ctn}$ correlated with overall survival, but not independent of grade and stage (23). Our study demonstrates that despite the fact that $p120^{ctn}$ expression was not significantly correlated with most well known prognostic markers, an independent association with prognosis was established. In patients with tumours classified as N0 by routine histopathological methods, the 5-yr survival for patients with normal $p120^{ctn}$ expression of the tumour was 63% *versus* 20% for the patients with reduced expression (P=0.02). $P120^{ctn}$ could therefore be useful in identifying patients with clinically negative nodes who are at risk for occult metastases.
It is unknown whether the observed reduction or complete loss of $p120^{ctn}$ expression represents mutation or simply transiently down-regulation. The fact that some of our cases show areas of loss and other areas with reduction or alteration of staining and the fact that the expression in some metastases is significantly different from the primary tumour, suggest that the absence of expression may be the result of down-regulation. At least, the observed loss of membranous staining in premalignant Barrett's epithelium suggest that this is an early event in the evolution of neoplasia, as seen in colorectal polyps and gastric dysplasia (28,42).
In our study reduced membranous expression was associated with a marked upregulation of cytoplasmic staining, as has been found in gastric cancer (28). The mechanisms responsible for this observed abnormal expression and the significance of this is not clear yet. Failure of E-cadherin and the catenins to localise to the membrane and/or cytoskeleton in spite of the abundant presence may be due to alterations in their phosphorylation status (43,44). Tyrosine phosphorylation of $p120^{ctn}$ like β- and γ-catenin by the cytoplasmic protein tyrosine kinases (*e.g.* src) and by a number of growth factors (upon binding to their membrane receptor), such as epidermal growth factor, hepatocyte growth factor/scatter factor, has been proposed as a mechanism for modulating the E-cadherin-catenin system (17,45-47). Increased phosphorylation of the catenins has been associated with cellular redistribution of E-cadherin, cell dissociation and increased motility (17,48,49). Indeed, elevated $p120^{ctn}$ tyrosine phosphorylation was detected in human tumour cells with down-regulated E-cadherin-mediated cell-cell adhesion (50,51). These data have suggested involvement of $p120^{ctn}$ in ligand-

induced mitotic signalling as well as cell transformation, and may indicate the upregulation of p120^{ctn} expression. Despite the homology of p120^{ctn} with other catenins, its function is likely to be different. It is expressed in a number of isoforms (up to 32) which appear to arise as a result of alternative splicing (16,52). Poorly differentiated cells fail to express particular isoforms that are typically observed in well-differentiated cell lines (53). This raised the possibility that unbalanced expression of isoforms in human carcinomas may influence cadherin function and contributes to malignant or metastatic cell phenotypes.

Of particular interest is our observation of focal nuclear localisation of p120^{ctn} in some tumours. Recently, van Hengel *et al.* reported that upon expression of p120^{ctn} cDNA lacking exon B, which encodes for an nuclear export signal, the isoforms were detectable within both the nuclei and cytoplasm of cancer cell lines (54). Moreover, it has been shown that endogenous p120^{ctn} could also be detected in the nucleus of human breast and colon cancer cell lines. These carcinoma cell lines express low to undetectable levels of E-cadherin. Upon expression of exogenous E-cadherin, p120^{ctn} was no longer detectable in the nucleus. Apparently, binding of p120^{ctn} to E-cadherin at the plasma membrane or in the cytoplasm restrains p120^{ctn} from entering the nucleus. Indeed, some but not all studies found that the membranous expression of p120^{ctn} correlates well with the expression of E-cadherin (21,23). Recently, a putative nuclear transcription factor, Kaiso, was shown to interact with p120^{ctn} (55). This suggests that p120^{ctn} might not only be involved in the modulation of E-cadherin function, but also in signal transduction, as has been shown for β-catenin (56).

In conclusion, we have found reduced membranous expression of p120^{ctn} in premalignant Barrett's epithelium reaching a high incidence in oesophageal and gastric cardia adenocarcinomas. Hopefully, future studies will reveal the role of this molecule in cell-cell adhesion and possibly cell signalling. Moreover, our results indicate that p120^{ctn} expression may be a useful prognostic marker in adenocarcinomas of the gastro-oesophageal junction

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CHAPTER 10

BIOCHEMICAL ANALYSIS AND SUBCELLULAR DISTRIBUTION OF THE E-CADHERIN-CATENIN COMPLEX IN ADENOCARCINOMAS OF THE GASTRO-OESOPHAGEAL JUNCTION

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Submitted

PRÉCIS

Perturbation in the expression, distribution and hence function of the members of the E-cadherin-catenin complex is seen in 50-70% of adenocarcinomas of the gastro-oesophageal junction.

INTRODUCTION

The cadherins are a family of calcium-dependent transmembrane adhesion proteins that interact in a homotypic fashion, playing a central role in the maintenance of tissue integrity, tissue morphology and cell-cell recognition (1,2). The importance of E(epithelial)-cadherin expression and function in normal development and tissue function is demonstrated by the lethality of E-cadherin knockout mice at an early stage in embryogenesis (3). E-cadherin is bound via series of undercoat proteins, the catenins, to the actin cytoskeleton. The catenin family comprises α-, β-, γ-catenin and a recently discovered 120 KDa protein, p120catenin (p120ctn). β- or γ-catenin bind directly to the cytoplasmic tail of E-cadherin in a mutually exclusive manner. α-Catenin then links β- or γ-catenin to the actin microfilament network of the cytoskeleton. The linkage between transmembranous E-cadherin, the catenins and the actin filaments is necessary to form strong cell-cell adhesion. Deletion of the intracellular catenin-binding domain of E-cadherin or alterations in the functionally active catenins, results in loss of the ability of E-cadherin to establish cell-cell adhesion (4-7).

Several studies have implicated E-cadherin in both the early and late stages of tumour initiation and progression. Various human cancer cell lines with an epitheloid, differentiated morphology were generally non-invasive and expressed E-cadherin, whereas cell lines with a fibroblast-like morphology were invasive and had often lost E-cadherin expression (8,9). Using a transgenic mouse model of pancreatic β-cell tumourigenesis Perl *et al.* demonstrated that loss of E-cadherin mediated cell-cell adhesion is causally involved in the transition from adenoma to carcinoma (10). Immunohistochemical studies in human cancers have frequently shown that a proportion of invasive carcinomas and carcinomas *in situ* show aberrant levels of E-cadherin and catenin expression in comparison to their related normal tissue (11-13). Therefore, changes in E-cadherin-catenin expression appear to be an important step in the development and progression of a malignant tumour.

Adenocarcinomas of the distal oesophagus and gastric cardia (*i.e.* gastro-oesophageal junction(GOJ)) are thought to arise from (short) segments of premalignant, metaplastic columnar epithelium, also referred to as Barrett's oesophagus. Most carcinomas carry a poor prognosis as local and systemic invasion is often seen at early stages. This is worrisome, since adenocarcinomas of the GOJ show an increase in incidence throughout the western world, especially among middle-aged white males, surpassing that of any other malignant tumour (14-17).

To elucidate the role of E-cadherin-catenin complex in adenocarcinomas of the GOJ we evaluated the level of expression and distribution of E-cadherin and the catenins in tumour tissue *versus* the normal mucosa of the same patients. E-cadherin and catenin are known to be present in the cell in different protein pools, membrane bound, free cytoplasmic and cytoskeleton bound. The expression of E-cadherin and catenins and their biochemical distribution between the Triton-X-100 soluble (membrane bound) fraction and the Triton-X 100 insoluble (cytoskeleton bound) fraction was assessed

using fractional protein extraction and western blot analysis. The spatial distribution of the proteins between the cell membrane, cytoplasm and nucleus was assessed using immunohistochemistry.

MATERIALS AND METHODS

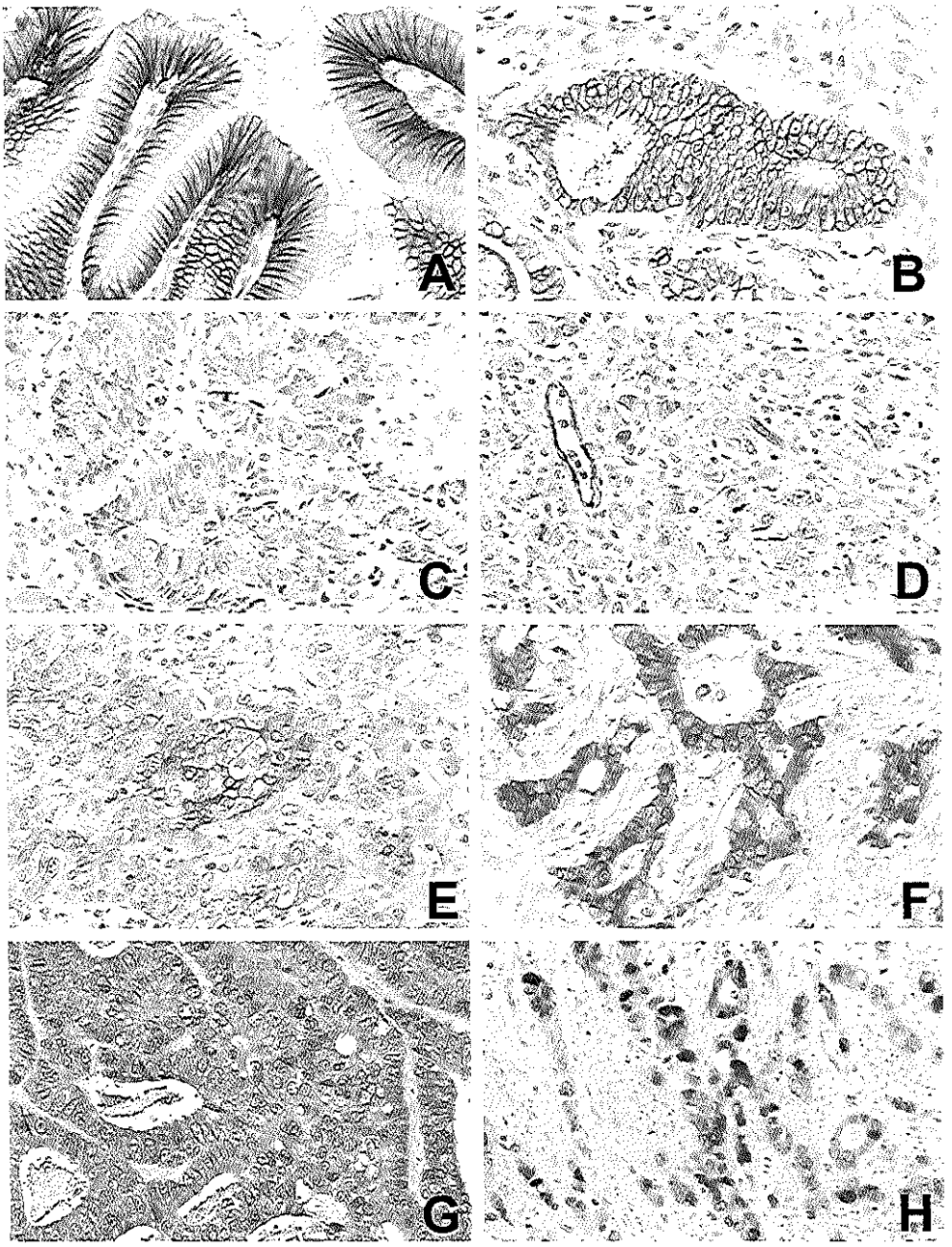
Patients and samples

Twenty-four patients with an adenocarcinoma of the GOJ (*i.e.* distal oesophagus or gastric cardia), treated at the University Hospital Dijkzigt, Rotterdam, the Netherlands, were included in our study. There were 20 males and 4 females with a median age of 63 years (range 43-78 years) at the time of operation. All patients underwent transhiatal resection of the oesophagus and proximal stomach and the gastro-intestinal tract was reconstructed with a gastric tube in all cases, as described previously (18). None of the patients received (neo) adjuvant therapy.

After routine pathological examination of the resection specimens, primary tumour samples and corresponding normal tissue was stored as archival paraffin blocks. From 11 patients also small pieces of the tumour and surrounding normal oesophageal or gastric mucosa were available as fresh frozen tissue. Fresh tissue was obtained immediately postoperatively and stored in liquid nitrogen at the Department of Pathology, Josephine Nefkens Institute, Rotterdam, the Netherlands.

Immuohistochemistry

Formalin-fixed paraffin-embedded tissue blocks of the primary tumour samples were cut into 4-μm thick sections, mounted on AAS-coated glass slides and stained using a standard avidin biotin immunoperoxidase technique. Sections were dewaxed in xylene and transferred to alcohol. Endogenous peroxidase was blocked by incubating the sections in 0.5% hydrogen peroxide in methanol for 20 minutes. To enhance immunoreactivity, rehydrated sections were treated with an antigen retrieval solution in a microwave oven. The slides were submerged in 0.01 M citrate buffer at pH 6.0 and were heated in a 700W microwave on full power for 15 minutes. Slides were then pre-incubated with 10% normal goat serum (DAKO, Glostrup, Denmark) in PBS/BSA 5% prior to incubation of the primary antibody at 4°C overnight. Primary antibodies used were E-cadherin (Euro-Diagnostica, Arnhem, The Netherlands) and α-catenin, β-catenin and γ-catenin (Transduction Laboratories, Lexington, USA). Subsequently, the slides were washed in PBS and incubated with biotinylated goat anti-mouse IgG (Biogenex, San Ramon, USA) for 30 min. After three washes in PBS, the slides were incubated with streptavidin-peroxidase complex(Biogenex) for a further 30 min. and finally developed with activated 3.3'-diaminobenzidine hydrochloride (Fluka, Neu-Ulm, Germany) with 0.08% H_2O_2 solution for 7 min. The slides were then counterstained with Mayer's Haematoxylin and dehydrated in alcohol before mounting. Negative controls sections were duplicate sections similarly stained in which the primary antibody was omitted and replaced by normal mouse immunoglobulins. Positive controls using normal gastric epithelium were also run with each batch, in addition to using non-involved normal oesophageal or gastric mucosa as an internal positive control.


A
B
C
D
E
F
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H

Western blotting

Expression of E-cadherin-catenin was analysed by Western blotting in 11 primary tumours and corresponding normal mucosa. Sections of 5-μm were cut on the cryostat and stained with H&E to be compared with the formalin-fixed paraffin embedded tissue sections. The frozen tissue samples were dissected to exclude as much stromal tissue as possible, and trimmed in order to get the same dimensions to ensure that an approximately similar amount of tissue was being analysed from each specimen. Approximately twenty-sections of 15-μm thickness were then cut from each sample and placed in a pre chilled eppendorf.

Tissue sections were solubilised by cytoskeleton buffer (19). 300μl of soluble fraction lysis buffer (0.5% Triton X-100, 50mmmol sodium chloride, 10mmol Pipes, 3mM magnesium chloride, 300mmol sucrose, protease inhibitor tablets (1 tablet/50 mL lysis buffer; Boehringer Mannheim, Mannheim, Germany) were added to each tube. Tubes were kept on ice for 20 min. with repeated vortexing, then were centrifuged at 13,000 rpm for 10 min, at 4°C. The resulting supernatant (with the Triton X-100 soluble fraction of the cell protein) was collected. The pellet was lysed in 200μl of the sodium dodecyl sulphate (SDS) lysis buffer (15mmmol Tris-Hcl, 5mmol EDTA, 2.5 mmol EGTA, 1% SDS) at 100°C for 15 minutes and then spun at 13,000rpm for 10 minutes at 4°C. The supernatant (with the Triton X-100 insoluble cell protein fraction) was collected. Samples were either analysed immediately or frozen at -70°C.

Protein concentration was measured using the Bradford protein assay kit (Biorad., CA, USA). Equal protein loading of corresponding lanes ensured accurate comparison of protein expression of the tumour and normal tissues by comparing band sizes and intensities. The calculated volumes of lysates were denatured and reduced with sample buffer (2% SDS, 10% glycerol, 50mM Tris-HCL pH 6.8, 5% β-mercaptoethanol, 0.25% bromophenol blue), boiled for 10 min., and then resolved by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) on a 8% polyacrylamide gel. The resolved proteins were transferred by electroblotting onto nitro-cellulose membranes (Millipore, Herts, UK). The membranes were blocked with 5% non fat dried milk (Marvel) in Tris buffered saline-Tween 20, pH 7.2) for 1 hour at room temperature and then incubated with the primary antibody diluted in TBS-T overnight at 4°C. Antibodies were used at a dilution of 1μg/mL for E-cadherin, β- and γ-catenin and 4μg/mL for α-catenin. The blot was then incubated with horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (Dako, Glostrup, Denmark) at 1μg/mL for 2 hours at room temperature. Detection was by enhanced chemiluminescence reagent (ECL, Amersham Life Sciences, Bucks, UK) followed by autography with Hyperfilm-MP (Amersham Life Sciences).

FIGURE 1 *E-cadherin immunoreactivity in normal gastric epithelium (A). E-cadherin immunoreactivity in a tumour with membranous localisation of the protein (B). Reduced membranous expression of E-cadherin (C); cytoplasmic staining for α-catenin. Also note the immunoreactivity of a blood vessel (D); reduced membranous and increased cytoplasmic staining of β-catenin (E and F); Strong cytoplasmic and homogeneously nuclear expression of β-catenin G); Scattered nuclear expression for γ-catenin (H).*

Evaluation of immunostaining and western blot

The stained sections were examined by two observers under light microscopy. The localisation and intensity of staining was always assessed relative to the normal mucosa in the same section. Tumours displaying well localised membranous staining and weak cytoplasmic staining were considered as normal. Weak or absent membranous staining, intense cytoplasmic and nuclear staining were considered abnormal (20).The expression of the E-cadherin-catenin complex in tumour tissue and corresponding normal tissue was analysed for the soluble and insoluble fraction. The ratio of the signal derived intensity of tumour and normal tissue was determined and compared between the soluble and insoluble fraction. Western blots were classified as abnormal when there was a difference in ratio between the soluble and insoluble fraction.

Statistical analysis

Categorical data were displayed in contingency tables when analysing protein expression with respect to pathological data and data obtained from Western blotting, and analysed by chi-square or Fisher's exact test, where appropriate. A p-value of <0.05 was accepted as statistically significant.

RESULTS

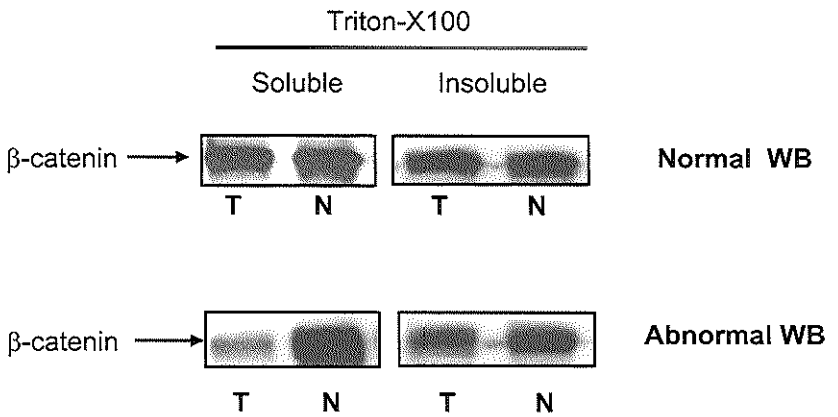
Immunohistochemistry: expression and localisation of E-cadherin-catenin

Normal squamous epithelium of the oesophagus and normal gastric epithelium showed a strong membranous expression of E-cadherin and the catenins localised at the intercellular borders (Figure 1A-B). In adenocarcinomas, abnormal expression of E-cadherin, α-, β- and γ-catenin, *i.e.* reduced membranous and/or increased cytoplasmic localisation of the protein, was seen in 13/24 (54%), 18/24 (75%), 16/24 (67%) and 15/24 (63%), respectively (Figure 1 C-F). Nuclear localisation of β-catenin was seen in 5/24 (21%) tumours and for γ-catenin in one tumour (Figure 1 G-H). There was no significant correlation between the expression of the E-cadherin and the catenins and tumour grade, residual tumour classification, pTNM-classification or tumour stage.

Western blotting: biochemical distribution of E-cadherin-catenin

All members of the complex were present in the Triton-X soluble and insoluble fraction from the normal mucosa and all tumours. In adenocarcinomas, an abnormal distribution of E-cadherin, α-, β- and γ-catenin between the soluble and insoluble fraction relative to the normal mucosa was seen in 4/11 (36%), 4/10 (40%), 5/11 (45%) and 5/11 (45%). Figure 2 shows a representative example of an abnormal western blot of β-catenin.

FIGURE 2. *Example of a Western Blot for β-catenin in tumour and corresponding normal tissue for the soluble and insoluble fraction. The ratio between the signal derived intensity for the tumour and normal tissue is about 1 for soluble and insoluble fraction (top, normal western blot). The abnormal western blot (bottom) shows a different ratio between the tumour and normal tissue for β-catenin in the soluble versus in the insoluble fraction.*



Correlation between immunohistochemistry and western blotting

There was a close correlation, albeit not significant, between immunohistochemisty and western blotting: both techniques detected 5/11 adenocarcinomas with an abnormal expression/distribution of E-cadherin. However, one tumour with a normal membranous expression of E-cadherin on immunohistochemistry, had an abnormal distribution of the protein between the soluble and insoluble fraction as shown by western blotting, and vice versa (Figure 3A). There was no correlation between immunohistochemistry and western blotting in detecting abnormal expression and localisation of the catenins (figures 3B-D).

FIGURE 3. *Correlation between immunohistochemistry and western blotting in detecting abnormal expression and distribution of E-cadherin (A) and the catenins (B-D).*

A

E-cadherin	*IHC* [a]		*Total*
	normal	*abnormal*	
WB [b]			
normal	5	1	6
abnormal	1	4	5
Total	6	5	11
			p=0.08 [c,d]

B

α-catenin	*IHC*		*Total*
	normal	*abnormal*	
WB			
normal	2	4	6
abnormal	1	3	4
Total	3	7	10
			p=0.9

C

β-catenin	*IHC*		*Total*
	normal	*abnormal*	
WB			
normal	4	2	6
abnormal	1	4	5
Total	5	6	11
			p=0.2

D

γ-catenin	*IHC*		*Total*
	normal	*abnormal*	
WB			
normal	3	3	6
	2	3	5
abnormal			
Total	5	6	11
			p=0.7

a, Immunohistochemistry; b, Western Blot; c, P-value; d, Fischer's Exact test

DISCUSSION

Unlike colorectal carcinomas, there is no uniform genetic model that fits the histologic progression from premalignant dysplastic Barrett's epithelium towards invasive adenocarcinoma of the GOJ (21). However, it has become increasingly apparent that the E-cadherin-catenin cell-cell adhesion complex plays an important role in tumour initiation and progression. Changes in expression of the E-cadherin and the catenins, as determined by immunohistochemistry, occurs already in dysplastic Barrett's epithelium and is considered an early event in carcinogenesis (22). Our findings of reduced membranous expression and/or increased cytoplasmic staining of E-cadherin-catenin in 54-75% of adenocarcinomas is similar to other studies (22-24). Reduced membranous localisation of E-cadherin and the catenins is associated with increased invasiveness, lymphatic involvement and prognosis (23). However, we did not find a correlation between abnormal expression of E-cadherin-catenin and unfavourable tumour characteristics, likely due to the relatively small numbers of tumours analysed in our study.

A significant proportion of tumours demonstrated strong nuclear expression of β-catenin and one tumour with nuclear localisation of γ-catenin. Nuclear accumulation of β-catenin is an early event in the neoplastic progression of Barrett's oesophagus since this is observed already in low grade dysplastic Barrett's epithelium(25,26). Nuclear localisation of both catenins indicates their role in signal transduction and regulation of gene expression (27). Free, cytoplasmic β-catenin translocates to the nucleus, binds transcription factors (Tcf/Lef) and stimulates transcription of target genes such as c-myc and cyclin D1 (28,29).

In the present study we also evaluated the biochemical and spatial distribution of E-cadherin, α-, β- and γ-catenin in the different cell compartments in cancerous tissues and normal adjacent mucosa from the same patient. We show in agreement with other studies that E-cadherin, α-, β- and γ-catenin are present in two protein pools, a Triton X-100 soluble membrane bound fraction and a Triton X-100 insoluble cytoskeleton bound fraction (30,31). Our results also demonstrate that complex disturbances of E-cadherin and catenins expression are common in adenocarcinomas of the GOJ. Evaluating the distribution of E-cadherin and catenins between the soluble and insoluble fraction showed an increase or decrease in expression in one of the two fractions relative to the normal mucosa. This shows that expression of the proteins does not always imply that they are functioning, as binding of the E-cadherin-catenin complex to the cytoskeleton is essential for its role in cell adhesion. Two studies demonstrated increased β-catenin in the soluble fraction and decreased β-catenin in the insoluble fraction in a subset of oesophageal squamous cell carcinomas and colorectal carcinomas and this was associated with increased cytoplasmic staining by immunohistochemistry (32,33). We confirm their findings, since in 4/5 tumours with an abnormal western blot for β-catenin, immunohistochemistry revealed reduced membranous and increased cytoplasmic staining. Moreover, 3/5 tumours had strong nuclear staining for β-catenin.

Failure of E-cadherin and the catenins to localise to the membrane and/or bind the cytoskeleton in spite of their abundance may be due to genetic or epigenetic changes in their structure and/or function. E-cadherin, α, β, and γ-catenin mutations have been reported in different tumours and cell lines (5,34-36). However, we and others could detect E-cadherin nor β-catenin gene mutations underlying the aberrant expression of the proteins (25,37,38). Post-translational regulation, perhaps by tyrosine phosphorylation, may be responsible for the distribution of the catenins in the different pools. The phosphorylation status of the catenins may be affected by intracellular membrane associated tyrosine kinases like src and receptor tyrosine kinases such as the epidermal growth factor (EGF) receptor. In response to transfection with v-src, both β-catenin is phosphorylated, and phosphorylation may interfere with cadherin function and disrupt adherens junctions, without affecting the overall expression of either the catenins or E-cadherin (39,40). Moreover, E-cadherin tyrosine de-phosphorylation was observed in primary bronchopulmonary and thyroid carcinomas displaying a spotty cytoplasmic pattern or a pericellular redistribution of the complex with no synthesis variations for E-cadherin or the catenins (41,42).
Imunohistochemistry and western blotting data correlated when expression and distribution of E-cadherin was analysed. E-cadherin is expressed in epithelia only. On the contrary, the catenins are also expressed in non-neoplastic cells, such as muscle, endothelium and nerves (Figure 1D). Therefore, contamination of the protein lysates with stromal tissue can influence the signal derived intensity of the western blots. This might explain why there was no correlation between the results of the immunohistochemistry and the western blotting with respect to the catenins. On the other hand, all tumour tissue samples were carefully selected to contain at least 75% tumour cells with minimal stromal tissue. Secondly, western blotting detected tumour samples with redistribution of the E-cadherin-catenin complex that were shown normal by immunohistochemistry. Therefore, both techniques might be of additional value in detecting discrete but important changes in distribution of the cell-cell adhesion complex in cancerous tissues.
In summary, our study showed that a perturbation in the expression, distribution and hence function of the members of the E-cadherin-catenin complex is frequently seen in adenocarcinomas of the gastro-oesophageal junction. Immunohistochemical technique and western blotting of the soluble and insoluble fraction of the proteins are both valuable tools in detecting aberrant expression and distribution of the complex and even might have additional value. Further research will have to focus on the possible mechanisms underlying the redistribution of this cell-cell adhesion complex in cancers. Ultimately, this might improve our understanding of the aggressive features of adenocarcinomas of the gastro-oesophageal junction, which show a sharp increase in incidence over the past decades in the western world.

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PART V

TUMOUR SUPPRESSOR GENES

CHAPTER 11

MOLECULAR GENETIC ANALYSIS OF THE VON HIPPEL-LINDAU (VHL) AND HUMAN PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA (PPARγ) TUMOUR SUPPRESSOR GENES IN ADENOCARCINOMAS OF THE GASTRO-OESOPHAGEAL JUNCTION

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PRÉCIS

Mutation analysis of the VHL and PPARγ genes was performed in 43 tumour specimens derived from adenocarcinomas of the gastro-oesophageal junction. No mutations were detected in both genes, despite frequent LOH (68%) at 3p25-26.

INTRODUCTION

Worldwide, adenocarcinomas of the gastro-oesophageal junction (GOJ), *i.e.* distal oesophagus and gastric cardia, show an increase in incidence over the past three decades (1,2). This is worrisome, since these carcinomas carry a poor prognosis, with 5-year survival rates of about 25% after surgery with curative intent (3). Adenocarcinomas of the GOJ are thought to arise from premalignant metaplastic epithelium, also known as Barrett's epithelium. This columnar epithelium has replaced the normal squamous lining of the oesophagus due to longstanding (duodeno)gastro-oesophageal reflux. Numerous molecular events involved in the progression from Barrett's oesophagus to adenocarcinoma have been characterised over the past decades, but there is still no uniform molecular pathway (4,5). Therefore, the pathogenesis of adenocarcinomas of the GOJ remains poorly understood.

Loss of a region on the short arm of chromosome 3 is frequently found in human carcinomas, including adenocarcinomas of the GOJ, and suggests the presence of a putative tumour suppressor gene within the region of chromosomal loss (6-16). Moreover, the tumourigenicity of carcinoma cell lines has been shown to be suppressed by the introduction of a human chromosome 3p (17). The region of frequent LOH on 3p in oesophageal adenocarcinomas was at 3p25-26 and encompasses the von Hippel-Lindau (VHL) tumour suppressor gene (14). This gene was linked to chromosome 3p in 1988 and identified at 3p25-26 in 1993 (18) (19). The gene, encoding a 213 amino acid protein, is composed of 3 exons. It is widely expressed during human embryogenesis and in normal and neoplastic human tissues (20). Germline mutations in *VHL* can cause VHL disease, an autosomal dominant heritable cancer syndrome characterised by the development of multiple benign and malignant tumours (21). It has been suggested that the *VHL* gene might also be the target gene of chromosome 3p allelic loss in adenocarcinomas of the GOJ but mutation analysis has not been reported (4,14,22,23). So far *VHL* mutations were only found in sporadic renal clear cell carcinomas (24,25). Interestingly, LOH at 3p25 and 3p23 has been shown to be associated with lymph node metastases in squamous cell carcinoma of the oesophagus and colorectal cancers and poor survival in patients with colorectal carcinoma, respectively (11,13,26).

A second candidate gene on 3p25 is the human peroxisome proliferation-activated receptor gamma (PPARγ). *PPARγ*, a member of the nuclear receptor superfamily, was first discovered in 1990 and was recently mapped on chromosome 3p24.2-25 by radiation hybrid mapping (27). It is involved in adipocyte differentiation, cholesterol homeostasis, lipogenesis, thermogenesis and glucose homeostasis (28). However, it has also tumour suppressor properties: PPARγ slows cell growth and induces cell differentiation in many different cell types (29-33). Inactivation by loss of function mutations in the exons 3 and 5 of *PPARγ* are associated with human colon cancer (34).

Given the frequent loss at chromosome 3p at 3p25-26 in adenocarcinomas of the GOJ encompassing two candidate tumour suppressor genes, we deemed it of interest to perform mutation screening of the *VHL* and *PPARγ* gene in these carcinomas.

MATERIALS AND METHODS

Tumour specimens

Fresh samples of adenocarcinomas of the GOJ (*i.e.* distal oesophagus and gastric cardia) were obtained from 26 resection specimens: 26 samples were derived from the primary tumours and 2 tumour samples were taken from lymph nodes who were macroscopically (and microscopically) infiltrated by tumour. Samples of tumour and normal gastric epithelium or squamous epithelium of the oesophagus were snap-frozen and stored in liquid nitrogen.

Two *in vitro* cell lines derived from oesophageal adenocarcinomas, JROECL19 and JROECL33 were obtained from the European Collection of Cell Cultures (ECACC), and 3 cell lines (P4C, M4C and P5C) were established in our laboratory (35). Two of these cell lines *in vitro* cell lines are derived form a primary tumour (P4C) and a lymph node metastases from the same patient. *In vivo* xenografts from 3 lymph nodes metastases (M2X, M9X and M30X) and 7 primary tumour xenografts (P21X, P23X, P27X, P33X, P34X, P35X, P47X) were also included in our analysis. *In vivo* xenografts were obtained after transplantation of tumour tissue to female nude mice, 4-6 weeks of age (35).

DNA isolation

DNA from cell lines was isolated according to standard procedures. Genomic DNA from xenografts, tumour samples, and normal tissue was isolated from 5-µm cryostat tissue sections by overnight proteinase K incubation in 50 mM Tris-HCL (pH 8.0) at 56°C, followed by phenol extraction and ethanol precipitation. DNA pellets were dissolved in 100 µL of 10 mM Tris-HCL (pH 7.8) and 1 mM ethylenediaminetetraacetic acid (EDTA). Microdissection was performed to ensure that the tumour tissue consisted at least of 75% tumour cells. Normal mouse DNA was used as negative control in all experiments in which xenograft DNA was investigated.

Analysis of the *VHL* and *PPARγ* gene by PCR-SSCP

The entire open reading frame (ORF) of the *VHL* gene, including both flanking 5' and 3' untranslated regions (UTR) and exon-intron boundaries, was investigated by PCR-SSCP analysis with 9 overlapping primer pairs (Table 1). In addition, PCR-SSCP analysis of *PPARγ* was performed on exon 3 with one primer pair and exon 5 with two overlapping primer pairs, as described by Sarraf *et al.* (34). Genomic DNA fragments were PCR-amplified in a Biometra® DNA thermal cycler (Biometra, Göttingen, Germany). PCR-amplification took place in a 15 µL reaction mixture containing 1.5 mM Mg^{2+}, 0.02 mM dATP, 0.2 mM dGTP, dTTP, dCTP each, 0.8 µCi α^{32}P-dATP (Amersham, Buckinghamshire, UK), 20 pmol of each

primer and 0.2 U *Taq* polymerase (Promega, Madison, WI, USA). Mineral oil was used to overlay each PCR reaction mixture. PCR was performed for 35 cycles (denaturing at 95°C for 30s, annealing at the appropriate temperature: 55°C or 57°C for 45s (Table 1) and extension at 72°C for 1 min.). A final extension step was carried out at 72°C for 10 min. Of each PCR product, 2 µL was diluted with 8 µL loadingbuffer (95% formamide, 10 mM EDTA, pH 8.0; 0.025% bromophenol blue and 0.025% xylene cyanol). Subsequently, samples were denatured at 95°C for 5 min. and chilled on ice before appropriate aliquots of the radiolabelled PCR products were loaded on an 8% non-denaturating polyacrylamide gel containing 10% glycerol. Electrophoresis was carried out at 7W overnight at room temperature in 1 x TBE running buffer. Acetic acid (10%) was used for fixation of the gel followed by drying on blotting paper (Schleicher & Schuell, Dassel, Germany) using a vacuum gel dryer. The gel was exposed to X-ray film overnight at -70°C, using intensifying screens. PCR products of exon 5 of the *PPARγ* gene were subjected to SSCP analysis with and without prior digestion with the restriction enzyme *FokI*. This enzyme digests the 383 bp PCR product in 2 fragments of 220 bp and 163 bp.

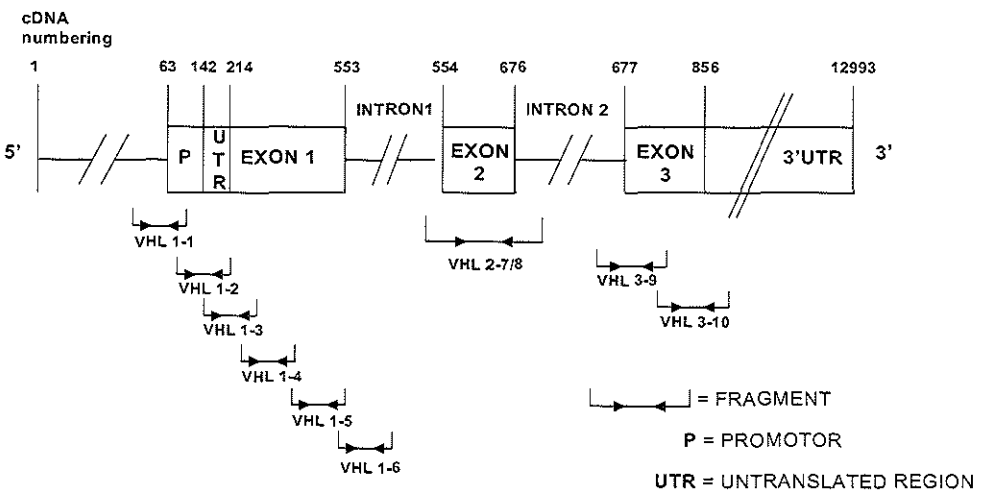
DNA samples from 3 patients with known *VHL* germline mutations served as positive controls for the SSCP analysis (36). We also included the A498 cell line, having a 4-base pair deletion in the *VHL* gene at nucleotides 639-642 (cDNA numbering) as previously reported (37).

TABLE 1. *Primers, product sizes and annealingstemperature for PCR-SSCP of VHL*

Exon	*Primers*	*SequenceProduct*	*size (bp)[a]*	*Annealings Temp. (°C)*
	VHL 1-1A	GAAATACAGTAACGAGTTGGCCTAGC		
1	VHL 1-1B	GTCGACCTCCGTAGTCTTCG	154	57°C
	VHL 1-2A	AGCGCGTTCCATCCTCTACCG		
1	VHL 1-2B	ACCTCGGCCTCGTCCCAGT	171	57°C
	VHL 1-3A	CCCGGGTGGTCTGGATCGCG		
1	VHL 1-3B	TTCCTCCGGGCCGGACTCTTC	167	57°C
	VHL 1-4A	AAGAGTACGGCCCTGAAGAAGAC		
1	VHL 1-4B	CTGCGATTGCAGAAGATGACCTG	178	57°C
	VHL 1-5A	GTGCTGCGCTCGGTGAACTC		
1	VHL 1-5B	CTCGGTAGCTGTGGATGCGG	157	57°C
	VHL 1-6A	AGCCCTACCCAACGCTGCCG		
1	VHL 1-6B	ACCCTGGATGTGTCCTGCCTCAAG	180	57°C
	VHL 2-7A	CCTTTGCTTGTCCCGATAGGTCA		
2	VHL 2-8D	CTTACCACAACAACCTTATC	187	55°C
	VHL 3-9C	GTACAGGTAGTTGTTGGCAAAG		
3	VHL 3-9B	TGGGTGGTCTTCCAGATCTTCG	201	57°C
	VHL 3-10A	ATTACAGGAGACTGGACATCGTC		
3	VHL 3-10D	TGAAACAGTGTAAGTTTCAACAG	151	57°C

[a] *bp=base pairs*

FIGURE 1. *The VHL gene structure. The relative positions of the VHL primers (VHL 1-1…VHL 3-10) on the gene are shown.*



Sequence Analysis

DNA with aberrantly migrating PCR-SSCP fragments was reamplified and purified over QIAquick spin columns (Qiagen, Hilden, Germany), cloned into the pGEM-T easy vector (Promega, Madison, WI, USA), and sequenced with α-^{35}SdATP, T7 and SP6 primers, and T7 DNA polymerase according to the dideoxy chain-termination method. Electrophoresis of the samples was carried out on an 8% denaturating polyacrylamide gel. After fixation and drying, gels were exposed to X-ray film at room temperature

LOH determination

In all tumour samples, LOH was determined with 2 microsatellite markers that map on 3p where both genes are located (8,38). Markers D3S1110 and D3S1317, were tested on 100ng of tumour and normal DNA in a PCR reaction as described previously (39). In addition, the VHL exon 1 and intron 2 polymorphisms were used for LOH determination by SSCP analysis. LOH was established by visual comparison of the intensity of allelic bands obtained from tumour samples with those of normal DNA. Furthermore, the correlation between LOH at 3p and tumour stage, tumour grade and 5-year overall survival was assessed.

Immunohistochemistry

In 42 tumours immunostaining for VHL was performed on 5-µm paraffin-embedded tissue sections by the avidin-biotin-peroxidase method. Sections were dewaxed, immersed in 10mM sodium citrate buffer (pH 6.0) and incubated at 100°C in a microwave oven for 15 min. The mouse anti-human VHL-antibody Ab-1 (Oncogene, Boston MA, USA) was applied overnight at a dilution of 1:100 in PBS and 0.1% Tween 20. Immunoreactivity was visualised with the peroxidase labelled StrAVIGen Multilink Kit (Biogenex, San Ramon CA, USA) and diaminobenzidine tetrahydrochloride (DAB, Seva, Heidelberg, Germany) and H_2O_2 as the chromogen. Human cerebellum (Purkinje cells) was used as a positive control.

Statistical analysis

Statistical analysis was performed using the SPSS package. The Chi-square or Fisher Exact tests were used to compare categorial data. Survival curves were constructed using the Kaplan-Meier Method, and compared with the log-rank test. P-values <0.05 were considered statistically significant.

RESULTS

Mutation analysis

The entire open reading frame, exon-intron boundaries and the 5' and 3' UTR of the *VHL* gene as well as exons 3 and 5 of the *PPARγ* gene were analysed for genetic alterations in 5 cell lines, 10 xenografts and 28 tumour samples including two lymph node metastases. We detected in the VHL gene two different aberrant SSCP patterns in 19 tumour samples. In all cases the matched normal DNA showed the same SSCP aberration. Upon sequencing, one pattern proved to be a previously described exon 1 polymorphism that was present in 19 paired tumour and normal tissue samples with primer combination VHL 1-1 (24) (Figure 2A). The second aberrant SSCP band shift was detected in one tumour sample with primer combination VHL 2-7/8 (Figure 2B). Upon sequencing, a basepair substitution in intron 2 of the *VHL* gene was detected (5' intron 2-gtactgatgt...ttccag-3' exon 3; deposited in NCBI/Nucleotide QUERY database libraries; accession no. AF010238). All three positive controls with an exon 1 *VHL* germline mutation showed an SSCP band shift using primer combination VHL 1-4 (Figure 3A). Two of the three patients were related, having a CGC→CCC G404 mutation and the other patient a CTG→CCG T401 mutation (Figure 3A) (36). We also detected the *VHL* exon 2 mutation in the A498 cell line using primer combination VHL 2-7/8 as an abnormal SSCP band shift (Figure 3B) (37).

PCR-SSCP analysis of exons 3 and 5 of the *PPARγ* gene did not reveal aberrant SSCP band shifts.

FIGURE 2. *A. Loss of heterozygosity (LOH) analysis by PCR-SSCP of the exon 1 polymorphism in primary tumours and xenografts with corresponding normal DNA. A non-informative case (N1) is shown, an informative case with no loss, two xenograft samples with LOH and two primary tumour samples with LOH. T=tumour, N=corresponding normal DNA. Arrowheads point to deleted alleles.* ***B.*** *LOH analysis by PCR-SSCP of the intron 2 polymorphism in xenograft P33X (T) with corresponding normal DNA (N1) and unrelated normal control DNA (N2). Arrowheads point to the deleted allele in the tumour DNA.*

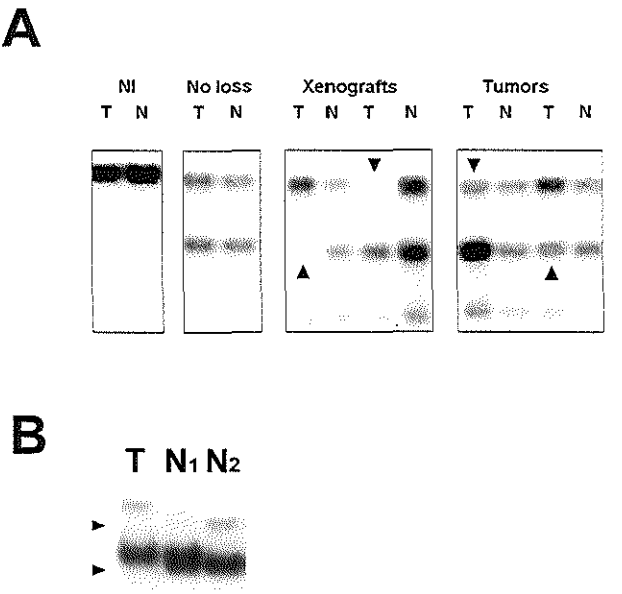
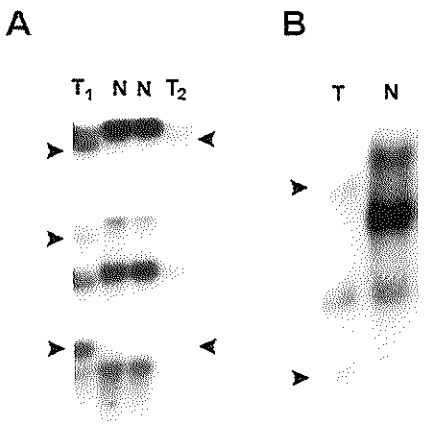


FIGURE 3. *PCR-SSCP analysis of the VHL gene. A. Shown are the aberrant migration patterns (arrowheads) of two tumour samples (T1 and T2) derived from two VHL patients with a previously described mutation in exon 1 of the VHL gene. N=corresponding normal DNA.* ***B.*** *An aberrant migration pattern (arrowheads) is shown of tumour DNA (T) derived form the A498 carcinoma cell line, harbouring a 4 base pair deletion in exon 2 of the VHL gene, versus normal control DNA (N).*



LOH determination

LOH analysis with exon 1 and intron 2 polymorphisms (Figure 2) and two microsatellite markers (D3S1110 and D3S1317) (Figure 4) revealed loss of at least one marker in 24 of 36 (67%) informative cases (Table 2). One tumour (21T) had a microsatellite instable phenotype.

FIGURE 4. *Loss of heterozygosity (LOH) analysis with marker D3S1110 in xenografts and primary tumours with corresponding normal DNA. T=tumour DNA, N=corresponding normal DNA. Arrowheads point to deleted alleles in the tumour DNA.*

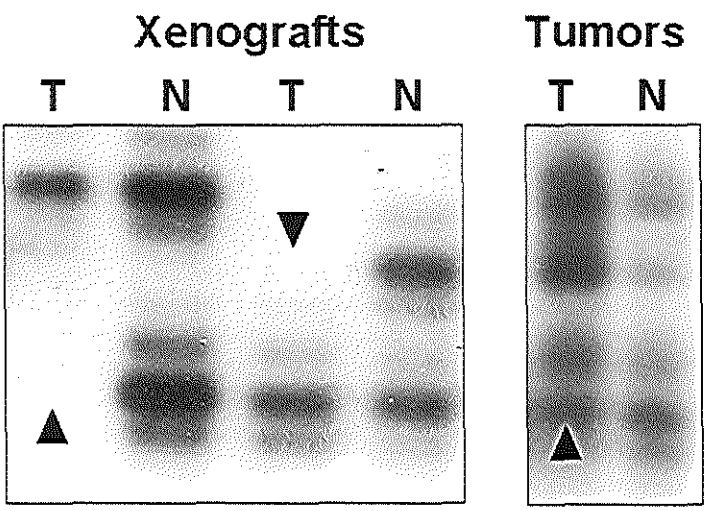
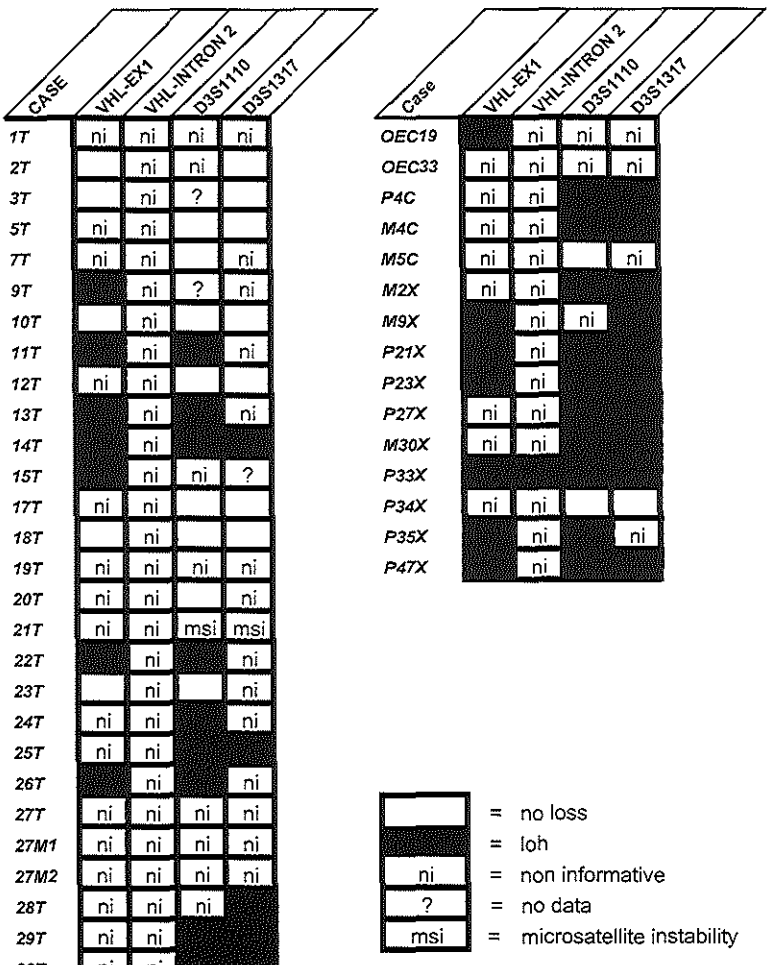


TABLE 2. *Patterns of allelic loss in adenocarcinomas of the gastro-oesophageal junction at locus 3p25-26. On the left are the primary tumor samples shown and on the right the cell lines and xenografts. Note that 27M1 and 27M2 are lymph node metastases from primary tumor 27T and P4C and M4C are cell lines derived from a primary tumor and a lymph node metastasis, respectively, from the same patient.*



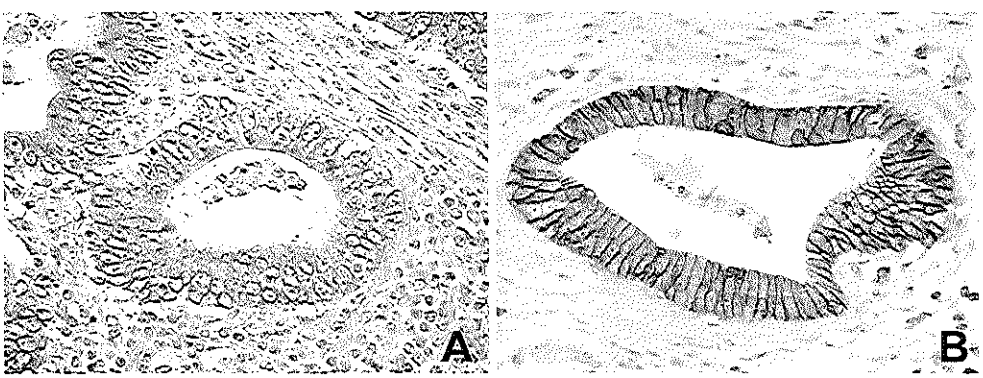
Correlation between LOH on 3p and Clinicopathological parameters

LOH at 3p25 was not significantly correlated with tumour stage, tumour grade or lymphangio-invasiveness of the tumour. However, there was a trend towards a higher frequency of lymph node metastases in tumours with LOH on 3p25: 22/25 (88%) tumours with LOH had lymph nodes involved by tumour whereas 7/12 (58%) tumours without LOH had lymph node metastases (p=0.08). The 5-years overall survival rate for patients whose tumours demonstrated LOH on 3p was 23%, whereas this was 24% for patients whose tumours had no LOH (p=0.9).

Immunohistochemistry

Diffuse cytoplasmic immunoreactivity for VHL was found in all 42 investigated tumours. The staining intensity varied from weak to very strong but the VHL expression in epithelial tumour cells was always much stronger than the expression in the surrounding stromal components (Figure 5A). There was no correlation between loss of the VHL locus and the VHL staining intensity. In 6 tumours strong membranous reactivity was observed in addition to the cytoplasmic expression (Figure 5B). Interestingly, 4 of these tumours showed no loss of the VHL locus, one tumour had LOH and one was not informative. VHL expression in the nuclei was not observed.

FIGURE 5. *Immunohistochemistry for VHL in adenocarcinoma of the gastro-oesophageal junction. Panel A shows a tumour with cytoplasmic immunostaining. Panel B shows a tumour with prominent membranous localisation of VHL.*



DISCUSSION

According to Knudson's two hit hypothesis, frequent loss of a chromosomal region in a number of comparable tumours is an indication for the presence of a tumour suppressor gene. Others and we have screened a panel of adenocarcinomas of the GOJ for chromosomal losses by comparative genomic hybridization and allelotyping (15,16,40-42). LOH at 3p is frequently and consistently reported throughout most studies. The rationale for this study was based on the study by Dolan *et al.* who reported that allelic loss was mainly seen within the VHL locus at 3p25-26 in 54% of the informative tumours (14). The other studies also showed loss of 3p but unfortunately did not specify the chromosomal region. *PPARγ* was recently shown to harbour somatic mutations in colon carcinomas (34). This gene is also located within the region of frequent LOH in adenocarcinomas of the GOJ, and therefore considered as another target tumour suppressor gene.

In our series of 43 GOJ adenocarcinoma tumour samples, only two aberrant SSCP band shifts were detected in tumour and corresponding normal DNA when the *VHL* gene was analysed. Upon sequencing these were proven to be polymorphisms. It is possible that *VHL* and *PPARγ* mutations remained undetected and thus our results underestimate the prevalence of *VHL* and *PPARγ* mutations. PCR-SSCP analysis has the advantage of being relatively rapid and therefore makes it a suitable method for mutational screening rather than completely sequencing all specimens. However, it is also known that PCR-SSCP does not identify all mutations, depending on the size of the amplicons being analysed by SSCP (43,44). Furthermore, some gene aberrations might have been missed in tumour samples due to admixture of normal stromal cells. However, tissue samples were microdissected to enrich for tumour cells. Secondly, LOH could be determined easily when tumour and normal DNA were compared suggesting that tumour samples had indeed little admixture of normal non-tumorous DNA. Moreover, 15 human carcinoma cell line and xenograft samples as a source of tumour cells without admixture of any normal human cells were also included in our analysis. All exons could be amplified with DNA derived from the xenografts and cell lines and SSCP did not reveal aberrant patterns. From this observation we can also conclude that possible homozygous deletions involving one or both genes are not likely to be present in these tumours. It still leaves the possibility that mutations are located outside the screened sequence, like in the 3' UTR gene regulatory element or within the intronic segments of *VHL*, but these have not been reported. No aberrant PCR-SSCP bands were detected for the *PPARγ* gene. We analysed only exon 3 and 5 of the *PPARγ* gene, since to date only mutations in exons 3 and 5 of *PPARγ* have been detected, greatly impairing the function of the protein.

Since we found a high frequency (67%) of allelic loss of the *VHL* and *PPARγ* gene locus at 3p25-26 without concomitant mutations of the remaining alleles, this might also point to another tumour suppressor gene on 3p involved in the genesis or

progression of adenocarcinomas of the GOJ. In this respect Clifford *et al.* (1998) made an interesting observation (6). They found that in renal cell carcinomas without *VHL* mutation the frequency of 3p12-21 LOH (93%) was significantly higher than the 3p25-26 LOH (43%). Since we do not have LOH data outside the 3p25-26 region we can not exclude other 3p tumour suppressor genes as the target of 3p loss. Tumour suppressor genes on 3p might include *β-catenin* on 3p22, *FHIT* on 3p14, *hMLH1* on 3p21 and the genetic locus NRC-1 at 3p12. LOH and homozygous deletion at the *β-catenin* locus have been observed in human malignancies (45,46). β-Catenin expression is altered in dysplastic Barrett's epithelium and adenocarcinomas (47). However, besides its tumour suppressive effect in establishing cell-cell adhesion (as a cytoplasmic partner of E-cadherin), the protein is a proto-oncogene involved in cell signalling (48). Activating mutations in *β-catenin* have been reported rather than inactivating mutations and LOH (49,50). The tumour suppressor gene *FHIT* is an interesting candidate for involvement in GOJ carcinogenesis since frequent deletions of this gene were reported in Barrett's oesophagus and adenocarcinoma (51). The mismatch repair protein hMLH1 plays a role in the control of replication fidelity. Tumours with inactivation of this protein are characterised by microsatellite instability (MSI). In the present study only one of 43 tumours showed a MSI phenotype which is in accordance with previous results (52). Therefore, it is unlikely that *hMLH1* is inactivated in GOJ tumours. Lovell *et al.* described a genetic locus on 3p12, designated NRC-1, which mediates tumour suppression (53). Data on involvement of NRC-1 in GOJ adenocarcinomas are lacking and the candidate gene still has to be identified. Alternatively, if LOH of 3p truly reflects loss of one VHL and PPARγ allele, this could point towards a gene dosage effect. Striking examples of the effect of gene dosage are the monoallelic *RET* mutations in Hirschsprung disease and monoallelic *PTEN* germline mutations in Cowden syndrome (34). Furthermore, epigenetic mechanisms, such as hypermethylation within the promotor region preventing gene transcription and leading to a reduction in VHL and PPARγ protein needs to be considered as well (34,54). However, all tumours showed normal levels of cytoplasmic VHL expression as has been reported in several other human carcinomas (20,55). Sakashita *et al.* reported also the expression of VHL in tumour cell nuclei (55). In our series no nuclear reactivity was found. In 6 tumours membranous VHL reactivity was found in addition to cytoplasmic expression. At least four of these tumours had retention of both VHL alleles and only one tumour had LOH at the VHL locus. The sixth tumour was homozygous for all tested markers. Membranous VHL expression has not been reported by others and its significance remains unclear.

In conclusion, these results show that *VHL* and *PPARγ* gene mutations do not appear to play a role in the tumourigenesis of adenocarcinomas of the GOJ, despite the high prevalence of LOH at 3p25-26. Because normal expression of the VHL protein is found in these tumours the target for LOH of 3p25-26 is most likely another gene. However, a gene dosage effect or epigenetic phenomena leading to

inactivation of *VHL* or *PPARγ* can not be ruled out. These possibilities warrant further investigation.

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CHAPTER 12

IDENTIFICATION OF A 8.6CM MINIMAL DELETION AT 14Q31.1-32.11 IN ADENOCARCINOMAS OF THE GASTRO-OESOPHAGEAL JUNCTION

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Submitted

PRÉCIS

Deletion of chromosome 14 at 14q31.1-32.11 occurs frequently, and implicates a putative tumour suppressor gene involved in tumourigenesis and progression of adenocarcinomas of the gastro-oesophageal junction.

INTRODUCTION

Adenocarcinomas arising from the lower oesophagus or the gastric cardia are commonly referred to as adenocarcinoma of the gastro-oesophageal junction (GOJ). As such, they can be regarded as a single entity (1,2). These tumours have shown a rapid increase in incidence over the past decades (2,3). The most important risk factor is gastro-oesophageal reflux leading to the replacement of the normal stratified squamous epithelium by columnar epithelium. This condition is known as Barrett's oesophagus. The prognosis for patients with adenocarcinoma of the GOJ is poor. Five-year survival after surgery with curative intent is approximately 20-25% (4,5). Little is known about the genetic alterations involved in the progression of Barrett's epithelium towards dysplasia and invasive adenocarcinoma. Recently, others and we identified chromosomal aberrations in these tumours by comparative genomic hybridisation (CGH) (6-8). Loss of chromosome 14q31-32.1 was detected in a significant number of cases, suggesting the presence of a tumour suppressor gene in that region (6). Deletion of part of chromosome 14 has also been reported in other malignancies such as colorectal carcinoma, adenocarcinoma of the stomach, gastrointestinal tract stromal cell tumours, ovarian cancer, bladder cancer, head and neck carcinoma, renal cell carcinoma and renal oncocytomas, malignant mesothelioma, neuroblastoma and meningioma (9-18). The goal of the present study was to define the common region of chromosome 14q loss in GOJ adenocarcinoma in more detail by LOH analysis using a set of polymorphic microsatellite markers. Thirty-seven samples comprised of 17 primary tumours and one dysplastic epithelium sample, 15 xenografts from 10 primary tumours and 5 lymph node metastases as well as 5 *in vitro* GOJ adenocarcinoma cell lines were used. The xenografts and cell lines facilitate detection of homozygous deletions.

MATERIALS AND METHODS

Patients

We analysed 37 tissue samples from 36 patients. All patients were diagnosed with adenocarcinoma of the gastro-oesophageal junction, *i.e.* distal oesophagus or the gastric cardia, and underwent transhiatal resection of the oesophagus and the proximal stomach with curative intent as described (1).

Tumour samples: frozen tumour tissue, cell lines and xenografts

The tissue samples were obtained from the resection specimens. Immediately after surgery small pieces of tumour, dysplasia and adjacent normal mucosa were taken, snap frozen and stored in liquid nitrogen until further analysis. Specimens included 17 primary tumours and one dysplastic epithelium sample adjacent to an adenocarcinoma, 10 primary tumour xenografts, 5 xenografts from lymph node metastases and 5 *in vitro* cell lines. Two cell lines, JROECL 19 and 33, established by Rockett *et al.* were obtained from the European Collection of Cell Cultures

(ECACC) and 3 cell lines (P4C, M4C and M5C) were established in our laboratory (19,20). Two of these *in vitro* cell lines are derived from a primary tumour (P4C) and a lymph node metastasis (M4C) from the same patient.

DNA extraction

DNA from cell lines was isolated according to standard procedures. Genomic DNA from xenografts and tumour samples was isolated from consecutive 5-µm cryostat tissue sections by overnight proteinase K digestion at 55°C followed by phenol extraction and ethanol precipitation. DNA pellets were dissolved in TE (10 mM Tris/HCl pH 7.8; 1mM EDTA). The tumour and dysplastic tissue samples contained at least 75% neoplastic cells.

Microsatellite marker selection

Chromosome 14q polymorphic microsatellite markers with a high degree of heterozygosity were selected from the Généthon human genetic linkage map (21). We used the primers from the database for marker amplification, except for the markers D14S67 and CCC1. D14S67 was amplified with primers A: CTACGCCTCTACAATTCT and B: GTAGTCAGGGTTTGCCAG and with primers X: GAGCCAATTTCTTAAAGTAAATC and Y: AGAAAACAGAACCAACAGGG. For marker CCC1 we used the primers: GCCATAAGCCTGAAGATTGG and GGCAGTTAAGAAGACACAGC. Additional information on the relative position of all the used markers on chromosome 14q was obtained from the genome database Ensembl from the Sanger Centre.

PCR amplification

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

Electrophoresis and interpretation of loss of heterozygosity

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

The signals derived from tumour and corresponding normal DNA were compared by visual inspection, independently performed by three investigators (BPLW, DAD and WNMD). DNA samples with controversial results were reamplified and in a number of cases we isolated and amplified DNA from a seperate part of the tumour. A case was classified as informative and having undergone allelic loss (LOH – loss of heterozygosity), informative and no LOH, not informative, or as having undergone allelic shift (microsatellite instability).

RESULTS

Thirty-seven tumours and one dysplastic Barrett's epithelium sample with corresponding normal DNA were analysed for LOH using 22 polymorphic microsatellite markers covering chromosome 14q. The relevant results are summarised in Figure 1. No LOH was found in 20 tumours and 2 tumours showed widespread microsatellite instability (MSI). LOH for at least one marker was observed in 15 of 37 tumours (41%). Eight tumours (P4C, M4C, Ba7, Ba19, M53X1, Ba32, P35X1 and Ba23) showed loss of all informative markers for the entire region 14q11-qter. In 7 tumours both loss and retention of heterozygosity was seen at informative markers. These patterns were used for defining the minimal region of overlapping deletion (MRO). With all xenograft and cell line DNA samples PCR products were obtained with all markers.
We defined a region of loss which was proximally (*i.e.* towards the centromere) bounded by marker D14S983 and distally (towards the telomere) by marker CCC1, the genetic size being approximately 8.6 cM. Informative LOH results are shown in Figure 2. Tumour Ba10 and the dysplasia from the same patient (Ba10d) were critical for defining the proximal boundary, whereas tumour Ba34 defined the distal boundary. This region has been cytogenetically mapped in band 14q31.1-32.11 and it was deleted in all tumours that showed loss of 14 q markers. Deletion of D14S1058 and D14S68 was found in all informative tumours with 14q loss. These markers are located at 95.89cM as is marker D14S67. Interestingly, tumour Ba27 had retention of marker D14S67 and LOH of all the other informative markers (Figures 1 and 2). There was no correlation between LOH on 14q and the location of the tumour (oesophagus or gastric cardia). GOJ tumours with 14q loss tended to have a higher tumour stage and more frequently distant metastases as compared to the tumours without 14q loss (Chi-Square test $p=0.13$ and $p=0.076$, respectively).

FIGURE 1. *All tumours with loss of 14q markers are shown. P4C and M4C are in vitro cell lines from a primary tumour and a metastasis, respectively, from the same patient. P35X1 is a xenograft from a primary tumour and M30X1, M53X1 and M55X1 are xenografts from metastases. The Ba numbers are primary tumours and Ba10d is the dysplastic epithelium sample belonging to primary tumour Ba10. Marker CCC1 is located between D14S67 and D14S977. The solid bar indicates the minimal region of overlapping (MRO) LOH.*

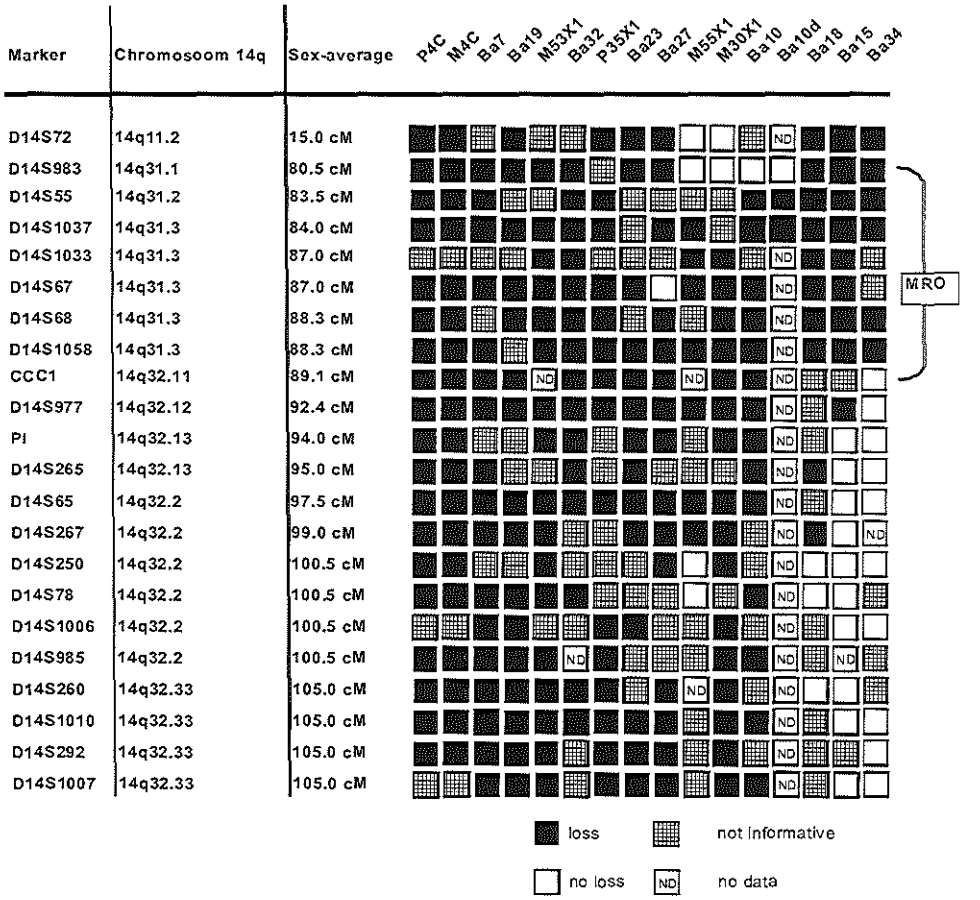
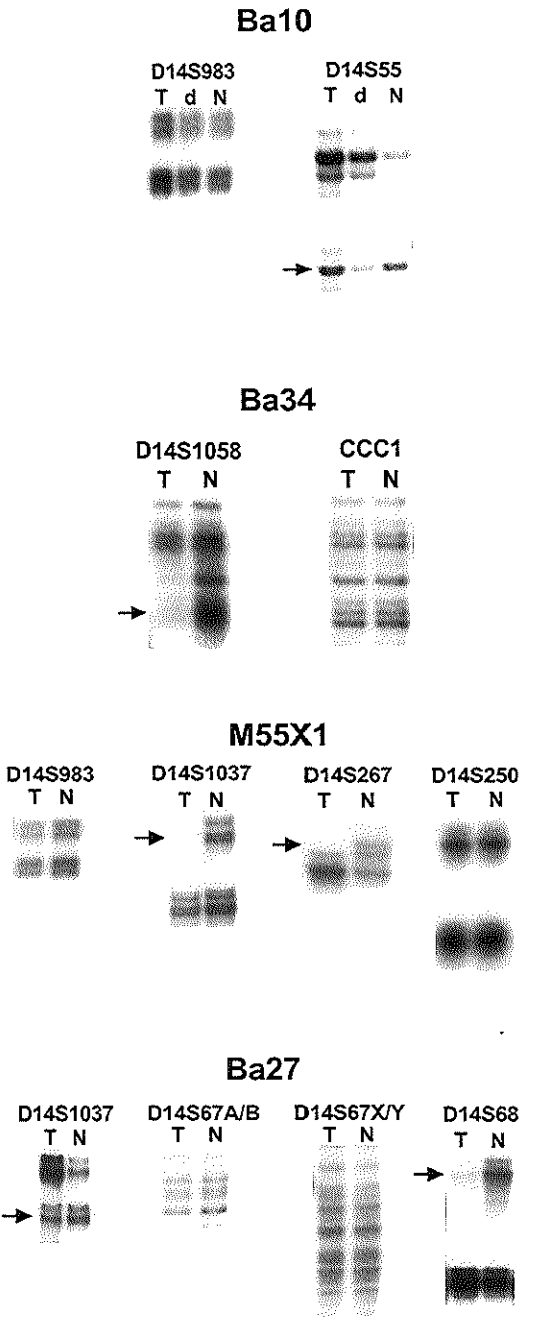


FIGURE 2. *Autoradiograms from 4 representative tumours (Ba10, Ba34, M55X1 and Ba27) showing allelic band patterns from critical markers on 14q. Arrows indicate allele loss. T=tumour DNA, d=Barret's dysplasia, N=normal DNA.*



DISCUSSION

In this study we confirmed our previous finding that allelic loss on chromosome 14q occurs in about half of all GOJ adenocarcinomas (6). This strongly suggests that this region contains one or more tumour suppressor genes involved in tumourigenesis. Furthermore, we identified the commonly deleted region of allelic loss. This is located at cytogenetic band 14q31.1-32.11 between marker D14S1983 and marker CCC1, with a genetic size of about 8.6 cM.

In tumour Ba27 the entire long arm showed loss of heterozygosity, except for a clear-cut case of retention at marker D14S67. This can be due either to retention of both D14S67 alleles in the tumour or to the presence of a homozygous deletion. In the latter case the PCR product obtained with marker D14S67 is then derived from the normal DNA present in the tumour sample. To rule out the possibility of a mutation in the primer region of the tumour DNA, the assay was repeated with a second set of D14S67 primers (X/Y). Also with these primers retention of both alleles in tumour Ba27 was evident (Figure 2). Whether the retention of both D14S67 alleles reflects a homozygous deletion is under investigation. In the xenografts and cell lines we did not detect homozygous deletions, since with all markers tested PCR products were obtained.

Genome-wide allelic loss analysis in colorectal carcinomas reported an overall mean LOH frequency of about 20% (22,23). The finding of more than 40% 14q allelic loss in adenocarcinomas of the GOJ points to possible involvement of this region in carcinogenesis of these tumours. Furthermore, loss of 14q32 has been reported in several other carcinomas like colorectal carcinoma (9,23,24), adenocarcinoma of the stomach (10,25) ovarian carcinoma (14), bladder carcinoma (18), head and neck (16), renal cell carcinomas and renal oncocytomas (15,17). Loss of 14q was reported to correlate with early age of onset, advanced stage and poor outcome of the disease (16-18,23,24). In our series we also found a trend that loss of 14q is more common in advanced stage tumours and in tumours with distant metastases. These findings indicate that inactivation of the putative 14q31-32 tumour suppressor gene is involved in disease progression. Furthermore, in concordance with this is our finding of no 14q loss (with markers D14S67, CCC1 and D14S68) in 11 Barrett's dysplasia samples, including 6 high grades (data not shown). However, the 14q loss found in tumour Ba10 was also detected in the tumour-adjacent dysplastic Barrett's epithelium (Ba10d, Figure 1 and 2).

To our knowledge no clear candidate 14q32 tumour suppressor genes have been suggested to date. Genes at the 14q32 region with possible tumour suppressor activity are the thyroid stimulating hormone receptor (*TSHR*), glutathione transferase zeta1 (*GSTZ1*) and the checkpoint suppressor 1 (*CHES1*) gene. Activating and inactivating mutations in the *TSHR* gene have been described and Gustavsson *et al.* reported decreased growth rate and inhibition of tumour formation after transfection of thyroid carcinoma cells with a wild type *TSHR* cDNA (26). GSTZ1 is an enzyme important in the detoxification of electrophilic molecules and has direct antioxidant activity. Deficiency of this gene can lead to the

accumulation of toxic compounds resulting in DNA damage (27). The *CHES1* gene suppresses a number of DNA damage-activated checkpoint mutations in yeast (28). Inactivation of CHES1 facilitates cell cycle progression in the presence of DNA damage. The used CCC1 polymorphic marker is located in the 3' UTR of the CHES1 gene and shows loss in 10 of 11 (91%) informative cases. The relevance of these genes in GOJ adenocarcinogenesis has to be determined.

Finally, in our previous CGH study we found that 14q loss occurred significantly more frequent in oesophageal adenocarcinomas than in gastric cardia tumours. In the present study this relationship was not confirmed. Also in a larger CGH study the significant relation was lost between the location of the tumour and loss of 14q (van Dekken, unpublished results). Besides phenotypical similarities, adenocarcinomas of the oesophagus and gastric cardia apparently also have similar genotype, as suggested by others (29,30).

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

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PART VI

SUMMARY, CONCLUSIONS AND EPILOGUE

CHAPTER 13

SUMMARY AND CONCLUSIONS

Worldwide, adenocarcinomas of the gastro-oesophageal junction, *i.e.* distal oesophagus and gastric cardia, show an increase in incidence over the past three decades. This is worrysome, since these carcinomas have a poor prognosis, with 5-year survival rates of about 25% after surgery with curative intent. Reflux of duodenal and gastric contents into the oesophagus leads to oesophagitis and ultimately premalignant metaplastic epithelium, also known as Barrett's oesophagus, can develop in a subset of patients. Adenocarcinomas of the oesophagus as well as gastric cardia are thought to arise from this precursor lesion through a multistep of malignant changes. The studies described in this thesis intend to provide a better insight in the epidemiological, clinical and genetic alterations of adenocarcinomas of the gastro-oesophageal junction.

CHAPTER 1 presents the state of art of the genetic alterations of Barrett's oesophagus and adenocarcinoma. Despite ongoing efforts to characterise the molecular changes in Barrett's oesophagus, its pathogenesis remains poorly understood. A wide variety of genetic events and mechanisms appear to play a role in the development and progression of Barrett's oesophagus-associated neoplastic lesions. A surprising degree of clonal heterogeneity in premalignant Barrett's epithelium has been found consistent with a complex pattern of evolution of neoplastic cell lineage's rather than a simple linear and uniform pathway of molecular progression. This is in contrast with colorectal carcinoma, where Vogelstein and others have proposed a model of genetic evolution. However, it has become clear that inactivation of tumour suppressor genes p53 and p16 (mutations and/or LOH) and cell cycle abnormalities or aneuploidy appear to be among the most important and well characterised molecular changes in the progression of Barrett's oesophagus to adenocarcinoma.

In the past decades, there has been increasing interest in a large family of transmembrane glycoproteins called cadherins, which are the prime mediators of calcium-dependent, cell-to-cell adhesion in normal cells. **CHAPTER 2** reviews the current literature about the E-cadherin-catenin complex in cancer. Loss of E-cadherin-mediated intercellular adhesion seems to be an important contributory factor in tumour pathogenesis. It is associated with loss of cell-contact inhibition of proliferation, thus enabling escape from growth-controlling signals. Loss of adhesion may also act by promoting tumour cell detachment from the primary site, so that malignant cells can spread to distant organs. Indeed, immunohistochemical studies in human cancers, including adenocarcinomas of the gastro-oesophageal junction, have frequently shown that a proportion of invasive carcinomas and *in situ* carcinomas show aberrant levels of E-cadherin and/or catenin expression in comparison to their related normal tissue. Expression of E-cadherin-catenin correlates with pathological characteristics of the tumour (tumour grade, depth of infiltration, venous permeation, lymph node involvement) and clinical variables such as disease relapse, disease-free survival and overall survival. Therefore, knowledge of the level of expression of the E-cadherin-catenin complex might serve as an additional prognostic marker in human carcinomas.

Besides its role in cell-cell adhesion, β-catenin interacts with other cellular proteins like the adenomatous polyposis coli (APC) protein. APC aids in the breakdown of free, unbound β-catenin that is not linked to E-cadherin. Elevated levels of free β-catenin due to decreased breakdown of β-catenin, results into a translocation of β-catenin into the nucleus of the cell. There it binds to transcription factors and, as a consequence, transcription of certain genes involved in promoting cell proliferation and migration is initiated. This suggests its dual role as a tumour suppressor (cell-cell adhesion when captured by E-cadherin-catenin complex) and as an oncogene (its role in signal transduction). Finally, this chapter reviews the possible mechanisms responsible for downregulation of this cell-cell adhesion complex: mutations, loss of heterozygosity as well as epigenetic mechanisms (promotor silencing through methylation) are all discussed.
From chapter 2 it is concluded that further elucidation of the mechanisms underlying the changes in E-cadherin and catenin function may lead to the development of novel therapeutic approaches based on biochemical and genetic manipulation.

PART II of this thesis deals with epidemiological and clinical aspects of adenocarcinomas of the gastro-oesophageal junction. In **CHAPTER 3**, we studied time trends in mortality and incidence rates of oesophageal and gastric carcinomas according to subsite and histology in the Southeast of the Netherlands since 1978. In males, the incidence of adenocarcinomas of the oesophagus and gastric cardia increased. In females, the increase in incidence of squamous cell carcinoma of the oesophagus appeared to be more marked than the rise in adenocarcinomas, whereas the incidence of gastric cardia carcinomas remained stable in the last 10 years. When incidence rates for adenocarcinomas of the oesophagus and gastric cardia were combined (defined here as adenocarcinomas of the gastro-oesophageal junction) for analysis of time trend, an increase in incidence for both sexes was observed over the study period (1978-1996): in males the incidence rose from 3.9 in 1978 to 6.7 per 100.000 person-years in 1996, and in females from 0.2 to 1.4 per 100.000 person-years. As discussed in chapter 3, several pitfalls may bias the observed trends in incidence rates for oesophageal and gastric cancer. But our data do tell us that there should be little doubt that the incidence of adenocarcinomas of the gastro-oesophageal junction, *i.e.* distal oesophagus and gastric cardia combined, has been rising in the 90's at least in males (and to a lesser extent in females) in the Southeast of the Netherlands.

In practise, it is often difficult to decide whether a tumour has originated from the gastric cardia or in the distal oesophagus. Furthermore, several studies suggest common risk factors and a similar pheno- and genotype for adenocarcinomas arising from the distal oesophagus and gastric cardia. However, current staging citeria, established by the Union Internationale Contre le Cancer (UICC) and the American Joint Committee on Cancer (AJCC), use separate classifications: cancer of the cardia is classified as gastric cancer and carcinoma of the (distal) oesophagus

is classified as oesophageal cancer. Therefore, in **CHAPTER 4** the question is addressed whether these tumours should be regarded and staged as one clinical entity. Pathology, pathological tumour node metastasis (pTNM) stage and 5-years survival data are presented on 252 patients who all underwent surgery for adenocarcinoma of the oesophagus (n=111) or gastric cardia (n=141). Macroscopic and microscopic characteristics of both groups were similar. Only Barrett's epithelium was seen more often surrounding oesophageal tumours than cardiac tumours. Although cardia carcinomas were more likely to be found at a more advanced T-stage (*i.e.* wall infiltration), the percentage of tumours with loco-regional lymph node involvement was similar (approximately 60%). Apparently, oesophageal carcinomas are detected at an earlier stage. The 5-years overall survival was 26% for oesophageal adenocarcinomas and 27% for gastric cardia carcinomas. When survival probability was analysed according to tumour stage, also no difference was found between both groups. In summary, this study supports the hypothesis that adenocarcinoma of the distal oesophagus and gastric cardia are one clinical entity and that both carcinomas should be staged according to uniform staging criteria On multivariate analysis, age of the patient, infiltration of the tumour (pT-classification), nodal involvement (pN-classification), distant metastasis (pM-classification), tumour grade (pG-classification) and residual tumour classification (pR) were all independent variables predicting survival.

As already discussed in chapter 1, still little is known about the cellular and molecular mechanisms that underpin the origin of adenocarcinomas of the gastro-oesophageal junction. This partly because of a lack of sufficient *in vivo* and *in vitro* model systems. **PART III** describes the establishment and characterisation of cell lines derived from adenocarcinomas of the oesophagus and gastric cardia. **CHAPTER 5** describes our experience with the establishment of *in vivo* and *in vitro* cell lines, which we started in 1997. Tumour samples derived from adenocarcinomas of the oesophagus and gastric cardia as well as their lymph node metastasis were xenografted subcutaneously to immunodeficient nude mice. In addition tumour tissue was also used for *in vitro* culture. Xenografting of 70 primary tumour samples and 17 metastasis resulted in initial growth of 22 and 6 tumours respectively (32%). Upon retransplantation, 6 long-term xenografts from primary tumours and 3 from metastasis were obtained. In vitro culture attempts of 34 primary tumours and 9 metastasis resulted in the establishment of 3 (7%) permanent *in vitro* growing cell lines. From one patient, a cell line of the primary tumour as well as the lymph node metastasis was established. The *in vivo* and *in vitro* cell lines were characterised by immunohistochemistry, microsatellite analysis and by counting the chromosome numbers in metaphase spreads to verify their epithelial and human tumour origin, respectively. This study shows that cell lines derived from adenocarcinomas of the gastro-oesophageal junction growing *in vivo* and *in vitro* can be successfully established and resemble histologically, immunohistochemically and genetically the tumours from which they originated.

These xenografts and cell lines are a valuable source of homogeneous tumour tissue for further investigations.

Recently, 5 human oesophageal and gastric cardia carcinoma cell lines were established and characterised in the United Kingdom. These cell lines were included in two of our studies on E-cadherin and β-catenin mutations in adenocarcinomas of the oesophagus (chapters 7 and 8 of this thesis). In two cell lines (JROECL 47 and 50) that are derived from one primary tumour, we detected an E-cadherin and a β-catenin mutation. These mutations could not be detected in the primary oesophageal tumour from which the cell lines were presumably established. Recently, identical E-cadherin and β-catenin mutations were described in colon cancer cell line HCT 116, established in 1981. This prompted us to investigate the derivation of the cell lines JROECL 47 and 50 by HLA typing, mutation analysis and microsatellite instability analysis, as described in **CHAPTER 6**. When the cell lines JROECL 47 and HCT 116 were xenografted on nude mice, this resulted in undifferentiated solid tumours, without glandular differentiation. *In vitro*, the morphology was also similar, with spindle shaped cells and similar growth rates. HLA typing revealed that cell lines JROECL 47 and HCT 116 all had the same HLA-DR allele which was different from the original primary oesophageal tumour from which the cell lines JROECL 47 and 50 were presumably established. Mutation analysis showed the same E-cadherin, β-catenin and k-ras mutations in the three cell lines. However, allelotyping showed different allele sizes between the three cell lines with 7 of 9 polymorphic markers, indicating a different origin of the cell lines. But all three cell lines had an extreme microsatellite instable phenotype. Obviously, separate cultures of HCT 116 resulted in different microsatellite alterations. Our assumption that cell lines JROECL 47 and 50 are admixtures of HCT 116 was confirmed by the European Cell Culture Tissue Bank (EACC). The conclusions that are drawn from this chapter are: (1) always check the derivation of cell lines since there is always a risk of contamination with other cell lines; (2) JROECL 47 and 50 are in fact admixtures of the human colon adenocarcinoma cell line HCT 116; (3) allelotyping of cell lines by microsatellite analysis is not appropriate for microsatellite instable cell lines.

In **PART IV** of this thesis the E-cadherin-catenin cell-cell adhesion complex in adenocarcinomas of the gastro-oesophageal junction is studied.
In **CHAPTER 7**, the hypothesis has been tested that genetic alterations in the E-cadherin gene play a role in the pathogenesis of adenocarcinomas of the oesophagus and that these can be held responsible for the reduced expression of E-cadherin by immunohistochemistry. All 16 exons of the E-cadherin gene were screened for mutations with the polymerase chain reaction single strand conformation polymorphism analysis (PCR-SSCP) technique in 57 tumour specimens, including 4 established cell lines and 4 xenografts. In two cell lines derived from the same patient, we detected a one base-pair deletion leading to a

premature stop codon. No other mutations were detected. Allelic loss (LOH) at the E-cadherin gene locus at 16q22.1 was also studied. LOH was found in 65% of the tumours. It is concluded that E-cadherin gene mutations are not involved in the subsequent progression of Barrett's epithelium to dysplasia and to adenocarcinoma of the oesophagus. The high percentage of 16q22.1 LOH without concomitant mutation of the remaining E-cadherin gene might point towards another tumour suppressor gene on 16q involved in the pathogenesis of these carcinomas. Alternatively, the high frequency of LOH at the E-cadherin locus could point to a gene dosage effect. Whether LOH then contributes to the reduced expression of E-cadherin remains to be determined.

As reviewed in chapter 2, β-catenin has recently also been identified as an oncogene. It plays a central role in the Wingless/Wnt (Wg/Wnt) signalling pathway. Phosphorylation of sites encoded by exon 3 of the β-catenin gene facilitates degradation of this protein by the APC gene product. Mutations in these sites or mutations in the APC gene lead to stabilisation of β-catenin, which then translocates to the nucleus where it modulates the transcription of genes involved in tumour formation. To explore the role of β-catenin mutations in adenocarcinomas of the oesophagus, we screened for genetic alterations in exon 3 in 69 tumour samples (**CHAPTER 8**). Neither mutations in exon 3 could be detected by PCR-SSCP analysis, nor did we find large interstitial deletions involving exon 3. In three selected tumours with strong homogenous nuclear staining for β-catenin, indicating activation of the Wg/Wnt signalling pathway, the APC mutation cluster region was also screened for mutations, but without success. Apparently, the abnormal cytoplasmic and nuclear localisation of β-catenin indicates that other mechanisms leading to elevated free β-catenin in these cancers must be involved.

A recently identified new member of the E-cadherin-catenin complex is p120-catenin ($p120^{ctn}$). Its role in malignancy is not clear yet. In **CHAPTER 9** the *in vivo* expression and cellular localisation of $p120^{ctn}$ is studied in 96 adenocarcinomas of the gastro-oesophageal junction relative to the normal mucosa. In addition, we evaluated the relationship between expression of $p120^{ctn}$ and clinicopathological features in order to examine the application of $p120^{ctn}$ as a prognostic marker. Immunohistochemical staining for $p120^{ctn}$ showed loss of the normal membranous pattern in 4/20 (20%) of specimens with Barrett's epithelium, in 65/96 (68%) primary tumours and in 11/13 (85%) lymph node metastasis. Reduced membranous expression of $p120^{ctn}$ in the primary tumours was significant associated with poor tumour grade and poor 5-year survival. The 5-year survival rate of patients with normal $p120^{ctn}$ expression was 54% compared to 12% for patients with reduced expression of $p120^{ctn}$. In the subgroup of patients with tumours classified as N0 by routine histopathological methods, the 5-year survival rate for patients with normal $p120^{ctn}$ expression of the tumour was 63% *versus* 20% for patients with reduced expression ($p=0.02$). Therefore, $p120^{ctn}$ could be useful in identifying patients at risk for poor survival and those patients with clinically negative lymph nodes who are at risk for occult metastasis. Of particular interest is

our observation of focal nuclear localisation of $p120^{ctn}$ in some tumours. This suggests that $p120^{ctn}$ might also be involved in signal transduction, as has been shown for β-catenin. This exciting finding warrants further investigation.
In **CHAPTER 10** the role of the E-cadherin-catenin complex in adenocarcinomas of the gastro-oesophageal junction was further elucidated. The spatial distribution of the proteins between the cell membrane and cytoplasm and nucleus was assessed using immunohistochemistry. E-cadherin and the catenins are known to be present in the cell in different pools: membrane bound, free cytoplasmic and cytoskeleton bound. The expression of E-cadherin and the catenins and their biochemical distribution between the Triton X-100 soluble (membrane bound) en Triton X-100 insoluble (cytoskeleton bound) fraction was assessed in tumour tissue *versus* the normal mucosa using fractional protein extraction and western blot analysis. Immunohistochemistry demonstrated redistribution of E-cadherin and the catenins from the membrane to the cytoplasm in 50-75% of the tumours. Five tumours showed nuclear expression of β-catenin. Western blotting showed a redistribution between the Triton X-100 soluble and insoluble fraction of E-cadherin and the catenins in 45-60% of the tumours in comparison to the distribution of the proteins in the normal mucosa. In conclusion: perturbation in the expression, distribution and hence function of the members of the E-cadherin-catenin complex is frequently seen in adenocarcinomas of the gastro-oesophageal junction. Immunohistochemistry as well as Western blotting of the soluble and insoluble fraction of the E-cadherin-catenin complex are both valuable techniques in detecting an abnormal E-cadherin-catenin complex. However, since there was no correlation between both techniques in detecting an abnormal complex, they even might have additional value.

PART V describes the genetic analysis of tumour suppressor genes in adenocarcinomas of the gastro-oesophageal junction. Tumour suppressor genes are cellular genes, which primarily are involved in cell proliferation, apoptosis, cell adhesion and gene expression regulation. In a normal cell, these genes inhibit cell growth and stimulate differentiation. In carcinomas, their inhibitory function is lost due to inactivation of both gene copies (alleles). Microsatellite allelotyping or loss of heterozygosity (LOH) analysis is a useful technique to define chromosomal regions of deletion in carcinomas. Frequent loss of one allele involving a chromosomal arm or locus suggests the presence, at or near that locus, of a tumour suppressor gene. **CHAPTER 11** focuses on the short arm of chromosome 3 (3p25.2). Candidate genes within this region are the VHL (von Hippel-Lindau) gene and the PPARγ (Human Peroxisome Proliferator-Activated Receptor gamma) gene. Mutational screening (PCR-SSCP) and LOH analysis of the 3p region was studied in 44 tumour samples from 41 patients in order to disclose the possible role of these two tumour suppressor genes in adenocarcinomas of the gastro-oesophageal junction. Despite the high percentage of LOH of 3p25 (63%), no mutations were detected in both genes. Epigenetic phenomena, mutations outside

the screened sequences, gene dosage effect or involvement of another tumour suppressor gene on 3p should be considered.
In **CHAPTER 12** evidence is presented for the existence of a candidate tumour suppressor gene on chromosome 14q at 14q31.1-32.11 involved in tumourigenesis of adenocarcinomas of the gastro-oesophageal junction. Thirty-seven paired tumour/normal tissue DNA samples derived from 36 patients (including 15 xenografts and 5 cell lines) were used to screen for allelic loss on 14q31.1-32.11 with 22 polymorphic microsatellite markers. Fifteen of the 37 (41%) samples clearly demonstrated LOH for at least one marker. No homozygous deletions were detected in the cell lines or xenografts. The minimal region of (uninterrupted) overlapping loss with a size of 8.6 cM is between markers D14S983 and CCC1 at cyogenetic location 14q31.1-32.11. LOH of 14q31.1-32.11 is probably a late event in tumourigenesis of adenocarcinomas of the gastro-oesophageal junction: (1) 11 DNA samples of dysplastic Barrett's epithelium did not show LOH; (2) tumours with 14q loss tended to have a higher tumour stage and more frequently lymph node metastases. However, from one tumour also corresponding dysplastic Barrett's epithelium was available for LOH analysis, and LOH at 14q32.1 was also present in this pre-neoplastic stage. Genes at the 14q31.1-32.11 region with possible tumour suppressor activity are the thyroid stimulating hormone receptor (TSHR1), glutathione transferase zeta 1 (GSTZ1) and the checkpoint suppressor 1 (CHES1) gene. However, the relevance of these genes in tumourigenesis of adenocarcinomas of the gastro-oesophageal junction has yet to be determined.

CONCLUSIONS

- The incidence of adenocarcinomas of the gastro-oesophageal junction, *i.e.* distal oesophagus and gastric cardia combined, has been rising in the 90's at least in males, and to a lesser extent in females, in the Southeast of the Netherlands (chapter 3).

- Adenocarcinomas of the distal oesophagus and gastric cardia are one clinical entity and should be staged according to uniform criteria (chapter 4)

- The following factors have independent predictive value in assessing survival probability in patients with adenocarcinoma of the gastro-oesophageal junction: age of the patient, depth of wall infiltration (pT-classification), locoregional and distant lymph node involvement (pN and pM-classifications), tumour grade (pG), residual tumour classification (radicality of the operation; pR) and p120-catenin expression (chapters 4 and 9).

- There is no uniform molecular pathway of progression from Barrett's oesophagus to invasive adenocarcinoma: there is a complex pattern of evolution

of neoplastic cell lineages rather than a simple linear pathway of progression (chapter 1)

- The establishment of *in vitro* and *in vivo* cell lines from adenocarcinomas of the gastro-oesophageal junction is well feasible and is a valuable tool for studying the genetics of this disease (chapter 5).

- The commercially available cell lines JROECL 47 and 50 established in the year 1997 from an oesophageal adenocarcinoma are in fact admixtures of colon carcinoma cell line HCT 116 (chapter 6).

- Perturbation of the E-cadherin-catenin complex, *i.e.* E-cadherin, α-, β-, γ- and p120-catenin, is frequently seen in the progression from Barrett's metaplasia towards adenocarcinoma (chapters 9 and 10).

- Immunohistochemistry should not be considered the "golden standard" for detecting aberrations of the E-cadherin-catenin complex (chapter 10).

- There is no evidence that somatic mutations in the E-cadherin, β-catenin, APC, VHL and PPARγ genes play an important role in the pathogenesis of adenocarcinomas of the gastro-oesophageal junction (chapters 7 and 8).

- Loss of heterozygosity of chromosome 14q at 14q31.1-32.11 implicates a putative tumour suppressor gene involved in tumourigenesis of adenocarcinomas of the gastro-oesophageal junction (chapter 11).

CHAPTER 14

EPILOGUE

GENERAL DISCUSSION, CLINICAL IMPLICATIONS AND FUTURE RESEARCH

INTRODUCTION

Due to long standing (duodeno)gastro-oesophageal reflux, the normal squamous cell lining of the oesophagus is replaced by metaplastic, columnar epithelium, so called Barrett's epithelium. A hallmark of Barrett's epithelium is the presence goblet cells. These cells are (under normal conditions) only present in the small and large intestine. Therefore, the name intestinal metaplasia is also applied for Barrett's epithelium. Barrett's epithelium carries a substantial risk for the development of adenocarcinoma. The risk of developing gastro-oesophageal adenocarcinoma is estimated to be 30 to 125-fold greater in patients with intestinal metaplasia than in patients without. Carcinogenesis is thought to be a multistep process: first, mild architectural abnormalities are seen and genetic abnormalities are already present. Although mild dysplasia is reversible, some patients go on to develop moderate and severe dysplastic Barrett's epithelium. Severe dysplasia carries a high risk for developing invasive carcinoma. In fact, high grade dysplasia coexists with invasive carcinoma in 30-60%. Therefore, some centers consider nowadays high-grade dysplasia as an indication for (prophylactic) oesophagectomy, whereas others follow patients on a regular basis with repeated endoscopies and biopsies.

EPIDEMIOLOGICAL ASPECTS

Studies from several parts of the western world suggest that the incidence of adenocarcinoma of the gastro-oesophageal junction is rising. Although data from the USA tell us that there is a dramatic increase in incidence (1), several other studies suggest that this rise might be overestimated (2). Changes in diagnostic tools, improved histological verification of tumours of the oesophagus and proximal stomach and other factors, as discussed in chapter 3 of this thesis, might have biased these observations. Despite these pitfalls, it can be concluded that the increase in incidence of adenocarcinomas of the gastro-oesophageal junction is a true increase, at least in the Southeast of the Netherlands.
Several risk factors have been mentioned, such as dietary factors, obesity, tobacco, alcohol, the use of H2-receptor antagonists and the use of medications that relax the lower oesophageal sphincter. The strongest risk factor however for oesophageal adenocarcinoma is gastro-oesophageal reflux disease (3). Whether the increase in incidence of adenocarcinomas reflects a true increase in gastro-oesophageal reflux disease (GORD) and consequently Barrett's oesophagus remains elusive. There are some early reports that this might indeed be the case. Again, one should remember that the prevalence of Barrett's oesophagus paralleled or even exceeded the increase of upper endoscopy since the 70's (4). Even more important, increased awareness of the endoscopic features of Barrett's oesophagus may have resulted in an increase in endoscopic diagnosis (5). Intruiging is the hypothesis that *Helibacter Pylori* infection, and as consequence chronic atrophic gastritis, protects against GORD and its complications. The decreased prevalence of *H. Pylori* could therefore explain (to some extent) the observed increase in incidence of

adenocarcinomas of the gastro-oesophageal junction. Finally, risk factors as yet unidentified for Barrett's oesophagus may be increasing and as the population ages, Barrett's oesophagus becomes more common and, hence, the population at risk for oesophageal carcinoma increases. Therefore, the question what causes the increase in incidence of adenocarcinomas of the gastro-oesophageal junction remains unanswered. The answer might come from the laboratory, where animal models and *in vitro* experiments will soon give more insight into the basic pathophysiological and genetic events that occur in the progression from Barrett's oesophagus to adenocarcinoma. Recently, a gene for severe pediatric gastro-oesophageal reflux disease was mapped to chromosome 13q14 (6). Perhaps this genetic locus also plays a role in the pathogenesis of Barrett's carcinoma. Untill now, heredity does not seem to contribute importantly to the occurrence of oesophageal cancer of any histological type (7).

CLINICAL ASPECTS

In line with chapter 4 of this thesis, recent publications show that adenocarcinoma of the oesophagus and gastric cardia is the same disease. Both carcinomas show similar epidemiology, intestinal metaplasia (its precursor lesion) is very common in the mucosa adjacent to early stage adenocarcinoma of the cardia and oesophagus and patient and tumour characteristics are similar (8). Furthermore, there was a similar prevalence of p53 mutations in adenocarcinomas of the oesophagus (53%) and cardia (58%). Also the spectrum (type) of mutations was similar and, once more, this provides strong genetic support for the concept that these tumours around the gastro-oesophageal junction have a common pathophysiology (9).
Adenocarcinoma of the gastro-oesophageal junction is an aggressive disease. Of the patients presenting to the physician with oesophageal carcinoma, about 60% undergo oesophagectomy. Thirty percent of the patients already has metastases on presentation of the disease and another 10% have metastases upon laparotomy, initially not detected during pre-operative work-up. In these patients, treatment is aimed at palliation: relieving symptoms of dysphagia by radiotherapy or stenting the oesophagus. For patients without an advanced stage of the disease, radical surgery is the treatment of choice and offers the best possibility of long-term survival. However, even after surgery with curative intent, the 5-year survival is about 20-30% (chapters 4 and 9). Most patients will develop metastatic disease, probably due to residual disease in the operation field and/or subclinical metastases at the time of surgery.
There are several strategies to improve the long-term survival in patients with adenocarcinoma of the gastro-oesophageal junction:
(1) surveillance endoscopy of patients with known Barrett's oesophagus to detect progression of disease towards cancer, and to allow surgery while cure is still likely (increase of early stage tumours).
However, little benefit is expected from surveillance programs, because most cancer patients present at an advanced stage outside of surveillance programs and

were not known to harbour Barrett's oesophagus. Moreover, controversy exists about the frequency of endoscopic surveillance in patients with Barrett's oesophagus and surveillance endoscopy in patients with Barrett's oesophagus seems not cost effective, as compared with mammography for the detection of breast cancer (10).

(2) to improve surgical technique; i.e. less morbidity/mortality together with improved radicality of the operation. Worldwide, controversy exists to what is the optimal surgical procedure for patients with (localised) disease. Transhiatal oesophagectomy (THO) without thoracotomy is the treatment of choice in the Netherlands. Radical or transthoracic resection of the oesophagus (TTO) with two-field lymph node resection is advocated by others, who believe that this approach gives the best chances for cure, despite the higher morbidity/mortality. In 1997, a randomized controlled trial started in two centers in the Netherlands (Amsterdam and Rotterdam). The aim of this trial is to determine whether TTO sufficiently improves the overall survival, the symptomatic recurrence free survival and the quality of life survival in comparison to THO (without thoracotomy) to compensate for the expected increase in peri-operative morbidity, mortality and costs of treatment. The follow up of the patients has just been completed and these important data will become available soon. Finally, the first experiences of minimally invasive (laparoscopic/thoracoscopic oesophagectomy were recently reported (11). Decreased morbidity, less post-operative pain, a shorter hospital stay and faster return to work are the claimed advantages of laparoscopic surgery, when compared with the "open" techniques. Whether this is true for minimally invasive oesophagectomy, without violating the oncological principles, needs to be investigated in prospective, randomized trials.

(3) to improve the adjuvant regimes, i.e. chemotherapy and radiotherapy. There seems to be no survival benefit for those patients who receive radiation or chemotherapy after initial surgical treatment. Recent publications, however, made clear that there might be a survival benefit for those patients who receive pre-operative or neoadjuvant chemotherapy followed by surgery compared to patients with surgery for adenocarcinoma alone (12). Hopefully, in the nearby future, data from more randomized trials will become available and will help us to define the best (neo)adjuvant regimens for patients with adenocarcinoma of the gastro-oesophageal junction.

GENETIC ASPECTS

As concluded in chapter 1, numerous molecular events involved in the progression from Barrett's oesophagus to adenocarcinoma have been characterised over the past decades. Despite the major advances in molecular biology, the pathogenesis of adenocarcinomas of the gastro-oesophageal junction remains poorly understood. A detailed understanding of the molecular mechanisms underlying the progression of Barrett's oesophagus through the metaplasia-dysplasia-carcinoma sequence lags significantly behind the analogous colorectal adenoma-carcinoma-sequence.

Nevertheless, a reasonable model of the molecular events that underpin the progression of this neoplastic sequence in Barrett's oesophagus has been proposed recently (13). Abnormalities within the cell cycle (aneuploidy), mutated tumour suppressor genes, especially p16 and p53, are among the most frequent reported aberrations in gastro-oesophageal adenocarcinomas and its precursor lesion.
In this thesis, the role of the cell-cell adhesion complex E-cadherin-catenin was studied in adenocarcinomas of the gastro-oesophageal junction (chapters 7-10). Immunohistochemical studies show progressive deterioration of E-cadherin based cell-cell adhesion as adenocarcinoma develops from metaplastic columnar epithelium. However, somatic mutations in the E-cadherin and β-catenin genes could not be detected. Therefore, other mechanisms responsible for perturbation of the adhesion complex will be hunted after in the near future. Epigenetic mechanisms, such as hypermethylation of the E-cadherin promotor, have been proven to play a major role in epithelial neoplasms (14). Furthermore, it has been demonstrated that the control of E-cadherin-mediated cell adhesion in benign and malignant epithelial cells is very complex and relies on interactions between various external factors and intracellular signalling pathways. The interaction of β-catenin with the adenomatous polyposis coli (APC) gene and its key role in the Wg/Wnt signalling pathway was a surprising and exciting finding. Stimulation of basic research in order to get a better understanding of the interactions involving components of the E-cadherin-catenin complex is crucial. Hopefully this will lead to the ability to predict and combat the events of tumour initiation and progression, and leading to a reduction in tumour metastases. It is important not to loose sight of the fact that the process of tumour invasion and metastases is complex, and targeting a single part of this process may not successfully stop it. Clearly, research to date has revealed the tip of the process, and much work still lies ahead. Some questions that still need to be answered are:

- What is the true role of $p120^{ctn}$ in cell adhesion? Does $p120^{ctn}$ nuclear staining imply a role of this protein in cell signalling? (chapter 9)
- Which are the target genes of the Wg/Wnt signalling pathway?
  Recently, a Japanese group suggested that E-cadherin represent a new downstream target gene (15)
- Which other components of the Wg/Wnt pathway are deregulated in adenocarcinomas of the gastro-oesophageal junction with nuclear localisation of β-catenin?
  Recently, axin mutations were found in colorectal and hepatocellular cancers with activating beta-catenin/TCF signalling (16). It speaks for itself to screen adenocarcinomas of the gastro-oesophageal junction with activation of the Wg/Wnt pathway for mutations in axin and other interacting proteins.
- Finally, perhaps the most important question: Can E-cadherin (and catenin) downregulation be blocked and subsequently tumour formation?

In the next decade, more novel proto-oncogenes and tumour suppressor genes will be discovered and the possible role in the carcinogenesis of adenocarcinomas of the gastro-oesophageal junction will be evaluated. As we have pointed out in chapters 11 and 12, loss of a chromosomal region (on 3p or 14q31.1-32.11) implies the presence of a (putative) tumour suppressor gene in the region of frequent loss. However, the sizes of these regions are still very large and many yet (undiscovered) genes may be linked to that particular region. The isolation and identification of new genes and mutation analysis of possible target genes is labour-intensive and tedious work. DNA microarrays are "DNA chips" which allow us to study simultaneously the expression of hundreds to thousands of genes and open reading frames "(ORF's) in one experiment. For example, one can compare the expression profile of genes in cancerous tissue *versus* normal epithelium, or non-dysplastic Barrett's epithelium *versus* dysplastic Barrett's epithelium. Using this technique, one could obtain information about genes and DNA sequences (yet unidentified genes) that play an important role in the progression from Barrett's oesophagus to adenocarcinoma.

Further characterisation of the genetic changes critical for the initiation and progression of Barrett's oesophagus into invasive adenocarcinoma is also important from a clinical point of view. Barrett's oesophagus develops in 5-20% of patients with gastro-oesophageal reflux disease and predisposes to oesophageal adenocarcinoma. As outlined above, the value of endoscopic biopsy surveillance is questioned. This is also due to the fact that most patients with Barrett's oesophagus do not develop cancer. There is also a substantial inter-observer variation in making the histological diagnosis of dysplasia. This makes validation of surveillance guidelines difficult because varying histopathological interpretations may lead to different estimated rates of progression. Potential (molecular) biomarkers have been identified that can be used in combination with routine histological staging to stratify patients' risk of progressing to adenocarcinoma of the gastro-oesophageal junction. In this respect, data from the Fred Hutchinson Cancer Center in Seattle seem promising (17). A systematic endoscopy protocol using histology and flow cytometry identifies subsets of patients with Barrett's oesophagus at low and high risk for progression to cancer.

Furthermore, meaningful clinical intervention in patients with adenocarcinoma of the gastro-oesophageal junction is still based on accurate histological descriptions. But this information can be supplemented by abnormalities in proto-oncogenes and tumour suppressor genes which can lead to a better prognostication and might be able to identify patients who are at high risk for recurrence of the disease and who might or might not benefit from (neo)adjuvant therapy. Some efforts in this regard have already met with some success (18,19). For example, the clinical significance of p53 mutations was investigated: patients with mutations were younger, had signs of more advanced disease, and had poorer prognosis than patients without mutations (19). As shown in chapter 9 of this thesis, $p120^{ctn}$ expression may also be a useful prognostic marker in adenocarciomas of the gastro-oesophageal junction. But if protein expression and mutation analysis is to be used

for clinical purposes, their status should preferably be evaluated during the initial work-up when only biopsy material is available. Some early reports found a discrepancy between the expression of a potential biomarker in the resection specimens of the primary tumour and the pre-operative biopsies. This certainly warrants further investigation, before "molecular staging" of adenocarcinomas of the gastro-oesophageal junction is introduced in the clinic.
Despite these promising developments, at present the best prognostic indicator for patients with these cancers remains pathological tumour stage (chapters 4 and 9), especially involvement by tumour of regional lymph nodes (pN-status). The fact that tumour recurrence and development of distant metastases, of patients who previously underwent a complete removal of the tumour, has led to the realisation that in many cases undetectable deposits of tumour cells must remain in the patient after removal of the tumour. Studies with newly developed sensitive methods for the detection of tumour cells in lymph nodes and in the sera of cancer patients, including immunohistochemistry and (reverse transcriptase) polymerase chain reaction, have indicated that tumour cells are detectable in a significant proportion of the patients who were judged to de be free of metastases by routine histopathologic methods (20-22). However, the clinical significance and usefullness have not been established yet.

SUMMARY AND CONCLUSIONS

Characterisation of the epidemiology, clinical aspects and molecular pathology of the metaplasia-dysplasia-carcinoma sequence of Barrett's oesophagus has only recently begun to resolve the complexities of the disease first described and debated nearly 50 years ago. As with many of the clinical and histopathological features of the disease, the molecular pathology of Barrett's oesophagus and adenocarcinoma does not readily or necessarily comply with the known molecular events that occur in other epithelial neoplasms. Yet, failure to unravel the complexities of the pathogenesis of columnar lined oesophagus is potentially devastating: the dramatic increase in incidence of oesophageal adenocarcinoma in Western communities. Advances in understanding the pathological and molecular mechanisms involved in the metaplasia-dysplasia-adenocarcinoma sequence will surely result in the development of markers of early stages of carcinogenesis and this, in combination with surveillance, has the potential to allow identification of patients at high risk of neoplastic change. This will in turn allow earlier intervention, either by molecular therapy, or perhaps more likely in the near future, early application of ablative or surgical methods of treatment. Elucidation of events at the molecular level will also have impact on the clinical management of adenocarcinomas of the gastro-oesophageal junction. For example, genetic markers can identify patients who are at risk for recurrence of the disease after surgical treatment. Expression analysis with DNA microarrays will certainly provide important clues to biological and clinical behaviour of cancers in the nearby future.

However, as more information becomes available concerning the molecular abnormalities encountered during the progression of Barrett's oesophagus to adenocarcinoma, the more complex the picture becomes.

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SAMENVATTING EN CONCLUSIES

De laatste drie decennia neemt de incidentie van het adenocarcinoom van de gastro-oesophageale overgang, hier gedefinieerd als adenocarcinoom van de distale slokdarm en maag-cardia, toe. Dit is een zorgzame ontwikkeling omdat de prognose van patiënten met een dergelijk gezwel teleurstellend is: de 5-jaars overleving na een in opzet curatieve operatie bedraagt slechts 25%. De voornaamste risicofactor voor het ontwikkelen van een adenocarcinoom van de gastro-oesophageale overgang is langdurige reflux (=terugvloed) van maagzuur en duodenumsappen in de slokdarm: het zogenaamde "zuurbranden". Dit leidt tot een ontsteking van de slokdarm en kan in circa 10-20 % van de patiënten leiden tot de vorming van metaplastisch cylinderepitheel, beter bekend als de "Barrett slokdarm". Er zijn sterke aanwijzingen dat adenocarcinomen van de gastro-oesophageale overgang ontstaan uit dit Barrett epitheel door middel van een aantal genetische en histologische veranderingen die in de tijd plaatsvinden. De studies die beschreven worden in dit proefschrift hebben tot doel om een beter inzicht te verwerven in de epidemiologische-, klinische- en genetische aspecten van adenocarcinomen van de gastro-oesophageale overgang.

DEEL I, HOOFDSTUK 1 beschrijft de genetische veranderingen die optreden bij het ontstaan van adenocarcinomen van de gastro-oesophageale overgang uit premaligne Barrett epitheel. Ondanks de enorme vlucht die de moleculaire biologie heeft genomen en die heeft geleid tot vele publicaties omtrent genetische afwijkingen die een rol spelen bij het ontstaan van deze tumoren, is er nog geen duidelijk beeld over de volgorde en het tijdspad waarin deze veranderingen plaatsvinden. Dit in tegenstelling tot dikkedarmkanker waar een duidelijk omschreven lineair genetisch model geldt. De laatste jaren is echter wel duidelijk geworden dat inactivatie van de p16 en p53 tumorsuppressorgenen (door mutaties en/of epigenetische fenomenen) en veranderingen in de celdeling of aneuploidy (=een afwijkende hoeveelheid chromosomaal DNA) een van de meest frequente en vroeg optredende genetische afwijkingen zijn die een rol spelen bij het ontstaan van het adenocarcinoom van de gastro-oesophageale overgang.
In de afgelopen decennia is de interesse in de werking van eiwitten die cel-cel adhesie tot stand brengen toegenomen. Het belangrijkste eiwitcomplex dat zorg draagt voor deze integriteit is het E-cadherine-catenine complex. Dit complex blijkt vaak te disfunctioneren in kankercellen waardoor cel adhesie verloren gaat. De kankercellen kunnen dan ongestoord delen, losraken van hun omgeving, en migreren naar de bloedbaan en lymfebanen alwaar ze aanleiding kunnen geven tot uitzaaiingen. **HOOFSTUK 2** geeft een overzicht van de recente literatuur over de rol van het E-cadherin-catenine complex bij het ontstaan van kanker. De mate van expressie van dit complex neemt af gedurende de ontwikkeling van Barrett epitheel tot invasief carcinoom. Een andere interessante bevinding is dat de mate van expressie nauw gecorreleerd is aan tumor karakteristieken en ook aan klinische

parameters, zoals de 5-jaars overleving na operatieve ingrepen. In het algemeen geldt dat een tumor met weinig expressie van het E-cadherine-catenine complex vaak een slechte graad van differentiatie heeft, een ongunstig tumor stadium heeft en daaruit voortvloeiend een slechte prognose. Gezien deze samenhang zal in de toekomst mogelijk de expressie van het E-cadherine-catenine complex een rol kunnen spelen bij het voorspellen van het (klinisch) gedrag van een tumor.
β-Catenine is een onderdeel van het E-cadherine-catenine complex. Dit eiwit bevindt zich in de cel gebonden aan het E-cadherine en draagt mede zorg voor de adhesie. Daarnaast speelt het β-catenine tevens een belangrijke rol bij signaal transductie. β-Catenine kan zich namelijk ook in de kern van de cel bevinden, gebonden aan transcriptiefactoren. Het β-catenine kan op die manier de transcriptie van genen betrokken bij de carcinogenese stimuleren. Dit maakt het β-catenine tot een bijzonder eiwit: enerzijds heeft het een tumorsuppressor functie (namelijk het tot stand brengen van cel-cel adhesie), anderzijds is β-catenine een oncogen (het stimuleerd de transcriptie van genen betrokken bij tumorvorming). Hoofdstuk 2 besluit met de conclusie dat het verder ontrafelen van de mechanismen die de veranderingen in de expressie van het E-cadherine-catenine complex veroorzaken en het identificeren van andere eiwitten die verder deel uitmaken van dit complex, van cruciaal belang zijn en kunnen leiden tot het ontwikkelen van nieuwe therapieën tegen kanker.

In **DEEL 2** van dit proefschrift wordt de epidemiologie en de klinische aspecten van adenocarcinomen van de gastro-oesophageale overgang besproken.
HOOFDSTUK 3 beschrijft de incidentie- en sterftecijfers van slokdarm en maagkanker in Zuidoost Nederland in de periode 1978-1996. De incidentie en sterftecijfers van slokdarmkanker stegen gedurende deze periode bij zowel manen als vrouwen. Daarentegen daalde zowel de incidentie van als sterfte aan maagkanker. Indien de incidentiecijfers worden uitgesplitst naar sublokalisatie en histologische type, dan blijkt dat de incidentie van adenocarcinomen van de distale slokdarm en maag-cardia toeneemt. Onder mannen steeg de incidentie van deze tumoren rond de gastro-oesophageale overgang van 3.9 in 1978 tot 6.7 per 100.000 persoonsjaren in 1996, en onder vrouwen van 0.2 tot 1.4 per 100.000 persoonsjaren. Omdat gedurende de onderzoeksperiode de incidentie van tumoren die niet nader gespecificeerd of geclassificeerd waren niet evident afnam, is het waarschijnlijk dat de geobserveerde toename in incidentie van het adenocarcinoom van de gastro-oesophageale overgang een "echte" toename is. Deze wordt met name gezien bij mannen van middelbare leeftijd sinds 1990.
In de praktijk is het vaak moeilijk om te bepalen of een tumor van de gastro-oesophageale overgang primair uitgaat van de slokdarm of van de maag-cardia. Uit de literatuur komt naar voren dat beide tumoren gemeenschappelijke risicofactoren hebben en een zelfde pheno- en genotype. De huidige klassificatie/stagerings criteria, zoals van de Union International Contre Le Cancer (UICC) en de American Joint Committee on Cancer (AJCC), gebruiken separate classificaties

voor beide tumoren. Kanker van de maagcardia wordt gestageerd volgens de principes van het maagcarcinoom, en kanker van de distale slokdarm wordt gestageerd volgens de principes van slokdarmkanker.
In **HOOFDSTUK 4** wordt onderzocht of beide tumoren gelegen rond de gastro-oesophageale overgang beschouwd kunnen worden als één entiteit en dientengevolge gestageerd kunnen worden volgens een zelfde classificatie. De pathologische kenmerken, TNM klassificatie en 5-jaars overleving werden onderzocht en vergeleken tussen 111 patiënten met een adenocarcinoom van de slokdarm en 141 patiënten met een adenocarcinoom van de maag-cardia. De macroscopische en microscopische kenmerken van beide groepen tumoren kwamen grotendeels overeen. Barrett epitheel werd echter frequenter gezien in de nabijheid van slokdarmtumoren. Hoewel het T-stadium van cardiacarcinomen ongunstiger was (d.w.z. diepere infiltratie in de wand van slokdarm-maag), was het percentage tumoren met loco-regionale kliermetastasen gelijk voor beide groepen (ongeveer 60%). Klaarblijkelijk worden slokdarm tumoren in een vroeger stadium gediagnostiseerd. De 5-jaars overleving na in opzet curatieve chirurgie was 26% voor de slokdarmgroep versus 27% voor de cardia groep. Ook de 5-jaars overleving uitgesplitst naar tumorstadia liet geen verschillen zien tussen beide groepen. Tenslotte werd een multivariante analyse verricht: leeftijd van de patiënt diepte van infiltratie (T-klassificatie), lymfkliermetastasen (N-klassificatie), metastasen op afstand (M-klassificatie), differentiatiegraad (G-klassificatie) en radicaliteit van de ingreep (R-klassificatie) zijn alle onafhankelijke variabelen die de 5-jaars overleving kunnen voorspellen. Samenvattend kan worden gesteld dat adenocarcinomen van de distale slokdarm en maag-cardia één klinische entiteit zijn en dientengevolge gestageerd dienen te worden volgens uniforme criteria.

Zoals reeds bediscussieerd in hoofdstuk 1, er is nog relatief weinig bekend over de cellulaire en genetische afwijkingen van adenocarcinomen van de gastro-oesophageale overgang. Het gebrek aan goede *in vivo* en *in vitro* modelsystemen is hier zeker debet aan. **DEEL 3** van dit proefschrift beschrijft de ontwikkeling en karakterisering van cellijnen afkomstig van adenocarcinomen van de slokdarm en maag-cardia.
HOOFDSTUK 5 beschrijft onze eigen ervaring sinds 1997 met het genereren en karakteriseren van cellijnen. Stukjes primaire tumor en lymfkliermetastasen, verkregen van resectiepreparaten van adenocarcinomen van de gastro-oesophageale overgang, werden subcutaan geïmplanteerd bij een immuun-deficiënte naakte muis (deze methode wordt ook wel *xenografting* genoemd). Daarnaast werd een deel van het verkregen tumor weefsel gebruikt voor het direct in kweek brengen. Subcutane transplantatie van 70 primaire tumoren en 17 lymfkliermetastasen resulteerde in initiële groei van respectievelijk 22 en 6 tumoren (32%). Na retransplantatie werden 6 permanent groeiende cellijnen (xenografts) verkregen van de primaire tumoren en 3 cellijnen van metastasen Het direct kweken van 34 tumoren en 9 metastasen resulteerde in het genereren van 3 (7%) permanente *in vitro* cellijnen. Van één patiënt lukte het om zowel de primaire tumor en de lymfkliermetastase te kweken.

Alle *in vivo* en *in vitro* cellijnen werden gekarakteriseerd door middel van immunohistochemie, microsatellietenanalyse en karyotypering om de epitheliale en humane origine te verifiëren.
Recent werden 5 humane cellijnen van slokdarm en maag-cardia adenocarcinomen in Engeland gegenereerd, gedeponeerd in een cellijnbank, en beschikbaar gesteld voor wetenschappelijk onderzoek. Twee van deze cellijnen (JROECL 47 en 50) werden door ons gebruikt voor E-cadherine en β-catenine mutatie analyses (zie hoofdstukken 7 en 8). In deze twee cellijnen, die beiden afkomstig zijn van één primaire tumor, vonden we een E-cadherine en een β-catenine mutatie. Echter, deze mutaties konden niet worden aangetoond in de primaire tumor. Identieke E-cadherine en β-catenine mutaties zijn beschreven voor een coloncarcinoom cellijn HCT 116, die in 1981 is gegenereerd. In **HOOFDSTUK 6** wordt de oorsprong van de slokdarmcellijnen JROECL 47 en 50 nader onderzocht met behulp van HLA-typering, mutatie- en microsatelliet analyses. Allereerst werd het groeipatroon van de cellijnen JROECL 47 en HCT 116 met elkaar vergeleken na subcutane transplantatie op een immuundeficiënte naakte muis. Beide cellijnen groeiden ongeveer even snel en bestonden voornamelijk uit ongedifferentieerde, spoelvormige tumorcellen. JROECL 47 en HCT 116 hadden allebei hetzelfde HLA-DR allel. Dit was een ander allel dan de primaire slokdarm tumor waaruit JROECL 47 en 50 waren gegenereerd. Mutatieanalyse van de E-cadherine, β-catenine en K-Ras genen toonde aan dat alle 3 de cellijnen dezelfde mutaties bezitten. Microsatellietanalyse daarentegen liet wel verschillen zien tussen de cellijnen. Men dient zich echter te realiseren dat alle 3 de cellijnen sterk microsatelliet-instabiel zijn. Daardoor is het mogelijk dat het kweken van separate klonen van coloncarcinoom cellijn HCT 116 resulteert in verschillende microsatellieten. De conclusies die kunnen worden getrokken uit dit hoofdstuk zijn: (1) de slokdarmkanker cellijnen JROECL 47 en 50 zijn in feite klonen van coloncarcinoom cellijn HCT 116; (2) controleer altijd de afkomst van de cellijn omdat er een (klein) risico is op contaminatie met andere cellijnen; (3) allelotypering is niet de juiste techniek om de microsatelliet-instabiele cellijnen te karakteriseren.

In **DEEL 4** van dit proefschrift wordt nader ingegaan op het E-cadherine-catenine cel-cel adhesie complex in adenocarcinomen van de gastro-oesophageale overgang.
In **HOOFDSTUK 7** wordt de hypothese getoetst dat genetische veranderingen in het E-cadherine gen een belangrijke rol spelen tijdens pathogenese van adenocarcinomen van de slokdarm en dat deze genetische veranderingen verantwoordelijk zijn voor de verminderde expressie van E-cadherine in deze tumoren. Alle 16 exonen van het E-cadherine gen werden gescreend op mutaties d.m.v. de "polymerase chain reaction-single strand conformation polymorphism analysis" (PCR-SSCP) techniek in 57 tumor DNA's, inclusief 4 cellijnen en 4 xenografts. In 2 cellijnen van dezelfde patiënt werd een mutatie gevonden die leidt tot een stopcodon in het DNA. Er werden geen andere mutaties gezien. Verlies van heterozygotie (LOH) van het E-cadherine locus op 16q22.1 werd gezien bij 65%

van de tumoren. Hieruit kunnen we concluderen dat E-cadherine mutaties geen belangrijke rol lijken te spelen tijdens de progressie van het Barrett epitheel naar het adenocarcinoom van de slokdarm. Het relatief hoge percentage verlies van heterozygotie van het E-cadherine locus op 16q22.1, zonder mutatie van het andere allel, wijst op de mogelijkheid van een ander kandidaat tumorsuppressorgen op 16q22.1 dat betrokken is bij de pathogenese van deze tumoren. Als LOH van 16q22.1 toch daadwerkelijk verlies van een E-cadherine allel weerspiegelt is het mogelijk dat een "dosage-effect" optreedt. Vooralsnog lijkt LOH van 16q22.1 geen relatie te vertonen met verminderde expressie van E-cadherine, maar dit verdient nader onderzoek.

Zoals reeds besproken is in hoofdstuk 2, is β-catenine tevens een oncogen. Het speelt een centrale rol in de Wingless/Wnt signaal transductie cascade. Fosforylering van aminozuren die gecodeerd worden door exon 3 van het β-catenine gen is noodzakelijk voor afbraak van vrij, ongebonden β-catenine door o.a. APC. Mutaties in exon 3 van β-catenine kunnen lijden tot verminderde afbraak van β-catenine en stabilisatie van het eiwit, dat vervolgens in de kern van de cel transcriptie van genen betrokken bij tumorvorming stimuleert. In **HOOFDSTUK 8** wordt de rol van β-catenine in adenocarcinomen van de slokdarm nader onderzocht. Mutatieanalyse door middel van PCR-SSCP werd verricht in 69 tumoren. Er werden geen mutaties in exon 3 gevonden, en ook geen deleties van het β-catenine gen. In 3 geselecteerde tumoren met homogene kernkleuring voor β-catenine, hetgeen activatie van het Wg/Wnt signaal transductie cascade impliceert, werden ook geen mutaties in APC gevonden. Klaarblijkelijk ligt een ander mechanisme ten grondslag aan de cytoplasmatische en kernkleuring van β-catenine in adenocarcinomen van slokdarm.

Onlangs is een nieuw eiwit geïdentificeerd dat deel uitmaakt van het E-cadherine-catenine complex: p120-catenine ($p120^{ctn}$). De functie van dit eiwit in normale en in kankercellen is nog onbekend. In **HOOFDSTUK 9** is de *in vivo* expressie en lokalisatie van dit eiwit onderzocht door middel van immunohistochemie in normaal slokdarm- en maagepitheel, in Barrett epitheel, in 96 adenocarcinomen van de gastro-oesophageale overgang en in 13 lymfkliermetastasen. Tevens werd de relatie tussen $p120^{ctn}$ expressie en pathologische en klinische parameters onderzocht. Verminderde membraanexpressie werd gezien in 20% van de Barrett preparaten, in 68% van de primaire tumoren en in 85% van de lymfkliermetastasen. Verminderde membraanexpressie van $p120^{ctn}$ was significant gecorreleerd aan slechtere differentiatiegraad van de tumor en een ongunstige 5-jaars overleving. De 5-jaars overleving van patiënten met normale membraanexpressie van $p120^{ctn}$ bedroeg 54% en de 5-jaars overleving van patiënten met verminderde membraanexpressie van $p120^{ctn}$ was 12% ($p=0.001$). In de subgroep van patiënten met tumorvrije lymfeklieren (N0-tumoren) was de 5-jaars overleving 63% voor de groep met normale expressie van $p120^{ctn}$ *versus* 20% voor patiënten met verminderde membraanexpressie $p120^{ctn}$ ($p=0.02$). De expressie van $p120^{ctn}$ lijkt derhalve een bruikbare prognostische parameter. Een interessante vondst is de

aanwezigheid van het p120^{ctn} in de kern van een aantal tumoren. Dit kan wijzen op de mogelijke betrokkenheid van p120^{ctn} in signaaltransductie cascades zoals ook voor β-catenine geldt. Dit verdient vervolgonderzoek.
In **HOOFDSTUK 10** wordt de rol van het E-cadherine-catenine complex in adenocarcinomen van de gastro-oesophageale overgang nogmaals nader onderzocht. Het E-cadherine en de cateninen zijn in verschillende fracties aanwezig in de cel: membraangebonden, in vrije vorm in het cytoplasma en gebonden aan het cytoskelet. De expressie van het complex en hun biochemische distributie tussen de Triton X-100 oplosbare (=membraangebonden fractie) en Triton X-100 onoplosbare fractie (=fractie gebonden aan het cytoskelet) werd onderzocht door gebruik te maken van gefractioneerde eiwit extractie gevolgd door Western blotting. Dit gebeurde bij 11 tumoren en corresponderend normaal weefsel De ruimtelijke verdeling van de eiwitten tussen de celmembraan en het cytoplasma werd onderzocht met behulp van immunohistochemie. Immunohistochemie toonde een verschuiving aan van E-cadherine en de cateninen van membraan naar cytoplasma in 50-75% van de tumoren. Kernexpressie van β-catenine werd gezien in 5 tumoren. Een verandering van de expressie en biochemische verdeling van E-cadherine en de cateninen tussen de Triton X-100 oplosbare en onoplosbare fractie in vergelijking tot normaal epitheel werd gezien in 45-65% van de tumoren. Dit hoofdstuk laat zien dat veranderingen in de expressie, in de ruimtelijke- en biochemische verdeling van het E-cadherine-catenine complex hetgeen leidt tot een verminderde adhesie, frequent aanwezig is bij adenocarcinomen van de gastro-oesophageale overgang. Immunohistochemie en Western blotting van de oplosbare en onoplosbare fractie van het E-cadherine-catenine complex zijn beide waardevolle technieken om een abnormaal E-cadherine-catenine complex te detecteren. Het is vooralsnog niet duidelijk welke van de twee technieken specifieker/sensitiever is in het detecteren van een abnormaal E-cadherine-catenine complex.

DEEL 5 van dit proefschrift beschrijft de genetische analyse van tumor suppressor genen in adenocarcinomen van de gastro-oesophageale overgang. Tumorsuppressorgenen zijn genen die primair betrokken zijn bij de regulering van celproliferatie, celdood celadhesie en genexpressie. In een normale cel remmen deze genen de cel groei en stimuleren ze differentiatie. In tumoren is hun remmende functie verloren gegaan ten gevolge van inactivatie of ten gevolge van verlies van beide gen kopieën (allelen). Microsatelliet analyse, ook wel verlies van heterozygotie (LOH) genoemd, is een eenvoudige techniek om tumoren te screenen op het verlies van delen van het chromosoom. Als er vaak verlies van een bepaalde chromosomale regio in tumoren wordt gevonden, dan is dat een suggestie voor de aanwezigheid van een tumor suppressor gen in of nabij het gebied van verlies.
HOOFDSTUK 11 richt zich op de korte arm van chromosoom 3 (3p25.2). Kandidaat tumorsuppressorgenen die hier liggen zijn het von Hippel-Lindau (VHL) gen en het humaan peroxisome proliferator-activated receptor gamma (PPARγ) gen.

Mutatiescreening van beide genen met behulp van de PCR-SSCP en LOH werd verricht in 44 tumor DNA's afkomstig van 41 patiënten met een adenocarcinoom van de gastro-oesophageale overgang. Ondanks het hoge percentage LOH van 3p25 in deze tumoren (63%), werden er geen mutaties gevonden in deze genen. Dit suggereert dat epigenetische mechanismen, mutaties buiten de onderzochte DNA sequenties, of een "gene dosage" effect een rol spelen. De mogelijkheid van de aanwezigheid van andere, nog niet geïdentificeerde, tumorsuppressorgenen op 3p dient ook overwogen te worden.

HOOFDSTUK 12 levert sterke aanwijzigingen voor de aanwezigheid van een tumorsuppressorgen op chromosoom 14q dat een rol speelt bij de pathogenese van adenocarcinomen van de gastro-oesophageale overgang. Verlies van heterozygotie (LOH) van het gebied 14q31.1-32.11 werd bepaald met 22 microsatelliet markers op tumor en normaal DNA verkregen van 36 patiënten (inclusief 15 xenografts en 5 cellijnen). LOH ofwel verlies van de chromosomale regio werd gevonden bij 15/37 (41%) monsters. Er werden geen homozygote deleties gezien in de xenografts en cellijnen. De minimale regio van overlappend verlies is 8.6cM en ligt tussen marker markers D14S983 en CCC1 op de cytogenetische band 14q31.1-32.11. Verlies van 14q31.1-32.11 is waarschijnlijk een relatief laat optredende genetische afwijking tijdens de ontwikkeling van een adenocarcinoom van de gastro-oesophageale overgang: (1) 11 DNA monsters afkomstig van dysplastisch Barrett epitheel vertoonden geen verlies van 14q32.1; (2) tumoren met LOH van 14q vertoonden veelal een ongunstiger tumorstadium en vertoonden vaker lymfkliermetastasen op afstand in vergelijking met de tumoren zonder verlies van 14q. Echter, van één tumor was er tevens DNA geïsoleerd van omringend dysplastisch Barrett slijmvlies. Ook in dit monster werd verlies van 14q aangetoond. Kandidaat tumorsuppressorgenen die in de regio 14q31.1-32.11 liggen zijn de thyroid stimulating hormone receptor (TSHR)1, het glutathione transferase zeta 1 (GSTZ1) en het checkpoint supressor 1 (CHES 1) gen. Of deze genen daadwerkelijk een rol spelen tijdens de progressie van Barrett epitheel tot adenocarcinoom dient nog onderzocht te worden.

CONCLUSIES

- De incidentie van het adenocarcinoom van de gastro-oesophageale overgang, d.w.z. van de distale slokdarm en maag-cardia, neemt toe in Zuidoost Nederland onder mannen en in mindere mate ook onder vrouwen (hoofdstuk3).

- Adenocarcinomen van de distale slokdarm en maag-cardia zijn één klinische entiteit en dienen gestageerd te worden volgens uniforme criteria (hoofdstuk 4).

- De volgende parameters hebben allen een onafhankelijke voorspellende waarde ten aanzien van de 5-jaars overleving van patiënten die een in opzet curatieve resectie ondergingen i.v.m. een adenocarcinoom van de gastro-oesophageale

overgang, te weten: leeftijd van de patient, infiltratie diepte van de tumor (pT-klassificatie), locoregionale en metastasen op afstand (pN- en pM-klassificaties), differentiatiegraad (pG-klassificatie), radicaliteit van de operatie (pR-klassificatie) en de $p120^{ctn}$ expressie (hoofdstukken 4 en 9).

- Er is geen allesomvattende moleculaire theorie omtrent het ontstaan van adenocarcinomen van de gastro-oesophageale overgang uit Barrett epitheel: er is juist een complex patroon van verschillende klonen van tumorcellen en geen simpel lineair model (hoofdstuk 1).

- Het ontwikkelen en karakteriseren van cellijnen van adenocarcinomen van de gastro-oesophageale overgang is goed mogelijk en deze cellijnen zijn onmisbaar voor verder studies naar het genetisch profiel van deze tumoren (hoofdstuk 5).

- De sinds 1997 commercieel verkrijgbare cellijnen JROECL 47 en 50 zijn niet afkomstig van een adenocarcinoom van de slokdarm maar zijn klonen van de coloncarcinoom cellijn HCT 116 (hoofdstuk 6).

- Verstoring van het E-cadherine-catenine complex, bestaande uit E-cadherine, α-, β-, γ- en p120-catenine, treedt vaak op tijdens de progressie van Barrett epitheel naar het adenocarcinoom van de gastro-oesophageale overgang (hoofdstukken 9 en 10)

- Immunohistochemie als onderzoeksmiddel is niet de "gouden standaard" om afwijkingen in het E-cadherine-catenine complex aan te tonen (hoofdstuk 10).

- Somatische mutaties in het E-cadherine, β-catenine, APC, VHL of PPARγ gen spelen geen belangrijke rol bij de pathogenese van adenocarcinomen van gastro-oesophageale overgang (hoofdstukken 7, 8 en 11).

- Verlies van heterozygotie van chromosoom 14q31.1-32.11 impliceert de aanwezigheid van een tumorsuppressorgen dat een rol speelt tijdens de carcinogenese van adenocarcinomen van de gastro-oesophageale overgang (hoofdstuk 12).

Appendices

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LIST OF PUBLICATIONS

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 23 augustus 1970 te Nijmegen. In 1987 behaalde hij het HAVO examen en in 1989 het VWO examen aan het Canisius College-Mater Dei te Nijmegen. Aansluitend begon hij met de studie geneeskunde aan de Universiteit Utrecht. De wetenschappelijke stage met betrekking tot hersenschade geïnduceerd door interstitiële bestraling werd verricht op het Brain Tumor Research Center, Department of Neurosurgery van mei tot november 1992 aan de University of California, San Francisco, USA (Prof. Dr. J.R. Fike). Gedurende de doctoraalfase was hij actief in het onderwijsmanagement en o.a. bestuurslid van het faculteitsbestuur. Tijdens de studie verrichtte hij onderzoek op het gebied van gastro-intestinale motiliteit en anti-reflux chirurgie op de afdeling chirurgie in het AZU (Prof. Dr. H.G. Gooszen). Tijdens de daarop volgende co-schappen werd het co-schap oogheelkunde verricht in het St Mary's Hospital te Londen (Verenigd Koninkrijk) en het co-schap Kindergeneeskunde gedurende 10 weken doorlopen aan de University of Wisconsin in Madison, USA. In mei 1997 behaalde hij zijn arts-examen. Van medio juni 1997 tot januari 2000 was hij werkzaam als arts-onderzoeker op de afdelingen chirurgie en pathologie (Prof. Dr. H.W. Tilanus en Dr. W.N.M. Dinjens). Gedurende deze periode werd onderzoek verricht naar de genetische veranderingen die optreden bij de progressie van Barrett epitheel tot adenocarcinoom van de gastro-oesophageale overgang. In de zomer van 1999, verrichtte hij gedurende 6 weken onderzoek naar het E-cadherine-catenine cel adhesie complex in het laboratorium van Prof. Dr. M. Pignatelli, verbonden aan de University of Bristol, Bristol, Verenigd Koninkrijk. Voor het experiment beschreven in hoofdstuk 12 ontving hij in 1999 de "Ronald Raven Award" van de "British Society of Surgical Oncology". Sinds februari 2000 is hij in opleiding tot chirurg in het Reinier de Graaf Gasthuis (Dr. P.W. de Graaf en Dr. L.P.S. Stassen) en zet deze voort in het Erasmus universitair Medisch Centrum Rotterdam (Prof. Dr. H.J. Bonjer).

Notes