Early-Onset Gastric Cancer
On the Road to Unraveling Gastric Carcinogenesis

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Early-Onset Gastric Cancer

On the Road to Unraveling Gastric Carcinogenesis

Maag kanker op een jonge leeftijd: Het ontrafelen van het maag carcinogenese vraagstuk

(met een samenvatting in het Nederlands)

Proefschrift

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About the Cover…

Napoleon died in exile on the island of St Helena and almost since the day of his death in 1821 there have been conspiracy theories about the cause. There have also been suggestions that chronic exposure to arsenic and medication errors were involved, while the theories that he had been poisoned was given a considerable boost in 1961, when a raised arsenic concentration was found in his hair.

Napoleon had requested that upon his death an autopsy be performed because he felt that his son would want to know whether or not cancer was present. The autopsy was performed by Dr. Francesco Antommarchi, a qualified anatomist, in the presence of 7 other physicians. Three separate accounts of the autopsy exist and agree on the main finding: a "cancerous ulcer" of the stomach, with a central perforation that led to peritonitis. There were enlarged, "hard" lymph nodes adjacent to the stomach. Although no histologic reports are available, the autopsy findings strongly suggest gastric cancer.

Theories that Napoleon was betrayed, poisoned, or a victim of inappropriate medical treatment have been undermined by new research based on the emperor's trouser collection. The research used a collection of 12 different pairs of trousers worn by Napoleon between 1800 and 1821, the year of his death in exile, and suggested a weight loss of 11 kg during the last year of his life, reducing his weight to 79 kg, consistent with a severe progressive illness. It lends credence to the idea that Napoleon died of stomach cancer, which was the cause of death specified in the original autopsy. (Lugli, A. Kopp Lugli and M. Horcic; *Human Pathology* (2005;36:320-4))

The Napoleon Bonaparte's family is said to be a striking example of a dominantly inherited predisposition to gastric cancer. (Sokoloff, B. (1938) Predisposition to cancer in the Bonaparte family. *Am J Surg* XL, 673-678)

The truth knocks on the door and you say "Go away, I'm looking for the truth" and so it goes away…

-Robert Pirsig

To Nick and Luana

1

Introduction

Stomach cancer has been recognized for several millennia. As early as 400 BC, Hippocrates first described cancer (which he named *karkinos* or*karkinoma*). Among his first descriptions was that of a patient with associated *melaina* or black vomiting, who likely had cancer of the stomach. In 1867, Waldeyer began intensive histologic investigations of mammary and gastric carcinoma. He believed that microscopic analysis strongly suggested that gastric cancer had its origins in the "pepsin and mucous glands" of the stomach, which led to his formulation of the *epithelial theory of cancer*. This theory, which states that all carcinomas are derived originally from epithelial cells, eventually became the central dogma of cancer biology, despite numerous competing theories over the years. Waldeyer was firmly convinced that "the development of cancer cells always originates in the preexisting, genuine epithelia of the organism… ."

The existence of a familial form of gastric cancer has been known since the 1800s when multiple gastric cancer cases were noted in the famous Bonapart family (see inside cover of this thesis). In the early 20th century, interest grew in determining the cause of cancer. In 1913, Johannes Andreas Grib Fibiger developed the first rodent model of stomach cancer, which suggested a link between chronic irritation and cancer. By 1940, gastric cancer was recognized as the second leading cause of cancer death in the United States, and attention turned to epidemiologic associations. Studies of migrant populations suggested that stomach cancer was associated with an environmental exposure occurring early in life. In the early 1970s, Correa formulated a multistep model of gastric cancer, which postulated a temporal sequence of pathologic changes that led from chronic (type B) gastritis to atrophic gastritis, intestinal metaplasia, and dysplasia and the eventual development of gastric cancer Based on epidemiologic studies of dietary histories, the first step in the Correa pathway – the development of gastric inflammation – was believed to be initiated by a diet rich in salt and nitrates/nitrites as well as deficiencies in fresh fruits and vegetables. Dietary factors and continued effects of chronic inflammation were felt to be responsible for the orderly progression from gastritis to atrophy, metaplasia, dysplasia, and, in a subset of patients, carcinoma.

Our understanding of gastric cancer underwent a marked shift with the rediscovery of *Helicobacter pylori*. Human gastric bacteria were first recognized in the early 19th century, but their identity and clinical significance were not appreciated until they were isolated and cultured from a human gastric biopsy by Marshall and Warren in 1982. Originally named *Campylobacter pyloridis*, the organism was shown to be a spiral-shaped, gram-negative, microaerophilic rod strongly associated with gastritis and peptic ulcer disease. It was recognized as a separate genus and renamed *Helicobacter pylori* in 1989. A causal relationship between *H pylori* and gastric cancer was first postulated by Marshall and Warren in 1983. The firm association between *H pylori* and gastric cancer has been demonstrated by a number of case control studies, which have retrospectively examined the *Helicobacter* infection status of gastric cancer patients. This association has begun to approach that shown for smoking and lung cancer, and *H pylori* was classified as a definite (type I) carcinogen in 1994 by the IARC, a branch of the World Health Organization. Although it is clear that most infected patients will remain asymptomatic and will not go on to develop peptic ulcer disease or gastric cancer from their infection, the causal link between *H pylori* and gastric cancer is now quite clear and includes not only epidemiologic associations but also animal model and interventional data.

Despite the vast array of data on gastric cancer, the exact mechanisms leading to this common cancer are far from understood. An outline of the environmental factors as well as important molecular and genetic findings in gastric cancer are reviewed in **chapter 2.** In this chapter not only is the background of gastric cancer extensively discussed but the concept of early-onset gastric cancer (EOGC) is also introduced here and the reasoning behind the study of

such a group of gastric cancers from patients aged 45 years and younger is explained. Evidence behind the hypothesis that the molecular phenotype of these cancers may differ from that of conventional gastric cancer occurring in patients over the age of 45 begins with investigating loss of heterozygosity and microsatellite instability in **chapter 3**. In **chapter 4** we examine the role of RUNX3 in EOGC more closely and in **chapter 5** we compare the expression of an array of proteins in both EOGC and conventional gastric cancers. In **chapter 6** we examine the COX-2 -765 G/C promoter polymorphism in EOGC, conventional gastric cancer and stump cancers and in **chapter 7,** the molecule cyclin E is highlighted with respect to survival in EOGC. A ubiquitin ligase, cdc4, which shows specificity to cyclin E comes to the forefront in **chapter 8.** In **chapter 9** and **chapter 10** we take a more general look at gastric cancer as a whole, with in chapter 9 a closer look at diffuse-type gastric cancers using representational difference analysis and in chapter 10 a comparison of genetic changes occurring in primary gastric cancers, xenografts and cell lines. **Chapter 11** takes a step toward the oesophagus and examines the molecular-genetic validity of the histological diagnosis of collision tumor and **chapter 12** takes a look into the small intestine and describes an inflammatory myofibroblastic tumor, an uncommon tumor also occurs in young patients. Finally in **chapter 13,** there is a brief summary and discussion of the thesis, with a Dutch summary in **chapter 14**.

2

Early Onset Gastric Cancer: On the road to unraveling gastric carcinogenesis

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Abstract

Gastric cancer is thought to result from a combination of environmental factors and the accumulation of specific genetic alterations due to increasing genetic instability, and consequently affects mainly older patients. Less than 10% of patients present with the disease before 45 years of age (early onset gastric carcinoma) and these patients are believed to develop gastric carcinomas with a molecular genetic profile differing from that of sporadic carcinomas occurring at a later age. In young patients, the role of genetics is presumably greater than in older patients, with less of an impact from environmental carcinogens. As a result, hereditary gastric cancers and early onset gastric cancers can provide vital information about molecular genetic pathways in sporadic cancers and may aid in the unraveling of gastric carcinogenesis.

This review focuses on the molecular genetics of gastric cancer and also focuses on early onset gastric cancers as well as familial gastric cancers such as hereditary diffuse gastric cancer. An overview of the various pathways of importance in gastric cancer, as discovered through *in-vitro*, primary cancer and mouse model studies, is presented and the clinical importance of *CDH1* mutations is discussed.

Background

Gastric cancer is the fourth most common malignancy in the world and ranks second in terms of cancer-related death.[1] Eastern Asia, the Andean regions of South America and Eastern Europe have the highest incidence of gastric cancer whereas low rates are found in North America, Northern Europe and most countries in South eastern Asia.

Several classification systems have been proposed, but the most commonly used are those of the World Health Organization (WHO) and of Laurén who describes two main histological types, diffuse and intestinal.[2] Intestinal adenocarcinoma predominates in the high-risk areas whereas the diffuse adenocarcinoma is more common in low-risk areas.[3] Although classification varies between Japan and the West, attempts have been made recently to standardize the systems used.[4] Early gastric cancer is a term to describe carcinomas limited to the mucosa or to both the mucosa and submucosa, regardless of nodal status. The prevalence of this lesion is higher in countries such as Japan where a screening programme is carried out.

Gastric cancer is thought to result from a combination of environmental factors and the accumulation of generalized and specific genetic alterations, and consequently affects mainly older patients often after a long period of atrophic gastritis. The commonest cause of gastritis is infection by *Helicobacter Pylori*, which is the single most common cause of gastric cancer[5, 6] and has been classified by the WHO as a class I carcinogen since 1994.[7] The risk of infection varies with age, geographical location and ethnicity, but overall 15-20% of infected patients develop gastric or duodenal ulcer disease and less than 1% will develop gastric adenocarcinoma. [7]

The pattern of gastritis has also been shown to correlate strongly with the risk of gastric adenocarcinoma. The presence of antral-predominant gastritis, the most common form, confers a higher risk of developing peptic ulcers; whereas corpus predominant gastritis and multifocal atrophic gastritis leads to a higher risk of developing gastric ulcers and subsequent gastric cancer.[8, 9] The response to *Helicobacter Pylori* infection and the subsequent pattern of gastritis depends on the genotype of the patients and in particular a polymorphism in interleukin 1 beta, an inflammatory mediator triggered by *Helicobacter Pylori* infection, is known to be of importance.[10] Multifocal atrophic gastritis is usually accompanied by intestinal metaplasia and leads to cancer via dysplasia, and thus intestinal metaplasia is considered a dependable morphological marker for gastric cancer risk. Unlike intestinal gastric cancer, the diffuse type typically develops following chronic inflammation without passing through the intermediate steps of atrophic gastritis or intestinal metaplasia.

The incidence of adenocarcinoma of the stomach is declining worldwide and this is mainly accounted for by the decline in the intestinal type. There has also been a change in the anatomical distribution of this malignancy over recent decades, with a fall in the incidence of mid and distal gastric cancer and a progressive increase in adenocarcinoma of the proximal stomach and cardia. This fall in incidence may be explained by the decline in *Helicobacter pylori* infection and associated atrophic gastritis. The possibility that the increasing incidence of adenocarcinoma of the cardia may be due to nitrosative chemistry is discussed by McColl *et al.*[11]

The exact mechanism underlying the malignant transformation of the gastric mucosa following *Helicobacter pylori* infection still needs to be clarified, but it is believed that the combination of a virulent organism, a permissive environment and a genetically susceptible host is necessary.[12, 13] Different strains of the bacteria vary in their carcinogenic potential, with those containing cag genes inducing a greater degree of inflammation. *Helicobacter pylori* can also produce the vacuolating cytotoxin VacA responsible for epithelial damage which contributes to gastric carcinogenesis.

Bacterial factors (motility, adhesion, urease, cag pathogenicity), components of the host immune response (Toll-like receptors, adaptive immunity, IL1-B polymorphisms, MHCII), dietary co-factors such as high salt and decreased ascorbic acid, gastrin hormonal responses and decreased acid secretion are all thought to play a role in malignant transformation of the gastric mucosa.[14] In addition, IL-8, heat shock proteins and proinflammatory cytokines, nitric oxide and oxidative stress have also been implicated in gastric carcinogenesis. All these factors interact to alter host cell signaling, derange apoptotic and proliferative signaling and promote the accumulation of genetic alterations leading ultimately to neoplasia as reviewed by Stoicov *et al*.[14] Interestingly, despite the importance of *Helicobacter pylori* as an initiating factor in gastric carcinogenesis, the molecular pathology of *Helicobacter pylori* and non-*Helicobacter pylori* cancers cannot be easily separated, and it has been reported that *Helicobacter pylori* -related and non-related gastric cancers do not differ with respect to chromosomal aberrations.[15]

Diet is also a known etiological factor in gastric carcinogenesis, especially for intestinal type adenocarcinoma. An adequate intake of fruit and vegetables appears to lower the risk with ascorbic acid, carotenoids, folates and tocopherols acting as antioxidants.[3] Salt intake strongly associates with the risk of gastric carcinoma and its precursor lesions, and this risk is increased in certain genetically predisposed individuals.[16] Other foods associated with high risk in some populations, include smoked or cured meats and fish, pickled vegetables and chilli peppers.[3] Alcohol, tobacco and occupational exposure to nitrosamines and inorganic dusts have been studied in several populations, but the results have been inconsistent.[3]

Epstein-Barr virus (EBV) which is observed in 7%-20% of gastric cancers and which occurs slightly more frequently in diffuse-type gastric cancers, has also been implicated in gastric carcinogenesis.[17] In addition, it is known that a Bilroth II operation, which leaves a remnant or gastric stump, increases the risk of gastric carcinoma more than 15 years after surgery, [18] possibly due to bile reflux.

Curative therapy of gastric cancer involves surgical resection (discussed in a review by Ushijima *et al* [19]), and most commonly takes the form of a total or subtotal gastrectomy, with an accompanying lymphadenectomy. However, substantial mortality associated with gastric cancer has prevailed despite technical advances in surgery and adjuvant therapy, and the overall 5-year survival rate in patients with resectable gastric cancer remains between 10% and 30%. Furthermore, the lack of early pathognomic symptoms often delays the diagnosis and although endoscopy is widely regarded as the most sensitive and specific diagnostic test for gastric cancer, infiltration of the gastric wall, cannot always be detected. Clinical features, diagnosis and treatment of gastric cancer are reviewed comprehensively by Dicken *et al*. [20]

Gastric cancer can be categorized into conventional gastric cancer, occurring in patients older than 45, early-onset gastric cancer (EOGC), occurring under 45 years old and gastric cancer occurring as part of a hereditary syndrome. This review will first deal with the molecular pathology of gastric cancer in the broad sense before focusing on the findings specific to EOGC and hereditary gastric cancer and how they can be used to examine gastric cancer as a whole.

Molecular Pathology of Gastric Cancer

Tumorigenesis is considered a multistep process involving generalized and specific genetic alterations that drive the progressive transformation of cells into cancer. Central to this transformation are genetic or epigenetic changes in the genome which specifically activate

oncogenes with a dominant gain of function, and produce alterations in tumor suppressor genes which cause loss of function. Hanahan and Weinberg [21] describe in a compelling review how virtually all mammalian cells carry a similar molecular machinery regulating their proliferation, differentiation, and death and suggest that there are six essential alterations in cell physiology that collectively dictate malignant growth. These comprise self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. They outline that each of these capacities acquired during tumor development represents the successful breaching of an anticancer defense mechanism hardwired into cells and tissues. In addition, they mention genetic instability as a precondition for tumorigenesis through disruption of key molecules in order to "fast-forward" their carcinogenic potential. This framework described by Hanahan and Weinberg [21] can be applied to gastric cancer to highlight the important advances in molecular knowledge in the field of gastric cancer through *in-vitro*, primary tumors and mouse model experiments. It is however important to bear in mind that in practice, many molecular functions can play a role in a number of these six critical processes, and certain molecules disrupted in cancer have wide-ranging functions.

Self-sufficiency in growth signals and oncogenes

The dependence of tumors on communication from neighboring cells can be relinquished by the autonomous production of growth factors, which in turn results in the disruption of critical homeostatic mechanisms. In this manner, alterations in growth factor receptors, integrins and downstream signaling pathways serve as oncogenes, driving the carcinogenic process.

In gastric cancer there have been a number of oncogenes implicated. K-sam, which belongs to the family of fibroblast growth factor receptors (FGFR) is frequently overexpressed in diffuse-type gastric cancers due to gene amplification.[22, 23] Growth factors of the epidermal growth factor (EGF) family and their respective receptors including c-erbB2 oncogene are preferentially overexpressed in intestinal gastric cancers.[24, 25] In addition, the c-met proto-oncogene which is the receptor for the hepatocyte growth factor (HGF) is frequently overexpressed in gastric cancers of both diffuse and intestinal type. [22, 26]

Interestingly, many oncogenes which are key players in other epithelial cancers do not play a central role in gastric cancer. For example, Ras proteins are present in structurally altered oncogenic forms in about 25% of human tumors. Despite a mutant *K-ras* oncogene mouse model which showed pancreatic periductal lymphocytic infiltration and gastric mucous neck cell hyperplasia, [27] mutation of this oncogene occurs very rarely in gastric cancer. Similarly, the role of the Wnt pathway which is central to colorectal carcinogenesis, remains unclear in gastric cancer. Activating mutations of β-catenin have been described in gastric cancer [28] and immunohistochemical abnormalities are present in 22-27% of gastric cancer [29, 30]; yet as outlined later, the importance of *APC* mutations in gastric cancer is not yet fully understood. Of note, the transcription factor c-myc which is a transcriptional target of many pathways including the Wnt signalling pathway, functions as an oncogene in gastric cancer, with overexpression causing impaired differentiation and promoting growth.[31] Overexpression of c-myc has been described in over 40% of gastric cancers.[30]

Proliferation of the gastric mucosa is regulated by numerous different mechanisms, one of which is endocrine regulation via the hormone gastrin. *Helicobacter* infection induces hypergastrinemia and this has been causally linked to increased proliferation and cancer. Infection in the insulin-gastrin transgenic mouse produces an early increase in acid secretion and over time progresses to

atrophy, achlorhydria, hyperplasia of mucous cell compartment, metaplasia, dysplasia and invasive gastric cancer by 8 months of age.[32] Conversely, gastrin deficiency has also been reported to cause gastric adenocarcinoma.[33] In addition, *Helicobacter pylori* infection also alters gastric mucosal signaling through the CagA protein which interacts with several major growth-regulating signal transduction pathways including the Ras/MEK/ERK pathway[34] and the Src family of protein kinases.[35]

Intestinal homeostasis is disrupted in tumor cells through numerous mechanisms. COX-2, one of the rate-limiting enzymes for prostaglandin synthesis from arachidonic acid, is frequently upregulated in gastric adenocarcinomas and its expression is thought to be a relatively early event in gastric carcinogenesis.[36] In fact *Helicobacter pylori* infection has been reported to induce overexpression of COX-2.[37, 38] The role of COX-2 in gastric carcinogenesis is reviewed by Saukkonen *et al*.[39] Recently, the molecule C/EBP-β, a transcription factor for COX-2, [40] has been shown to play a role in gastric cancer.[30, 41]

Intestinal homeostasis is maintained under normal circumstances by molecules such as mucin core proteins (MUC), the expression of which has been found to vary in the different types of intestinal metaplasia.[42] In addition, due to the recent attention given to the activation and silencing of developmental pathways in cancer initiation and progression, focus has been drawn to the *Drosophila caudal*-related homeobox transcription factors Cdx1 and 2 which are important for early differentiation and maintenance of intestinal epithelial cells. Notably, ectopically-expressed Cdx2 was found to induce gastric intestinal metaplasia in two separate mouse models.[43, 44] However, progression to dysplasia and cancer occurred in only one of these models and the neoplastic role of Cdx2 remains speculative. Interestingly, both Cdx1 and Cdx2 have been shown to be expressed in intestinal metaplasia and gastric carcinomas in the human stomach.[45]

Insensitivity to growth-inhibitory signals

Cancer cells must evade antiproliferative signals if they are to survive, and the inactivation of tumor suppressor genes is a common event in gastric carcinogenesis. This can occur through mutations, deletions and epigenetic events. Methylation is an epigenetic process causing chromatin structure modulation, transcriptional repression and the suppression of transposable elements, and so is functionally equivalent to alterations such as mutations and deletions. However, a major difference is that epigenetic inactivation can be abrogated by DNA methylation inhibitors, and may be reversible. Hypermethylation in gastric cancer is extensively reviewed by Sato *et al*.[46] A genome-wide scan for aberrant methylation revealed silencing of nine genes in gastric cancers[47] and even in non-cancerous gastric mucosa, aberrant methylation can be present.[48] Of note, nitric oxide has also been shown to induce methylation. [49]

As outlined below, a vast array of tumor suppressor genes have been implicated in gastric cancer including *TP53*, *p16*, *APC*, *TGF*-β and related molecules, *TFF1*, *SOCS1*, *testin*, *FHIT* and *RUNX3*. On the other hand, tumor suppressor genes such as *PTEN*, despite playing a vital role in many carcinomas, do not have an important role in gastric carcinogenesis.[50]

The tumor suppressor gene *TP53* encodes for a nuclear protein, which plays a key role in tumor progression by regulating DNA repair, cell division and apoptosis. Low apoptosis rate and high cell proliferation are thought to be important factors for gastric cancer development and inactivation of p53 may be central to gastric carcinogenesis. Mutation and/or LOH at the *TP53* locus has been reported in approximately 30-40% of gastric cancers, but can also be found in intestinal metaplasia.[51]

The important cell cycle regulator, p16 (transcribed from *CDKN2A*) is lost in many gastric cancers, particularly cardia tumors,[30] and methylation has been shown to be of importance in the downregulation of this gene.[52] Additionally, EBV-associated gastric cancers have been shown to be more frequently associated with promoter methylation of *CDKN2A*. [53]

Adenomatous polyposis coli (*APC*) is a tumor suppressor gene which is mutated in sporadic and familial colorectal tumors. Under normal circumstances, APC binds to β-catenin and induces its degradation. Mutations of APC or β-catenin result in stabilization and accumulation of β-catenin, which can then translocate to the nucleus, where it acts as an oncoprotein, through transcription of target genes. This is a well-established mechanism in colorectal cancer, however less is known about the relative importance of this pathway in gastric cancer. Whereas some reports document relatively frequent occurrence of mutations,[54, 55] others find no mutations.[56, 57] The complexity is further increased by a report finding an inverse relationship between *APC* gene mutation in gastric adenomas and the development of adenocarcinoma.[58] Interstingly, *CDH1* and *APC* mutations have been reported to be synergistic in intestinal tumor initiation in mice[59] whereby double heterozygous animals showed a significant 5-fold increase in gastric tumor numbers, compared with Apc1638N animals.

Another feature in gastric carcinogenesis is the loss of growth inhibition by transforming growth factor (TGF)-β due to mutation of the Type II TGF-β receptor,[60] which leads to increased cell proliferation and reduced apoptosis. In addition, the cytoplasmic Smad4 protein, which transduces signals from ligand- activated TGFβ receptors to downstream targets, may be eliminated through mutation of its encoding gene. Loss of the locus encompassing *SMAD4* (18q21.1) and *DCC*(18q21.3) locus has been long known, [61] but more recently, haploid loss of this locus has been shown to initiate gastric polyposis and cancer in *Smad4+/-* mice.[62] Loss of the remaining *Smad4* wild-type allele was detected only in later stages of tumor progression, suggesting that haplo-insufficiency of Smad4 is sufficient for tumor initiation. Furthermore, bone morphogenetic protein (BMP)-2, a member of the BMP family belonging to the TGF-β superfamily has been shown to inhibit cell growth, and induced cell differentiation in normal and cancerous gastric cell lines.[63] Epigenetic silencing of the *BMP2* through methylation in gastric carcinomas has recently been described and noted to occur more frequently in diffuse type than intestinal type gastric cancers.[64]

Trefoil factor 1 (TFF1, also known as pS2) is synthesized and secreted by the normal stomach mucosa and by the gastrointestinal cells of injured tissues. The link between mouse Tff1 inactivation and the fully penetrant antropyloric tumor phenotype[65] prompted the classification of *TFF1* as a gastric tumor suppressor gene. Accordingly, altered expression, deletion, and/or mutations of the *TFF1* gene have been observed in human gastric carcinomas.[30, 66] The *Tff1* knock-out mice were subsequently shown to have overexpression of Cox-2 [67] and this inverse link between TFF1 and COX-2 has been confirmed in other studies.[30] TFF1 expression is in part regulated by interleukin-6 (IL-6), but the downstream intracellular signaling mechanisms of the IL-6 family of cytokines are not well understood. Mouse models have been used in an attempt to elucidate the function of the signal transducers and activators of transcription 1 and 3 (STAT1/3) and the Src-homology tyrosine phosphatase 2 (SHP2)-Ras-ERK, which are the two major signaling pathways emanating from gp130, the IL-6 family co-receptor in the gastrointestinal tract. [68] Gp130(757F) mice, with a 'knock-in' mutation abrogating SHP2-Ras-ERK signaling, developed gastric adenomas by three months of age. In contrast, mice harboring the reciprocal mutation ablating STAT1/3 signaling, or deficient in IL-6-mediated gp130 signaling showed impaired colonic mucosal wound healing. These gastrointestinal phenotypes are highly similar to

the phenotypes exhibited by mice deficient in trefoil factor 1 (pS2/TFF1) and intestinal trefoil factor (ITF)/TFF3 respectively. In further studies, mice lacking the SHP2 binding site on the gp130 were found to develop invasive gastric cancer by 30 weeks of age, [69] highlighting the need for balanced IL-6 signaling in maintaining gastric homeostasis. More recently, a gp130 mutant mouse model with exaggerated Stat3 activation [70] was found to share histological features of gastric polyps in ageing mice with monoallelic null mutations in Smad4 and the investigators suggest a novel link for cross-talk between STAT and SMAD signaling in gastric homeostasis. Downstream, the phosphorylated STAT protein translocates into the nucleus with subsequent activation of target genes. One of the STAT-activated genes, suppressor of cytokine signalling-1 (*SOCS-1*), is thought to be an important tumor suppressor gene in gastric cancer and can be inactivated though hypermethylation.[71, 72]

Since 1996, *FHIT*, a fragile locus exhibiting susceptibility to carcinogen-induced alterations, has been implicated in gastric carcinogenesis. [73] The consequent absence or reduction of FHIT protein expression is consistent with the proposal that the *FHIT* gene is a preferential target for environmental carcinogens and this may also account for the geographical differences found in *FHIT* aberrations.[74] More recent data showed that *FHIT* knock-out mice [75] develop tumors in the lymphoid tissue, liver, uterus, testis, fore-stomach and small intestine, together with structural abnormalities in the small intestinal mucosa suggesting that *FHIT* plays important roles in systemic tumor suppression and in the integrity of mucosal structure of the intestines. In another recent knock-out mouse model a tumor suppressor function for Testin was proposed [76] and it was suggested that *TES* may be a one-hit TS gene, as is *FHIT*.[77]

RUNX3 is another gene which has been hotly debated regarding its possible tumor suppressor function in gastric carcinogenesis. The debate arises due to the conflicting mouse models reported in the literature [78, 79] which are discussed by Levanon et al.[80] More recently, it has been found that *RUNX3* can be overexpressed in gastric tumors and that copy numbers of the *RUNX3* locus are seldom reduced in gastric cancer.[81]

Finally, insensitivity to growth-inhibitory signals can also be facilitated by *Helicobacter pylori* infection and it has been found that *Helicobacter pylori* decreases levels of the cyclin-dependent kinase inhibitor p27(kip1) in gastric epithelial cell, [82] which results in a decrease in apoptotic response to infection.[83] In addition, a recent mouse model lacking p27kip1 demonstrated that loss of p27 and *Helicobacter pylori* colonization cooperate to produce gastric cancer.[84]

Apoptosis

Acquired resistance toward apoptosis is a hallmark of most and perhaps all types of cancer.[21] Many of the signals that elicit apoptosis converge on the mitochondria, which respond to proapoptotic signals by releasing cytochrome C, a potent catalyst of apoptosis. Members of the Bcl-2 family of proteins, which are either proapoptotic (Bax, Bak, Bid, Bim) or antiapoptotic (Bcl-2, Bcl-XL, Bcl-W) govern mitochondrial death signaling through cytochrome C release and some of these proteins have been implicated in gastric cancer.[85] In addition, p53 can elicit apoptosis by upregulating expression of proapoptotic Bax in response to DNA damage. In fact, mutation of p53 results in the removal of a key component of the DNA damage sensor which can induce the apoptotic cascade. The ultimate effectors of apoptosis include an array of intracellular proteases termed caspases. Two "gatekeeper" caspases, −8 and −9, are activated by death receptors such as FAS or by the cytochrome C released from mitochondria respectively, and the Fas Ag pathway of apoptosis is recognized as the leading cause of tissue destruction during *Helicobacter pylori* infection. Early in infection, Fas antigen-mediated apoptosis depletes parietal and chief cell

populations, leading to architectural distortion. As infection progresses, metaplastic and dysplastic glands appear, which are resistant to Fas-mediated apoptosis. Fas antigen-deficient (lpr) mice infected with helicobacter, develop gastric cancer as early as 7 months after infection.[86] Nitric oxide, while usually discussed in the context of DNA damage and mutagenesis, can also directly influence mitochondrial pathways of apoptosis[87] and also potentially plays a role in multiple levels of cell signal transduction during *Helicobacter pylori* infection. Furthermore, bacterial factors may also directly induce apoptosis.[88]

Limitless replicative potential and telomeres

Growth signal autonomy, insensitivity to antigrowth signals, and resistance to apoptosis all lead to an uncoupling of a cell's growth program from signals in its environment.[21] Evolving premalignant cell populations also acquire unlimited replicative potential during tumor progression, and this is often through telomere maintenance. Telomeres are located at the ends of chromosomes and are responsible for the maintenance of chromosomal integrity. During cell division, these telomeres become shortened. However, in transformed cells, shortening of the telomeres is inhibited by reactivation of telomerases, preventing these cells from undergoing physiological senescence. Telomere maintenance is evident in virtually all types of malignant cells usually via upregulating expression of the telomerase enzyme resulting in unlimited multiplication of cells. There is a vast array of molecules involved in telomere maintenance and in gastric cancer expression of Protection of Telomeres-1 (POT1) is associated with telomere length and correlates with tumor stage.[89]

Angiogenesis

In order to facilitate an increase in size, tumors need to develop angiogenic ability. This is achieved by signalling through integrins and adhesion molecules on endothelial cells as well as through cell-matrix and cell-cell contacts. A large number of angiogenic factors have been identified in human malignancy, and gastric cancer is no exception. These include vascular endothelial growth factor (VEGF),(possibly induced via *Helicobacter Pylori*), [90] basic fibroblast growth factor (bFGF) and IL-8, [91] which are derived from tumor cells and participate mainly in neovascularisation within gastric cancer tissue. In addition, extracellular proteases receive signals from proangiogenic integrins, and help dictate the invasive capability of angiogenic endothelial cells. The ability to induce and sustain angiogenesis seems to be acquired in discrete steps during tumor development, via an "angiogenic switch." Tumors appear to activate this switch by changing the balance of angiogenesis inducers and inhibitors.

Tissue Invasion and Metastases

Tumor metastases are the cause of 90% of human cancer deaths. Successful invasion and metastasis requires all the attributes which are needed for initial carcinogenesis, combined with alterations in proteins involved in the tethering of cells to their surroundings in a tissue. The most widely observed alteration in cell-to-environment interactions in cancer involves E-cadherin, a homotypic cell-to-cell interaction molecule ubiquitously expressed on epithelial cells and playing a central role in gastric cancer (as discussed in detail under hereditary gastric cancer). Invading and metastasizing cancer cells travel through a range of tissue microenvironments to which they adapt by producing a changing spectrum of integrin α or β subunits on their cell surfaces. The activation of extracellular proteases and the altered binding specificities of cadherins, CAMs, and integrins are central to the acquisition of invasiveness and metastatic ability and MMP2 has been shown to be of particular

importance in gastric cancer.[92] Through comparison of gastric cancer SAGE libraries, 54 candidate GC-specific genes have been identified including melanoma inhibitory activity (MIA) and matrix metalloproteinase-10 (MMP-10), which is important in metastasis and correlated with poor prognosis.[93]

Genomic instability

Under normal circumstances, the occurrence of mutations is prevented by the maintenance of genomic integrity by an array of DNA-monitoring and repair enzymes and karyotypic order is guaranteed by checkpoints that operate at critical times in the cell's life. Yet cancers occur relatively frequently in the human population, causing some to argue that the genomes of tumor cells must acquire increased mutability in order for the process of tumor progression to reach completion in several decades time. Derangement of specific components of the genomic "caretaker" systems has been used as an explanation and the loss of function of these key players is believed to result in genome instability and the generation of mutant cells with selective advantages.[21]

A variable number of numerical or structural genetic aberrations have been reported in gastric cancer cells, including those involving changes in chromosomes and DNA copy number, but the significance of these changes and the underlying genetic changes are unknown. Loss of Heterozygosity studies and comparative genetic hybridization (CGH) analyses have identified several loci with significant allelic loss, indicating possible tumor suppressor genes important in gastric carcinoma. Common targets of loss or gain include chromosomal regions 1q, 3p, 4, 5q, 6q, 9p, 17p, 18q and 20q. [61, 94-97] It has been shown that different histopathologic features can be associated with distinct patterns of gains and losses, supporting the notion that gastric tumors evolve through distinct genetic pathways.[98] Persistent inflammation caused by *Helicobacter pylori* is also known to cause genetic instability through the generation of mutagenic substances such as reactive oxygen species [99] and reactive nitrogen species [37] which may act to directly damage the host cell DNA. *Helicobacter pylori* has also been implicated in limiting the defense against such insult by decreasing the antioxidant properties of the gastric mucosa.[100] Such a direct gastric mutagenic through oxidative DNA damage in *H. pylori* infection, has been shown in transgenic mouse models. [101]

Genetic instability at the level of microsatellite instability (MSI) occurs in many sporadic human tumors and the relation between microsatellite instability and gastric carcinoma has received considerable attention. This is due to the discovery that MSI may be found in sporadic carcinomas that are characteristic of hereditary nonpolyposis colorectal cancer (HNPCC) [102], a syndrome where germline mutations of the mismatch repair genes are present. The levels of MSI found in gastric carcinomas from both Western and Eastern populations is probably in the region of up to 15%.[103] Wu *et al*. demonstrated that the subset of sporadic gastric cancer with high frequency MSI (MSI-H) showed a distinct clinicopathologic and genetic profile from those with a low frequency (MSI-L) or microsatellite stable (MSS) genotype. [104] However, whereas the role of microsatellite instability and DNA mismatch repair gene defects in HNPCC is unquestionable and well established, the relevance of this phenomenon in gastric cancer is far from clear and currently has limited clinical value.[103] Somatic mutations of mismatch repair (MMR) genes such as *hMLH1* or *hMSH2* are extremely rare in sporadic gastric cancers, with only one mutation found, in *hMSH2*. [105] However, MSI positive tumors can still lack *hMLH1* protein expression and many studies suggest that hypermethylation of the *hMLH1* promoter region may be the principal mechanism of gene inactivation in sporadic gastric carcinomas with a high frequency

of MSI.[106, 107]. The role of microsatellite instability in gastric carcinoma is comprehensively reviewed by Hayden *et al.* [103]

As is evident from the preceding text, multiple genetic and epigenetic alterations in oncogenes, tumor-supressor genes, cell-cycle regulators, cell-adhesion molecules, DNA repair genes and genetic instability as well as telomerase activation are implicated in human stomach cancer. However, particular combinations of these alterations differ in the two histological types of gastric cancer.[98] The diffuse phenotype in gastric cancer (hereditary and sporadic) is related to reduced E-cadherin expression [108] and loss of E-cadherin is probably the fundamental defect in diffuse type gastric carcinoma, providing an explanation for the observed morphological phenotype of discohesive cells with loss of polarity and gland architecture. Recent findings with E-Cadherin, C/EBP-β, TFF1 and COX-2 expression emphasize the fact that diffuse and intestinal cancers differ at a molecular level.[30] However, the onset of carcinogensis is strongly associated with *Helicobacter pylori* infection as reviewed by Nardone *et al* [109] and indeed there is close correlation between diffuse GC and *Helicobacter pylori* infection, similar to that found with intestinal type cancer.[110] Studies have also shown decreased E-Cadherin expression in the gastric mucosa of infected individuals.[111] Therefore, even if the intestinal and diffuse type GCs are characterized by a different genetic pathway, they depend upon the same triggering factor.

In addition to the wealth of research looking at specific genes of interest in gastric cancer, gene expression array data has also revealed a vast amount of information on gastric cancer. However, putting these pieces together into a chronological narrative remains daunting, and a recent approach involving a meta-analysis of previous expression array data hints at how complicated the "gastrome" can be.[112] There is by no means a clear-cut pattern of mutations in gastric cancers, and the genetic research can often be hampered by the diversity of changes that are induced by *Helicobacter pylori* infection, diet, ageing and other environmental factors. Tumors are unquestionably riddled with genetic changes yet we are faced with an unsolvable puzzle with respect to a temporal relationship. In order to solve this problem, one approach is to investigate tumors that are less influenced by these environmental factors. Gastric cancers occurring in young patients, known as early-onset gastric cancers, provide an ideal background on which to try and uncover the initiating stages in gastric carcinogenesis. In addition hereditary cancers can often illuminate discrete mutations that can initiate the pathway of gastric carcinogenesis.

Hereditary Cancer and E-Cadherin

The existence of a familial form of gastric cancer has been known since the 1800s when multiple cases of gastric cancer were noted in the Bonaparte family.[113] Approximately 1-3% of gastric cancers arise as a result of inherited gastric cancer predisposition syndromes, one of which is hereditary diffuse gastric cancer, caused by a germline mutation in the *CDH1* gene, encoding E-Cadherin. Gastric cancer in its hereditary form can also be caused by germline mutations of the *TP53* tumor suppressor gene which occurs in the Li-Fraumeni syndrome.[114] In addition, *BRCA2* gene mutations are associated with familial aggregations of not only breast but also of stomach, ovarian, pancreatic, and prostate cancers. [115, 116] A proportion of hereditary nonpolyposis colorectal cancer (HNPCC) kindreds (the so-called Lynch II families) are associated with a high frequency of extracolonic carcinomas, most commonly affecting the endometrium and stomach [117] and these are known to harbor microsatellite instability. [118] In addition, gastric cancer occurs infrequently in polyposis syndromes such as familial adenomatomous polyposis

(FAP) [119] and Peutz-Jegers syndrome. [120, 121] The American Society for Gastrointestinal Endoscopy recommends endoscopic surveillance for high-risk individuals (history of gastric adenoma, FAP, HNPCC, Peutz-Jeghers syndrome, and Menetrier's disease) every 1 to 2 years.

Approximately 30% -40% of all hereditary diffuse gastric cancer (HDGC) families carry *CDH1* germline mutations.[122] The other 60%-70% of HDGC remain genetically unexplained and are probably caused by alterations in other genes. It has been suggested there may be a need for p53 mutation screening in families with hereditary gastric cancer lacking *CDH1* germline mutations.[123] No evidence has been found for a role of germline mutations in *SMAD4* and *Caspase-10* in these families. [123] E-cadherin is a member of the cadherin family of homophilic cell adhesion proteins that are central to the processes of development, cell differentiation, and maintenance of epithelial architecture.[124] It is the predominant cadherin family member expressed in epithelial tissue and is localised at the adherens junctions on the basolateral surface of the cell. Mutations in *CDH1* were initially identified in 1998 in three Maori families from New Zealand that were predisposed to diffuse gastric cancer. [125] Since then, similar mutations have been described in more than 40 additional HDGC families of diverse ethnic backgrounds.[122] Preliminary data from these families suggest that the penetrance of *CDH1* gene mutations is high, ranging between 70% and 80%.[126] In order to qualify for a diagnosis of HDGC, the following criteria must be met [127]: two or more documented cases of diffuse gastric cancer in first or second degree relatives, with at least one diagnosed before the age of 50 years; or three or more cases of documented diffuse gastric cancer in first or second degree relatives, independent of age of onset. Death from gastric cancer in these families has occurred in individuals as young as 14 years and the majority of affected persons die aged less than 40 years. There also appears to be an increased frequency of cancers occurring at other sites such as the breast, colorectum, and prostate in these mutation carriers.[126] However, inclusion of associated cancers into the definition of HDGC is not yet recommended.[127]

Abnormalities of CDH1

CDH1 is a tumor suppressor gene and loss or inactivation of the remaining normal allele is a required initiating event in susceptible individuals with a germline mutation. Analysis of all reported genetic abnormalities in *CDH1* found in HDGC reveals that the majority are inactivating mutations (splice site, frameshift, and nonsense) rather than missense mutations. Furthermore, *CDH1* germline mutations are evenly distributed along the E-cadherin gene, in contrast with the clustering in exons 7-9 observed in sporadic diffuse gastric cancer.[128] Loss of heterozygosity as the "second hit" does not appear to be frequent in HDGC. Instead, hypermethylation of the *CDH1* promoter is likely to be a common cause of down-regulation or inactivation of the second *CDH1* allele in HDGC tumors.[129] The verdict has not yet been reached concerning the possible carcinogenic role of coexistent infection with *Helicobacter pylori* on a *CDH1* mutated background, and it remains possible that *Helicobacter pylori* infection as well as dietary and other environmental influences may modify the disease risk in these susceptible individuals.[130]

Clinical Management

There remains some uncertainty about clinical management and disease outcome after genetic testing for *CDH1* mutations, and the psychosocial burden it poses on family members is well recognised.[127] Once a *CDH1* mutation has been identified in an asymptomatic individual, they are presented with the options of endoscopic surveillance or prophylactic gastrectomy. The aim of surveillance is of course to identify an early curable lesion but the value of endoscopy is

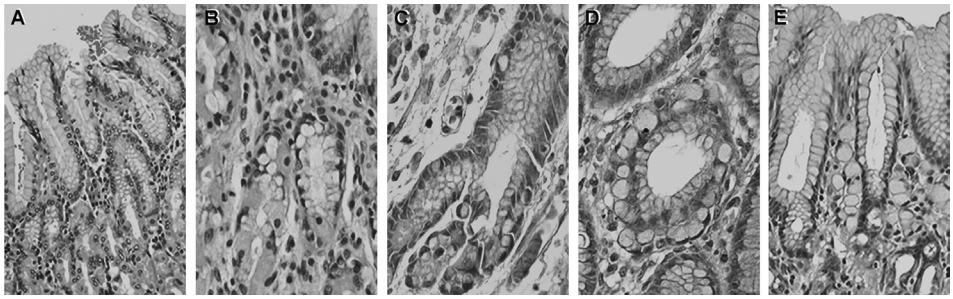

Figure 1 Proposed model for the development of diffuse gastric cancer in E-cadherin mutation carriers: background changes of gastric mucosa encompassing mild chronic gastritis and foveolar hyperplasia (A); in-situ signet-ring cell carcinoma (foveolae and glands with intact basement membrane totally or partially lined by signet ring cells) (B and C); "early"(C) and overt (D) pagetoid spread of signet-ring cells below the preserved epithelium of glands/foveolae; early invasive intramucosal signet-ring cell carcinoma (E). ***(See page 193 for colour figure)***

unproven due to the difficulty of detecting intramucosal lesions.[131] Some reports have found an antral predominance of HDGC[132] whereas other reports show no antral predominance in HDGC and alarmingly, have calculated the likelihood of detecting HDGC from five random biopsies at between 1-50%.[133] Current clinical recommendations for surveillance, propose a 30 minute endoscopy every six months by an endoscopist experienced in the diagnosis of early gastric cancer. [131] In an effort to improve the diagnostic yield of surveillance endoscopy in the upper gastrointestinal tract, techniques such as chromoendoscopy are advised.[134] In addition, all patients having surveillance should be entered into a research protocol comparing different endoscopic methods.[131] Obviously there is a great need for the development of molecular markers in the serum or in gastric brushing in order to overcome the sampling bias inherent in current random biopsy sampling methods.

Prophylactic gastrectomy is clearly a huge undertaking and not without significant psychological and clinical effects on the patient. To date, it has been demonstrated that prophylactically resected stomachs from different families all carried multifocal signet ring cancer. [135, 136] Importantly, surveillance using endoscopy (with chromoendoscopy in some cases) and multiple mucosal biopsies failed to identify intramucosal carcinoma in all of the published cases surveyed. Thus, the estimated risk reduction of gastric cancer by gastrectomy is significant. However, it also follows that since there is an estimated 70% penetrance, a universal policy of prophylactic gastrectomy would result in 30% of HDGC mutation carriers receiving an unnecessary operation. On the other hand, it is not known whether such lesions are present in all individuals with CDH1 mutations, and whether all pathologic changes would develop into clinically significant lesions. [126] The age at which genetic testing should be performed is not yet clear from the current evidence, as at least five subjects have been reported to have developed this lethal cancer before the age of 18 years. However, since the implications of the diagnosis are far reaching, some believe that genetic screening should be reserved until the patient is able to give informed consent.[131]

Model of development of HDGC

In situ carcinoma lesions have been identified in gastrectomy specimens from patients with CDH1 mutation [133, 135] whereby foveolae and glands with intact basement membrane are totally or

partially lined by signet ring cells. Some *in situ* lesions are restricted to the neck zones (Figure 1 B and C). On the basis of the findings of these studies, a model for the development of diffuse gastric cancer in E-cadherin mutation carriers was proposed, as depicted in Figure 1, and encompassing the following lesions: *in situ* signet-ring cell carcinoma (B and C), pagetoid spread of signet-ring cells below the preserved epithelium of glands/foveolae (C – "early" pagetoid spread and D – "overt" pagetoid spread), and invasive carcinoma (E – early invasive intramucosal signet-ring cell carcinoma). The discrepancy between the numerous invasive carcinoma foci and the low number of in situ carcinoma lesions suggests that invasion of the lamina propria by signet ring cells may occur without a morphologically detectable in situ carcinoma. HDGC develops in the setting of background changes of gastric mucosa encompassing mild chronic gastritis, foveolar hyperplasia (Figure 1A), tufting, globoid change and vacuolization of superficial epithelim.[133]

The gastric mucosa in *CDH1* germline mutation carriers is normal until the second *CDH1* allele is inactivated. It is postulated that this downregulation occurs in multiple cells in the gastric mucosa, accounting for the multifocal tumor lesions which develop and [133] environmental and physiological factors such as diet, carcinogen exposure, ulceration and gastritis are suggested to promote this downregulation event. The tumor then expands slowly until additional genetic events, probably in combination with an altered microenvironment, lead to clonal expansion and tumor progression. Interestingly, because the second hit does not involve somatic, irreversible, mutation of the second *CDH1* allele, but rather more frequently occurs via methylation [129], it is plausible that the early stage lesions may be reversible. Identification of patients with germline *CDH1* mutations paves the way for studies to increase our understanding of the mechanisms by which these mutations ultimately lead to sporadic cancer as well as HDGC. The genetic changes occurring after the inactivation of *CDH1* remain to be elucidated.

Early Onset Gastric Cancer

Gastric cancer is rare below the age of 30; thereafter it increases rapidly and steadily to reach the highest rates in the oldest age groups, both in males and females. The intestinal type rises faster with age than the diffuse type and is more frequent in males than in females. Early onset gastric cancer (EOGC) is defined as gastric cancer presenting at the age of 45 or younger. Approximately 10% of gastric cancer patients fall into the EOGC category,[137] although rates vary between 2.7%[138] and 15%[139] depending on the population studied. Young patients more frequently develop diffuse lesions which often arise on the background of histologically "normal" gastric mucosa. It is postulated that genetic factors may be more important in EOGC than in older patients as they have less exposure to environmental carcinogens,[140] thus these cancer could provide a key tool in the unraveling the genetic changes in gastric carcinogenesis. *Helicobacter pylori* may still play a role in the development of gastric cancer in young patients, [141, 142] although this is likely to involve a much smaller percentage of patients than in the older age group.

Approximately 10% of young gastric cancer patients have a positive family history,[137] some of which are accounted for by inherited gastric cancer predisposition syndromes, and as discussed under hereditary gastric cancer, the underlying genetic events are not always known but can involve *CDH1* germline mutations.[143, 144] The 90% without a family history emphasizes that the occurrence of gastric cancer in young patients remains largely unexplained.

The clinicopathological features of gastric carcinoma are said to differ between the young and elderly patients and it has been claimed that young patients have a poorer prognosis.[145] Others

Table 1

Characterisitics of EOGC	Reference
more common in females	138,148
diffuse type cancer more common	138,148
often multifocal	151
no intestinal metaplasia	138,148
lack of MSI	149,152,153
infrequent Loss of heterozygosity	153
Low COX2 expression	31
infrequent loss of TFF1 expression	31
no loss of RUNX3	82
gains at chromosomes 17q, 19q and 20q	157

report that tumor staging and prognosis for young patients is similar to older patients and depends on whether the patients undergo a curative resection.[137, 139, 146] Young patients with gastric cancer in the United States are more likely to be black, Asian or Hispanic.[147] Relative to older patients, young patients have a female preponderance, a more frequent occurrence of diffuse cancer and less intestinal metaplasia.[137, 147, 148] This predominance of females is considered by some to be due to hormonal factors.[149] Cancers in young patients are more often multifocal than in older patients [150] as is also seen in HDGC.[133]

Thus early onset gastric cancers are known to have a different clinicopathological profile than conventional gastric carcinomas. This suggests that they represent a separate entity within gastric carcinogenesis and indeed evidence at a molecular genetic level supports this (Table 1). It is known that microsatellite instability which usually occurs at a frequency of 15% in older gastric carcinomas is consistently absent in young patients [148, 151, 152] and this is despite analysis of distal tumors (where MSI is usually commoner) and inclusion of mixed and intestinal type tumors (diffuse tumors generally have less MSI).[153] However, it may be possible that geographical factors play a role.[154] A lack of microsatellite instability excludes the mutator phenotype as an important predisposing factor in the development of early-onset gastric cancer. This contrasts with the situation in colorectal cancer where 58% of patients without HNPCC aged under 35 years showed evidence of microsatellite instability.[155] EOGC also contrasts with colorectal cancer with respect to the tumor suppressor gene *APC* which causes the familial adenomatosis polyposis syndrome. The role of *APC* in EOGC is limited and nuclear expression of β-catenin has not been found to differ between EOGC and conventional gastric cancers.[30]

The molecular expression profile of EOGC and conventional gastric cancers have been found to differ and EOGC have a COX-2 Low, TFF-1 expressing phenotype.[30] A higher incidence of aberrant E-Cadherin expression in EOGC regardless of histological type [148] has also been reported, although a more recent report which compared EOGC with conventional cancers showed that aberrant expression of E-Cadherin correlated significantly with diffuse type.[30] The expression of low molecular weight isoforms of cyclin E are also known to differ between EOGC and conventional cancers, being present in 35% of EOGCs, compared to in 8% of conventional gastric cancers and 4% of stump cancers. In addition, immunohistochemical staining of low molecular weight isoforms of cyclin E were found to be an independent positive prognostic indicator in early-onset gastric cancer (unpublished data).

Recent literature regarding *RUNX3* has excluded it as having a tumor suppressor function in EOGC,[81] although as some of the cell lines used in this study were from conventional gastric cancers, the implications may be more far-reaching and include conventional gastric cancer. Gains at chromosomes 17q, 19q and 20q have been found in EOGC with comparative genomic hybridization [156] and LOH findings have also shown that losses are infrequent in EOGC. [152]

As we can see, EOGCs differ from conventional gastric cancers, not only at a clinicopathological level, but also at a molecular genetic level. If this is indeed due to the fact that the environment plays a smaller role in the triggering of the carcinogenic pathway, the investigation of this group of cancers may reveal genetic changes which assist in the task of putting forward a multistep pathway for gastric cancer.

Future Prospectives

In summary, observations of human cancers and animal models implicate numerous genetic changes in gastric cancer. However, the multistep pathway of carcinogenesis which occurs in some epithelial cancers and which has allowed accurate clinical and pathologic characterization is not yet elucidated in gastric cancer. Gastric cancer exhibits heterogeneity in histopathology and molecular changes that have impeded the uncovering of a temporal molecular pathway. Gastric cancers often occur without any consistent mutational abnormality and with a considerable variation in pathogenesis ranging from a stepwise progression of changes (gastritis -> metaplasia -> dysplasia -> invasive carcinoma) to tumors arising in the absence of a precursor lesion.

Further study of hereditary gastric cancers and early onset gastric cancer as unique subsets of gastric cancer may aid us in the search for a gastric cancer pathway. The rarity of hereditary gastric cancer often hampers research in this field. On the other hand, early-onset gastric cancers, although relatively scarce, provide an ample number of cancers if they can be collected at a nationwide level. Recent developments of techniques adapted to paraffin material will maximize the number of cancers available for research and the use of SNP Chips, expression arrays, kinase arrays and other new technologies, combined with EOGC material may set us well on the road to unraveling gastric carcinogenesis.

Abbreviations

World Health Organisation (WHO), early-onset gastric cancer (EOGC), fibroblast growth factor receptors (FGFR), epidermal growth factor (EGF), hepatocyte growth factor (HGF), Adenomatous polyposis coli (APC), transforming growth factor (TGF), bone morphogenetic protein (BMP), Trefoil factor 1 (TFF1), signal transducers and activators of transcription (STAT), Src-homology tyrosine phosphatase 2 (SHP2), suppressor of cytokine signalling-1 (SOCS-1), Protection of Telomeres-1 (POT1), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), melanoma inhibitory activity (MIA), matrix metalloproteinase-10 (MMP-10), microsatellite instability (MSI), hereditary nonpolyposis colorectal cancer (HNPCC), familial adenomatous polyposis (FAP), hereditary diffuse gastric cancer (HDGC),

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3

Early onset gastric carcinomas display distinct molecular characteristics within gastric carcinogenesis

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Abstract

Gastric cancer is thought to result from a combination of environmental factors and accumulation of specific genetic alterations, consequently affecting mainly older patients (>50 years of age). Less than 10% of patients present with the disease before 45 years of age, and these young patients are thought to develop carcinomas with a different molecular genetic profile to that of sporadic carcinomas occurring at a later age.

Forty early onset gastric carcinoma resected specimens were characterised for microsatellite instability (MSI) and loss of heterozygosity status using 22 polymorphic microsatellite markers. Twenty-four biopsies were additionally evaluated for the presence of MSI. No MSI was observed in any of the cases analysed. Losses were infrequent, but were most common for the D1S234 (26.1%) and D1S1676 (17.4%) markers, flanking the *RUNX3* gene; for the p53ALU (23.1%) and TP53 (15.4%) markers, near the *TP53* gene; and for the D16S2624 (17.2%) marker, near the E-cadherin (*CDH1*) gene. All cases presenting loss of *CDH1*, as well as 6/7 cases with loss of *TP53*, displayed aberrant staining of the corresponding proteins, pointing to a functional role of these proteins in early onset gastric carcinogenesis. No germline *CDH1*, *TP53* or *RUNX3* mutations were detected in any of the cases analysed. No correlation was observed between non-functional E-cadherin and the histological type of the tumours analysed. Finally, the presence of Epstein-Barr virus was not detected in any of the cases analysed. Based on these results, early onset gastric carcinomas appear to have distinct characteristics from gastric carcinomas occurring at a later age.

Introduction

The incidence of gastric cancer has been diminishing over the last decade, largely due to an improvement in sanitation, but it still is the second most common cause of cancer-related death in the world [1]. Gastric cancer result from a combination of environmental factors and accumulation of specific genetic alterations. Environmental factors such as the microbial agents *H. pylori* and the Epstein-Barr virus (EBV) may play an important role in gastric carcinogenesis [2-4]. When associated with host genetic predisposition, these factors may increase the likelihood of genetic alterations [5, 6].

So far, few genes have been implicated in hereditary gastric carcinogenesis. Loss of function of the adhesion molecule E-cadherin has been shown to be strongly associated with diffuse-, but not intestinal-type gastric carcinoma [7, 8]. Gastric cancer can also occur as part of the hereditary non-polyposis colorectal cancer (HNPCC) syndrome [9, 10], whereby alterations in the mismatch repair genes (*hMLH1*, *hMSH2* and *hMSH6*) are responsible for colorectal, gastric and endometrial tumour formation. Disrupted function of mismatch repair genes manifests as microsatellite instability, and has been reported in 15-39% of sporadic gastric cancers [11, 12]. It has further been shown that gastric cancer occurs, albeit rarely, as part of the Li-Fraumeni syndrome [13, 14].

Many reports have studied genomic instability and the resulting allelic imbalance of sporadic gastric carcinomas through comparative genomic hybridisation (CGH) and loss of heterozygosity (LOH) studies. Aberrations most commonly found in gastric carcinogenesis include gains at 1p32-p36, 17p13, 17q24 and 20q13, regions known to contain the oncogenes *MYC*, *API4* (*SURVIVIN*) and *PCNA*, and losses at 3p12, 3p25, 5q14-q23, 16q22, 17p13 and 18q12, encompassing the demonstrated tumour suppressor genes (TSGs) *FHIT*, *APC*, *CDH1*, *TP53* and *SMAD4* [15-17]. Recently, *RUNX3* (1p36) has been postulated as a TSG and was shown to be inactivated in gastric cancers [18].

Gastric cancer affects mainly older patients (>50 years of age), with only a small percentage (<10%) of patients presenting with the disease earlier than 45 years of age. However, it is thought that these young patients develop carcinomas with a different molecular genetic profile to that of sporadic carcinomas occurring at a later age (referred to throughout as "conventional" carcinogenesis) [19-21]. Although genetic characterisation of gastric carcinomas has been the subject of several studies, few have addressed this issue regarding early onset gastric cancer (EOGC). This study therefore aims at defining EOGC by studying markers and protein expression previously used to characterise gastric carcinomas.

Methods

Patient Material

Formalin-fixed, paraffin-embedded tissue of 40 gastric carcinomas and 24 tumour-derived biopsies from patients aged 45 years or younger were retrieved from the Pathology Department of the Academic Medical Centre (Amsterdam, The Netherlands), other hospitals in the Netherlands following a search in a nation-wide database, the Department of Pathology at the Jorvi Hospital (Espoo, Finland) and Johns Hopkins Medical Institute (Baltimore, USA). The tumours were classified by an experienced gastrointestinal pathologist (GJAO) according to the Laurén classification as intestinal (n=10), diffuse (n=21) and mixed-type (n=9) gastric adenocarcinomas. Patient information is depicted in Table 1. Only case Y11 had a history of familial gastric carcinomas.

Table 1 Patient and tumour characteristics

Resection	Sex (M/F)	Mean Age (Range)	Biopsy*	Sex (M/F)	Mean Age (Range)
Diffuse	6/15	30.2 (18-42)	Diffuse	4/9	29.1 (25-36)
Intestinal	6/4	36.3 (27-45)	Intestinal	6/1	30.1 (20-45)
Mixed	3/6	33.4 (26-43)	Mixed	1/2	28.7 (23-38)

*: no histotype could be made for one of the biopsies

This study was conducted in accordance with the ethical standards of our institutional medical ethical committee on human experimentation, as well as in agreement with the Helsinki Declaration of 1975, as revised in 1983.

RNA In Situ Hybridisation for EBV Detection

In situ hybridisation for EBER1 nuclear RNA transcripts was performed as previously described [22], except as follows: the fluorescein label of the hybridised probe molecules was detected by incubation for 30 minutes with rabbit anti-FITC (Dako; 1/500 diluted in PBS containing 10% human AB serum). Staining was revealed by the Power Vision kit (ImmunoLogic), followed by visualisation using DAB as chromagen. The DT-35 probe was used as a positive RNA *in-situ* hybridisation control [23].

Helicobacter pylori Detection

Helicobacter pylori vacA s region genotype and the presence of the *cagA* gene were determined by PCR followed by agarose gel electrophoresis, as previously described [24].

Microdissection and DNA Isolation

Tumour tissue was microdissected from deparaffinised haematoxylin-stained 5µm tissue sections. The percentage of tumour cells ranged from 60 to 90%. For each case non-neoplastic tissue was obtained from a tumour-free lymph node or, when not available, from normal gastric mucosa. DNA isolation was performed as previously described [25]. For confirmation of losses, as well as for recent cases, DNA was isolated using the PUREGENE® DNA Isolation Kit (Gentra Systems, Minneapolis, USA) according to manufacturer's instructions. DNA concentrations were measured using PicoGreen® dsDNA Quantitation Kit (Molecular Probes, Leiden, The Netherlands).

Microsatellite Analysis

The markers used for microsatellite analysis are depicted in Table 2. One of the primers for each marker was fluorescently labelled. PCR was performed with annealing temperatures ranging from 50°C to 64°C for 35 to 40 cycles using Platinum *Taq* (Life Technologies, Inc., Rockville, MD) in a PTC-100 or PTC-200 cycler. Analysis was carried out using an automated ABI 377 or ABI 3100 sequencer (Applied Biosystems) with a Genescan™ 350ROX size standard (Applied Biosystems) and the manufacturer's Genescan® 2.1 software.

Scoring of LOH and MSI

Normal samples with two distinctly sized alleles of a particular marker were termed informative. For all informative markers the allelic imbalance factor was calculated as described by Cawkwell

Table 2 Markers for LOH and MSI analysis

Marker	Position	Repeat Type	Putative Tumour Suppressor Gene/Remarks
D1S1676	1p36	Tetranucleotide	*RUNX3/AML2/CBFA3/PEBP2aC*
D1S234	1p36	Dinucleotide	*RUNX3/AML2/CBFA3/PEBP2aC*
D3S1478	3p21	Dinucleotide	*FHIT*; *hMLH1*
D5S346	5q21	Dinucleotide	*APC*; MSI consensus marker
D5S107	5q11.2-qter	Dinucleotide	*APC*
D9S171	9p21	Dinucleotide	$p14^{ARF}$; $p16^{INK4A}$/*CDKN2A*; $p15^{INK4B}$/*CDKN2B*
D10S2491	10q23	Dinucleotide	*PTEN*
D13S260	13q12.3	Dinucleotide	*BRCA2*
D14S68	14q24.3-qter	Dinucleotide	frequently deleted region in Barret carcinomas
D16S2624	16q22.1	Tetranucleotide	*CDH1*
D17S855	17q	Dinucleotide	*BRCA1*
p53ALU	17p13	Pentanucleotide	*TP53*
TP53	17p13	Dinucleotide	*TP53*
D18S64	18q21.3	Dinucleotide	*DCC*; *DPC4/SMAD4*; *SMAD2*
D18S858	18	Trinucleotide	*DCC*; *DPC4/SMAD4*; *SMAD2*
D19S883	19p13.3	Dinucleotide	*LKB1/STK11*
D19S886	19p13.3	Dinucleotide	*LKB1/STK11*
D19S565	19p13.3	Dinucleotide	*LKB1/STK11*
D21S49	21q22.3	Dinucleotide	*TFF1*; *TFF2*; *TFF3*
BAT25	4q12	Mononucleotide	MSI consensus marker
BAT26	2p16	Mononucleotide	MSI consensus marker
BAT40	1p13.1	Mononucleotide	MSI consensus marker

The sequences of the primers and their corresponding locations on the chromosomes were obtained from the Genome Data Base (*http://www.gdb.org*), the Cooperative Human Linkage Centre (*http://lpg.nci.nih.gov/CHLC*), or Genéthon (*http://www.genethon.fr*)
The primers used for the p53ALU marker are as follows, represented 5′ to 3′:
Forward: GAATCCGGGAGGAGGTTG
Reverse: AACAGCTCCTTTAATGGCAG
The primers used for the TP53 marker are as follows, represented 5′ to 3′:
Forward: AGGGATACTATTCAGCCCGAGGTG
Reverse: ACTGCCACTCCTTGCCCCATTC

and colleagues [26]. LOH was assumed if the allelic imbalance factor was >1.6 or <0.6. Observed losses were confirmed using at least 10 ng of DNA to prevent induced LOH.

MSI was classified according to international criteria. Tumours were scored as stable (MSS) when no shifts were observed, as MSI-low (MSI-L) when shifts were seen in <40% of markers and as MSI-high (MSI-H) with instability in ≥40% of markers. MSI had to be confirmed at least once, to ensure reproducibility.

Immunohistochemistry

Immunohistochemistry (IHC) was performed as previously described [27]. The following antibodies were used: DO-7 and BP53-12 (Neomarkers) for p53 (1:2000 dilution); HECD-1 (Thamer) for E-cadherin (1:2000 dilution); MLH-1 (Pharmingen) for MLH1 (1:100 dilution); MSH-2 (Oncogene Research Products) for MSH2 (1:200 dilution); and MSH-6 (Transduction Labs) for MSH6 (1:400 dilution). All incubations were performed overnight at 4ºC except for p53 (one hour incubation at room temperature). p53 immunoreactivity was scored as positive when

Table 3 Primer sequences and PCR conditions for RUNX3 mutation detection

Exon	Primers	PCR annealing temperature (°C)	SSCP conditions
1	F: TTCATTCATTCCCCGTGGCACT R: CCAGGGCCCTGGGCTATTGT	59	*Sty* I overnight digestion 0.8X MDE, 20ºC
2	F: GCCGCTGTTATGCGTATTC R: CACTGCTCCCGAGGCTCT	58*	0.8X MDE, 8ºC
3	F: CAATGCTGAAATGGCGAGGC R: TAAGCTGTCCCCCTGCATCC	61	12% Acrylamide/10% glycerol, 20ºC
4	F: GCCAACCACCTGCCTCTATT R: AGGGGGCTCGGTGGCACTT	59	0.8X MDE, 20ºC
5	F: TCTGGGGAAGCAACGGCTGA R: CAGCCCCTCCCTCCGTG	58	12% Acrylamide, 20ºC
6	F: CTGACCTCTTCCCTGCTGTC R: CCCATCACTGGTCTTGAAGG	59	*Sty* I overnight digestion 0.8X MDE, 20ºC

*: amplification carried out with BIO-X-ACT Polymerase (Bioline Ltd, London, UK)

staining was seen in 10% or more tumour cells. E-cadherin immunoreactivity in the cytoplasm was considered aberrant and membranous staining in 30% or more tumour cells was graded as positive.

PCR/SSCP

Non-neoplastic DNA was used for mutation detection PCRs.

CDH1 was amplified and analysed by PCR/SSCP as described previously [28].

RUNX3 primer sequences and PCR/SSCP conditions are depicted in Table 3. SSCP was performed as described for *CDH1*.

The *TP53* mutational hotspot (exons 5 to 8) was amplified using the primers from the OPERON kit (Operon Technologies, Inc., Atlantic City, USA) using manufacturer's instructions.

Sequencing Analysis

Reamplified abnormal SSCP bands were sequenced on an ABI 377 or 3100 automated sequencer (Applied Biosystems) using the ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and the original primers. All *TP53* amplicons were sequenced using the same procedure. All sequence alterations detected were confirmed by a second independent PCR.

Statistical Analysis

For statistical analysis the χ^2 test was used. A value of $p<0.05$ was considered statistically significant.

Results

To study molecular alterations in EOGC, 40 gastric carcinomas were characterised through analysis of polymorphic microsatellite markers and IHC, as well as mutation detection on relevant cases. Twenty-four biopsies were additionally evaluated for the presence of MSI. Detailed patient information is depicted in Table 1.

MSI status was assessed by PCR through the analysis of three polymorphic markers (BAT 25, BAT 26 and BAT 40). All cases studied (38 EOGCs and 24 biopsies) were microsatellite stable for the three markers. Furthermore, IHC analysis of the mismatch repair proteins MLH1, MSH2

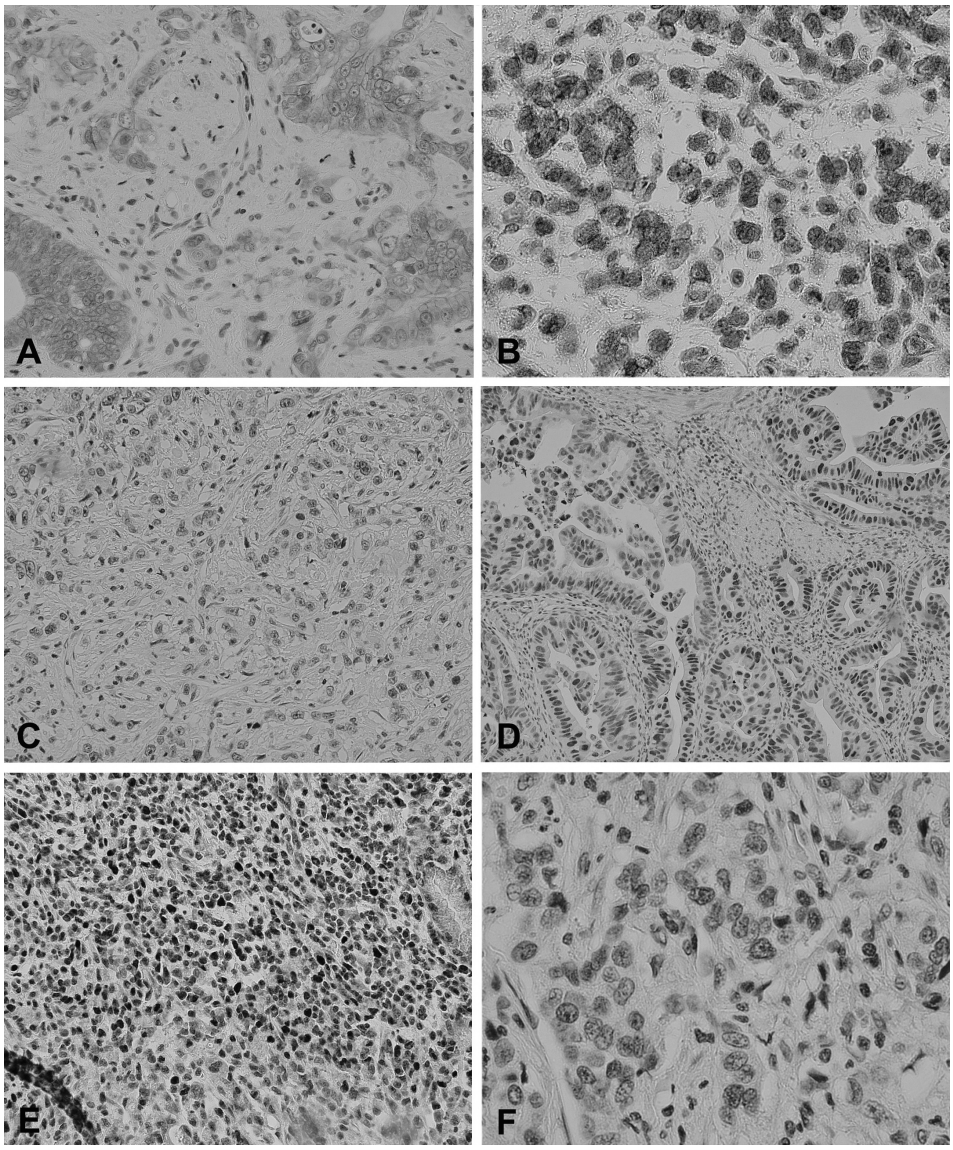

Figure 1 IHC results for E-cadherin (A, B), p53 (C, D) and MSH6 (E, F). Normal protein expression was detected for E-cadherin in case Y13 (A), for p53 in case Y15 (C), and for MSH6 in case Y65 (E). Abnormal protein expression was detected as follows: E-cadherin cytoplasmic expression was seen in case Y28 (B), nuclear p53 overexpression was observed in case Y11 (D), and absence of MSH6 expression was seen in case Y89 (F). ***(See page 194 for colour figure)***

Table 4 Results of LOH analysis

	Histology	D1S234	D1S1676	D3S1478	D5S346	D5S107	D9S171	D10S2491	D13S260	D14S68	D16S2624	D17S855	p53ALU	TP53	D18S64	D18S858	D19S883	D19S886	D19S565	D21S49
Y01	Diffuse	R	R	R	R	NI	NI	R	NI	R	LOH	R	NI	R	NI	R	R	R	R	R
Y03	Mixed	IC	IC	R	R	NI	NI	R	R	R	R	R	R	R	R	R	R	NI	NI	R
Y04	Intestinal	LOH	LOH	NI	NI	R	NI	R	R	NI	LOH	NI	NI	LOH	NI	R	R	NI	NI	NI
Y05	Mixed	R	R	R	R	R	NI	R	R	R	LOH	R	NI	NI	LOH	R	R	R	R	R
Y11	Intestinal	LOH	LOH	R	LOH	LOH	NI	R	R	NI	R	R	NI	LOH	NI	LOH	R	NI	NI	NI
Y13	Intestinal	NI	R	NI	R	R	R	R	R	R	NI	R	NI	R	NI	NI	NA	R	NI	R
Y15	Diffuse	LOH	LOH	NI	R	R	R	NI	NI	R	R	R	R	NI	R	R	R	R	R	NI
Y22	Diffuse	NA	NA	NI	NI	R	R	R	R	R	NI	R	NI	NI	R	NI	NI	R	NI	R
Y23	Diffuse	R	R	R	R	R	R	R	R	NI	R	R	R	R	NI	NI	R	R	R	NI
Y27	Diffuse	R	R	NI	R	R	R	R	R	NI	R	R	NI	NI	R	R	R	R	R	R
Y28	Diffuse	R	R	R	R	R	R	R	R	R	R	R	NI	R	R	R	R	NI	R	R
Y29	Diffuse	R	R	R	R	R	R	R	NI	NI	R	R	R	NI	NI	NI	R	NI	NI	R
Y42	Diffuse	LOH	R	R	R	R	NI	R	R	R	R	NI	LOH	R	R	R	NA	R	R	R
Y43	Mixed	NI	NI	R	R	R	R	R	R	NI	R	NI	NI	R	NA	NA	R	IC	NI	R
Y44	Intestinal	R	R	R	R	R	R	R	R	R	R	R	NI	R	R	R	R	NI	NI	R
Y45	Intestinal	NI	R	R	R	R	LOH	R	R	NI	LOH	R	NI	NI	NI	NI	R	NI	R	LOH
Y50	Mixed	NI	NI	R	R	R	R	LOH	R	NI	R	R	NI	NI	R	R	R	R	R	R
Y55	Intestinal	LOH	R	R	R	R	NI	R	R	NI	R	R	NI	NI	NI	R	R	NI	R	R
Y57	Diffuse	R	R	NI	R	R	R	R	R	R	NI	R	NI	R	NI	R	R	R	NI	LOH
Y61	Diffuse	R	R	NI	R	R	R	R	NI	NI	NI	NI	NI	NI	R	R	R	R	R	R
Y20	Diffuse	R	R	R	R	R	R	R	NI	NI	NI	R	LOH	R	R	R	NA	R	R	R
Y26	Mixed	NA	NA	NI	R	R	NI	R	R	R	IC	NI	R	R	R	NI	R	R	R	NI
Y39	Diffuse	R	NI	R	R	R	R	R	NI	R	LOH	R	NI	R	R	R	R	R	R	R
Y48	Diffuse	NI	NI	R	R	NI	R	R	R	R	NI	R	R	R	R	LOH	R	NI	NA	R
Y51	Diffuse	NA	NA	R	R	NI	R	R	R	NI	R	R	IC	R	R	NI	NI	R	R	R
Y60	Diffuse	R	NI	R	R	R	R	NI	R	R	R	R	R	NI	R	NI	R	NI	R	NI
Y64	Diffuse	NA	NA	R	NI	R	R	NA	R	R	R	IC	NI	IC	IC	NA	IC	IC	R	IC
Y65	Diffuse	R	R	NI	NI	R	NI	R	R	LOH	R	R	NI	R	R	R	R	NI	NI	NI
Y66	Diffuse	R	R	R	R	R	NI	R	R	R	NI	R	LOH	R	R	NI	NI	R	R	NI
Y67	Intestinal	R	R	R	NI	R	R	R	R	NA	R	NI	NI	LOH	NI	NI	NA	R	R	R
Y69	Intestinal	R	NI	R	NI	NI	R	R	R	R	R	R	NI	R	LOH	R	R	R	NI	R
Y70	Mixed	NA	NI	NI	R	R	R	R	R	NI	R	R	NI	NI	NI	NI	R	IC	NI	R
Y71	Intestinal	NI	R	R	R	R	R	R	R	R	NI	R	NI	R	R	R	R	R	R	R
Y77	Mixed	LOH	LOH	R	NI	R	LOH	R	NI	R	R	R	R	R	NI	NI	NI	R	R	R
Y85	Mixed	IC	NI	R	R	R	NI	R	R	R	R	NI	NI	R	NI	R	NA	R	IC	R
Y86	Diffuse	NA	NA	R	R	R	R	R	R	R	R	R	R	NI	R	R	R	R	R	NI
Y87	Diffuse	R	NI	R	NI	R	R	R	NI	NI	NI	R	NI	R	NI	R	NA	R	R	NI
Y88	Diffuse	NI	R	LOH	R	NI	NI	R	R	R	R	NI	R	NI	R	R	R	R	R	R
Y89	Intestinal	NI	IC	R	R	R	NI	R	NI	NI	NI	R	NI	LOH	IC	R	NA	R	R	NI
Y90	Mixed	IC	NI	R	NI	R	NI	R	R	R	R	R	NI	R	R	R	R	NI	R	R
	Loss	6/23	4/23	1/30	1/31	1/34	2/26	1/37	-	1/24	5/29	-	3/13	4/26	2/23	2/26	-	-	-	2/28

R: retention of heterozygosity; LOH: loss of heterozygosity; NI: non-informative; IC: inconclusive; NA: no amplification possible

and MSH6 was performed for the same panel. All cases were positive for the presence of MLH1, MSH2 and MSH6 in the tumour cells, with the exception of one case where immuno negativity of MSH6 suggested a mutation in *hMSH6* (Figure 1F).

All 40 EOGCs were subsequently analysed for LOH at sites previously shown to be implicated in gastric carcinogenesis or in syndromes containing gastric cancer in their phenotype, as well as at loci thought to harbour TSGs. The markers used, as well as their chromosomal location, are depicted in Table 2. LOH was infrequently observed within the panel studied: 20/40 (50%) tumours showed no losses at any of the loci studied, and only 7/40 (17.5%) tumours displayed losses at two or more loci. The most frequent losses were detected for the D1S234 (6 losses in 23 informative cases, 26.1%) and D1S1676 (4/23, 17.4%) markers, flanking the *RUNX3* gene; for the p53ALU (3/13, 23.1%) and TP53 (4/26, 15.4%) markers, near the *TP53* gene; and for the D16S2624 (5/29, 17.2%) marker, near the *CDH1* gene. The remaining markers analysed showed little or no LOH. Table 4 depicts the complete results of the LOH analysis.

To substantiate the role of p53 and E-cadherin in EOGC, the expression of these proteins was determined by IHC. The immunohistochemical data, related to histological phenotype and LOH information of the corresponding gene, is depicted in Table 5. No antibody was commercially available for RUNX3 detection.

Six out of seven (85.7%) EOGCs exhibiting loss of markers proximal to *TP53* also presented aberrant p53 expression (demonstrated by p53 overexpression (Figure 1D), suggestive of a *TP53* mutation), consistent with loss of functional p53 in these tumours. Overall, p53 overexpression was detected in 13/38 (34.2%) cases. A significant association was observed between aberrant p53 expression and intestinal-type gastric carcinomas ($p<0.05$).

E-cadherin expression in the tumour cells was compared to that of the normal mucosa. All five cases with LOH for the marker proximal to *CDH1* displayed abnormal E-cadherin expression (four cases showed cytoplasmic staining (Figure 1B), one case showed total lack of protein expression), consistent with loss of protein function. In a total of 38 cases analysed, 23 (60.5%) displayed abnormal protein expression (14/23 showing negative membranous staining; 17/23 showing cytoplasmic staining). No association was found between aberrant staining and tumour histological type.

In order to determine if familial mutations were the cause of the aberrations found in the cases with LOH, mutation detection by PCR/SSCP was performed for *CDH1*, *TP53* and *RUNX3* for the cases with corresponding LOH. No alterations were found in the mutational hotspot (exons 5 to 8) in any of the seven cases exhibiting LOH near *TP53*. Analysis of the complete *CDH1* gene was possible for two of the five cases (Y4 and Y5) with LOH near *CDH1*. Amplification of all the exons was not possible for the remaining three cases due to poor material quality. The three alterations that were found (Table 6) are all previously described polymorphisms [28]. Analysis of the complete *RUNX3* gene was possible for two of the six cases (Y4 and Y11) with LOH near *RUNX3*; no alterations were found.

Infection by EBV, previously demonstrated to affect patterns of loss in gastric carcinomas [25], was determined in 31 EOGC cases with intact RNA as demonstrated by EBER 1 *in situ* hybridisation. The presence of EBV was not detected in any of the cases analysed.

Finally, *H. pylori* infection was detected in 11 out of 29 (37.9%) cases analysed. This value is in accordance with literature data concerning the Netherlands population [29].

Discussion

This study attempted to characterise EOGC based on parameters previously shown to be relevant to the onset of gastric carcinogenesis: microsatellite instability, regions harbouring LOH reflecting inactivation of possible TSGs, and infection by EBV.

Microsatellite analysis of *BAT 25*, *BAT 26* and *BAT 40* revealed no MSI in any of the 62 cases analysed in this study. Expression analysis of the proteins responsible for mismatch repair confirmed these results, with only one case (Y89) being negative for MSH6. Of note, Kariola *et al* demonstrated in a panel of HNPCC patients that carriers of *MSH6* mutations effectively repaired GT mismatches [30]. These observations suggest that the mismatch repair mechanism is not affected by loss of MSH6. These results lend support to previous reports: Hayden *et al* found no MSI in 10 British cases younger than 40 years [31] and one MSI-H case in 16 Portuguese patients under 40 years [32] using 12 polymorphic microsatellite markers; Shiao *et al* found no MSI-H cases in a panel of 101 Italian EOGCs (≤40 years) analysed at five microsatellite loci [33]; Lim *et al* detected one MSI case in 76 EOGCs (<30 years) analysed by the BAT 26 marker [34]. Despite geographical differences in the prevalence of MSI in "conventional" gastric cancers (15-39%) [11], our data, supports previous reports indicating that whereas some gastric cancers occur due to defects in the mismatch repair system, EOGCs do not arise via this mechanism.

Numerous publications have reported regions of LOH that occur frequently in gastric carcinogenesis, the most prominent being those at 1p, 5q and 17p [35-38], including *APC* and *TP53*. Indeed, loss of function of p53 has been linked to gastric carcinogenesis [13, 14, 38, 39]. An overall analysis of the LOH results obtained in this study demonstrated fewer regions of loss in EOGC when compared to reports on "conventional" gastric tumours [35-38, 40, 41]. Five loci,

Table 5 Correlation between tumour histotype, and E-cadherin and p53 LOH status and protein expression

	E-cadherin				**p53**					
	Expression		**LOH**		**Expression**		**LOH (p53ALU)**		**LOH (TP53)**	
Histology	Normal	Abnormal	Loss	Retention	Normal	Abnormal	Loss	Retention	Loss	Retention
Diffuse	9	11	2#	19	15	5	3#	7	0	12
Intestinal	5	5	2#	8	4	6*	0	0	4&	3
Mixed	1	7	1#	8	6	2	0	3	0	6

*: statistically significant ($p<0.05$)
#: all cases display abnormal protein expression
&: 3/4 cases display abnormal protein expression

Table 6 Mutation detection results (CDH1)

Intron/Exon	**Codon**	**Nucleotide change***	**Cases**
Promoter	-	-284 C→A	Y4 and Y39
Intron 4	-	+10 G→C	Y5
Exon 13	-	2076 T→C	Y4 and Y5

*: According to the *CDH1* sequence deposited in the Ensembl library (ID number ENST00000261769); the A of the ATG initiator codon corresponds to position 1

corresponding to the locations of *TP53*, *CDH1* and *RUNX3*, all showed LOH in over 10% of cases analysed, suggesting a role for these genes in EOGC.

For *TP53*, two markers (p53Alu and TP53) near the gene were used. A total of seven cases exhibited deletions (Table 4). The observation that no case exhibited loss of both markers simultaneously may be explained by chromosomal instability previously demonstrated to be prevalent at the 17p13 locus [42, 43]. In order to substantiate the role of p53 within EOGC, expression of the protein was also assessed. All seven LOH cases presented with abnormal p53 expression (Table 5), representative of a non-functional protein. IHC analysis of the complete EOGC panel revealed a statistically significant association between p53 abnormalities and intestinal-type EOGCs ($p<0.05$). This strengthens previous suggestions of a higher prevalence of chromosomal instability in intestinal-type gastric carcinomas when compared to those of the diffuse-type [44, 45]. Intestinal-type gastric carcinomas tend to have a higher proliferative rate than those of diffuse-type [39], and this has been shown to be correlated with abnormal p53 expression [46]. The percentage of cases presenting abnormal p53 expression is in keeping with previous reports for gastric carcinogenesis [39, 47].

The marker proximal to the *CDH1* locus was lost in a significant proportion of informative cases (5/29, 17.2%) (Table 4). IHC analysis of E-cadherin showed all five cases to display abnormal protein expression (Table 5). The EOGCs revealed a total of 23/38 (60.5%) cases bearing abnormal E-cadherin expression. This level of abnormal expression appears higher than that found for "conventional" gastric carcinomas [47, 48]. Abnormal E-cadherin expression and *CDH1* alterations have previously been linked with diffuse-type gastric carcinoma: Kozuki *et al* correlated abnormal expression of E-cadherin, and other cell-cell adhesion components with undifferentiated gastric carcinomas [49]. Guilford *et al* described *CDH1* germline mutations in eight hereditary gastric cancer families, all of which presented with diffuse-type gastric carcinomas [8, 28]. Given these data, it is interesting to note that in the present panel, while loss of protein expression occurred more frequently than in "conventional" gastric carcinomas, no association was seen between cases harbouring E-cadherin abnormalities and histological type of EOGCs, at odds with a strong association between E-cadherin loss and "conventional" gastric carcinomas of the diffuse type [48-50].

Losses at 1p36 have been shown to be common events in gastric carcinogenesis [37, 41]. LOH in this region was among the highest found for the present panel. This locus harbours *RUNX3*, demonstrated by Li *et al* to be lost in a series of gastric carcinomas and gastric carcinoma-derived cell lines [18]. Both 1p36 markers, flanking the *RUNX3* gene, showed frequent LOH (Table 4), and four cases harboured losses of both markers. The frequent loss might imply a relevant role for *RUNX3* in EOGC development, in accordance with previous reports, where loss of the gene was shown to be associated with stimulated proliferation and suppressed apoptosis of gastric epithelial cells [18]. There is, however conflicting evidence, as the expression of RUNX3 in the gastric mucosa of mice differed significantly between strains analysed [51]. Furthermore, the gastric hyperplasia observed in the *Runx3*$^{-/-}$ mice used in Li's study was not observed in the mouse strain studied by Levanon and colleagues [52]. As the possibility of gross deletions or rearrangements at 1p36 in gastric tumours – resulting in LOH – cannot be ruled out, it remains to be seen whether genes other than *RUNX3*, located in the region, are important in EOGCs.

Interestingly, infection by EBV has been correlated with induction of RUNX3 expression [53]. Previous studies [3, 25] have also suggested that infection by EBV may play a role in the development of gastric carcinomas. However, all cases with intact RNA (31/40) were negative for

EBV, excluding it as an important factor in EOGC. Levels of infection (37.9%) by *H. pylori*, were similar to those found in the general population [29].

To address the possibility that patients showing LOH at the relevant loci developed the disease early on due to a familial predisposition, screening for germline mutations was performed for *TP53* (mutated within the Li-Fraumeni syndrome), *CDH1* (mutated within hereditary diffuse gastric cancer) and *RUNX3*.

Although a complete screen could not be performed for *CDH1* and *RUNX3*, none of the cases yielded germline mutations for the genes studied, strengthening the argument that these patients (with the possible exception of Y11) did not develop gastric carcinoma due to a familial predisposition.

The results obtained in this study point to EOGC being a separate entity within gastric carcinogenesis. By contrast to older age groups, many of the TSGs addressed in this study, defects in the mismatch repair mechanisms, and infection by EBV do not appear to play a predominant role in EOGC. Identification of pathways consistently disrupted in EOGC will help explain the nature underlying the early presentation of the tumour. It remains to be elucidated whether *RUNX3* is important in EOGC, or indeed whether another gene on 1p36 may be important for this subtype of gastric carcinogenesis.

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4

Exclusion of *RUNX3* as a tumour-suppressor gene in early onset gastric carcinomas

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Abstract

Recent studies claim a critical role for *RUNX3* in gastric epithelial homeostasis. However, conflicting results exist regarding *RUNX3* expression in stomach and its potential role as a tumour-suppressor gene (TSG) in gastric carcinogenesis. Our aim was to evaluate the role of *RUNX3* in early onset gastric carcinomas (EOGCs). We analysed 41 EOGCs for RUNX3 aberrations using loss of heterozygosity (LOH), fluorescence *in situ* hybridisation (FISH) and immunohistochemistry (IHC) analyses. LOH of markers flanking *RUNX3* was relatively common, indicating that loss of the gene may play a role in gastric carcinogenesis. However, FISH analysis of selected cases and a panel of 14 gastric carcinoma-derived cell lines showed widespread presence of multiple copies of centromere 1. While *RUNX3* copy numbers were generally equal to or fewer than those of centromere 1, at least two copies were present in almost all cells analysed. Accordingly, a subpopulation of tumour cells in 12/37 cases showed RUNX3 protein expression. However, expression was not detected in the adjacent non-tumorous mucosa of any case. Together, these observations indicate that chromosome 1 aberrations occur frequently in EOGCs and are reflected in the LOH and IHC patterns found. Our findings refute a role for *RUNX3* as a TSG in EOGCs.

Introduction

In spite of the steady decline in its occurrence, gastric carcinoma is the second deadliest malignant neoplasm worldwide (Parkin et al., 1999). The disease has a high mortality, with a five-year survival rate of approximately 20% (Jemal et al., 2004). It is therefore crucial to acquire a better understanding of this disease, and to identify the genes responsible for its onset.

Our group has recently reported a significant percentage of early onset gastric carcinomas (EOGCs, defined as cases presented earlier than 45 years of age) to show loss of heterozygosity (LOH) at the 1p36 locus (Carvalho et al., 2004), where a known regulator in major developmental pathways, *RUNX3*, is found. Instability within this region has been previously reported in the context of gastric carcinoma (Kokkola et al., 1998; Sakakura et al., 1999; Wu et al., 2002). Interestingly, the 1p36 region has been thought to harbour one or several tumour-suppressor genes (TSGs) for colon cancer since it was shown that the introduction of a normal human 1p36 chromosome fragment into colon carcinoma cells markedly suppressed their tumorigenicity (Tanaka et al., 1993).

RUNX3, together with *RUNX1* and *RUNX2*, make up the RUNX family of genes, which play decisive roles in definitive haematopoiesis, osteogenesis, neurogenesis and thymopoiesis (Inoue et al., 2002; Komori et al., 1997; Levanon et al., 2002; Okuda et al., 1996; Taniuchi et al., 2002; Woolf et al., 2003). Their dysregulation has been linked to several pathologic conditions, including leukaemia and cleidocranial dysplasia (Lee et al., 1997; Mundlos et al., 1997). In adults, RUNX3 is mainly expressed in the haematopoietic system with high levels of mRNAs and proteins in spleen, thymus and blood (Bangsow et al., 2001).

Mouse models have shown the Runx3 protein to form complexes with Smad3 that transmit activin signals, making Runx3 a target in the TGF-β pathway (Hanai et al., 1999; Li et al., 2002). Further, *Runx3* has been shown to be important in murine embryonic development, as *Runx3* $^{-/-}$ mice display several neurological, immunological and gastrointestinal defects (Brenner et al., 2004; Ehlers et al., 2003; Fainaru et al., 2004; Inoue et al., 2002; Ito & Miyazono, 2003; Levanon et al., 2002; Li et al., 2002; Woolf et al., 2003). Noteworthy, all inbred *Runx3* $^{-/-}$ mice die at birth, or very soon thereafter.

Recent studies have linked *RUNX3* to gastric epithelial homeostasis and gastric carcinogenesis. Li *et al.* (Li et al., 2002) recently reported hemizygosity of *RUNX3* in gastric cancer specimens, as well as reduction of *RUNX3* expression in tumour cells when compared to normal mucosa. *Runx3* $^{-/-}$ mice were also generated as part of the study of Li and colleagues. All mice died within 10 days, and their gastric mucosa was found to be hyperplastic. In light of the role of *Runx3* in the TGF-β pathway (Crawford et al., 1998; Fainaru et al., 2004), this observation was explained, at least in part, by resistance to the growth-inhibitory activity of TGF-β and to TGF-β-induced apoptosis (Li et al., 2002).

Contrasting results were obtained by Brenner and colleagues. *Runx3*$^{-/-}$ mice, generated from a different genetic background (Brenner et al., 2004; Levanon et al., 2002) to that of Li and colleagues, survived for up to 24 months, did not display any early-onset gastric hyperplasia and none went on to develop gastric carcinoma. Additionally, these Runx3-deficient mice registered early-onset colitis, and 80% went on to develop late-onset gastric hyperplasia, which the authors contended to be secondary to the onset of colitis. The authors also report a lack of expression of RUNX3 in normal gastric mucosa through immunohistochemistry (IHC) and RNA *in situ* hybridisation.

The current study therefore aimed at clarifying the role of RUNX3 in early onset gastric carcinogenesis.

Materials and Methods

Patient Material

Formalin-fixed, paraffin-embedded tissue of 41 gastric carcinomas from patients aged 45 years or younger were retrieved from the Pathology Department of the Academic Medical Centre (Amsterdam, The Netherlands), other hospitals in the Netherlands following a search in a nation-wide database, the Department of Pathology at the Jorvi Hospital (Espoo, Finland) and Johns Hopkins Medical Institute (Baltimore, USA). The tumours were classified by an experienced gastrointestinal pathologist (GJAO) according to the Laurén classification as intestinal- (n=10), diffuse- (n=22) and mixed-type (n=9) gastric adenocarcinomas. Patient information is depicted in Table 1. Only case Y11 had a first-degree relative diagnosed with gastric carcinoma.

This study was conducted in accordance with the ethical standards of our institutional medical ethical committee on human experimentation, as well as in agreement with the Helsinki Declaration of 1975, as revised in 1983.

Fluorescent activated cell sorting (FACS)

Cases Y5, Y11 and Y102 were flow-sorted as described previously (Corver et al., 2005).

Cell culture

Cell lines TMK1, NUGC4, HSC39, HS746T, ST42, KATOIII, GP220, AGS, MKN45, MKN7, MKN74 and MKN28 were cultured in RPMI 1640 (GIBCO BRL, MD) supplemented with 10% Foetal Calf Serum (FCS, GIBCO BRL, MD, USA) and 2% Penicillin-Streptomycin (GIBCO BRL, MD). Cell lines HM02 and HM51 were cultured in DMEM (GIBCO BRL, MD) supplemented with 10% FCS (GIBCO BRL, MD) and 2% Penicillin-Streptomycin (GIBCO BRL, MD).

FISH

Pre-treatment of Y4 paraffin sections was performed as described previously (Haralambieva et al., 2002).

FACS-sorted cells from paraffin sections of cases Y5, Y11 and Y102 were applied to Superfrost Plus glass slides and air-dried overnight at room temperature. The slides were treated with 1 M $Na_2B_4O_7$ for 30 minutes, washed in phosphate-buffered saline pH 7.4 (PBS), fixed in 1% formalin/PBS for 10 minutes, washed in PBS, dehydrated and dried.

Nuclear spreads of the 14 cell lines were prepared as described previously (Miki et al., 2004). The nuclear spreads were air-dried and baked overnight at 60°C. The slides were treated with

Table 1 Patient and tumour characteristics

Histology	Sex (M/F)	Mean age (range)
Diffuse	6/16	30.2 (18-42)
Intestinal	6/4	36.3 (27-45)
Mixed	3/6	33.4 (26-43)

Table 2 Results of LOH and IHC analysis

	Histology	D1S234	D1S1676	RUNX3 IHC (tumour cells)	Type of non-tumorous adjacent gastric mucosa present
Y01	Diffuse	R	R	-	Non-specialised gastric mucosa
Y03	Mixed	IC	IC	-	Cardia- and fundic-type gastric mucosa
Y04	Intestinal	LOH	LOH	-	Fundic-type gastric mucosa
Y05	Mixed	R	R	-	Fundic-type gastric mucosa
Y11	Intestinal	LOH	LOH	+	None
Y13	Intestinal	NI	R	-	None
Y15	Diffuse	LOH	LOH	-	Fundic-type gastric mucosa
Y22	Diffuse	NA	NA	-	Transitional- and antral-type gastric mucosa
Y23	Diffuse	R	R	-	Fundic-type gastric mucosa
Y27	Diffuse	R	R	-	Cardia- and fundic-type gastric mucosa
Y28	Diffuse	R	R	-	None
Y29	Diffuse	R	R	-	Transitional- and antral-type gastric mucosa
Y42	Diffuse	LOH	R	-	Antral-type gastric mucosa
Y43	Mixed	NI	NI	-	Transitional- and antral-type gastric mucosa
Y44	Intestinal	R	R	-	Non-specialised gastric mucosa
Y45	Intestinal	NI	R	-	None
Y50	Mixed	NI	NI	+	None
Y55	Intestinal	LOH	R	-	Antral-type gastric mucosa
Y57	Diffuse	R	R	-	Fundic-type gastric mucosa
Y61	Diffuse	R	R	+	Non-specialised gastric mucosa
Y20	Diffuse	R	R	-	Transitional- and antral-type gastric mucosa
Y26	Mixed	NA	NA	ND	ND
Y39	Diffuse	R	NI	-	Antral-type gastric mucosa
Y48	Diffuse	NI	NI	-	Fundic-type gastric mucosa
Y51	Diffuse	NA	NA	ND	ND
Y60	Diffuse	R	NI	-	Non-specialised gastric mucosa
Y64	Diffuse	NA	NA	-	Antral-type gastric mucosa
Y65	Diffuse	R	R	-	Fundic-type gastric mucosa
Y66	Diffuse	R	R	+	Non-specialised gastric mucosa
Y67	Intestinal	R	R	+	Fundic-type gastric mucosa
Y69	Intestinal	R	NI	+	Fundic-type gastric mucosa
Y70	Mixed	NA	NI	+	None
Y71	Intestinal	NI	R	-	Antral-type gastric mucosa
Y77	Mixed	LOH	LOH	+	None
Y85	Mixed	IC	NI	+	None
Y86	Diffuse	NA	NA	ND	ND
Y87	Diffuse	R	NI	+	None
Y88	Diffuse	NI	R	+	Fundic-type gastric mucosa
Y89	Intestinal	NI	IC	-	None
Y90	Mixed	IC	NI	+	Fundic-type gastric mucosa
Y102	Diffuse	R	R	ND	ND
	Loss	6/24	4/24		

R: retention of heterozygosity; LOH: loss of heterozygosity; NI: non-informative; IC: inconclusive; NA: no amplification possible; ND: not determined

100 µg/ml RNase (Roche, Basel, Switzerland) in 2XSSC (standard saline citrate) pH 7.0 for 20 minutes at 37°C, followed by digestion with 0.01% pepsin (Sigma-Aldrich) for 10 minutes at 37°C.

Labelling, hybridisation and detection were performed as described previously (Haralambieva et al., 2002), with the exception of the final probe concentration (30 ng/µl). The probes used were PAC LLNLP704N11132 (RZPD, Berlin, Germany) (*RUNX3*) and pUC1.77 (centromere 1).

Slides were analysed with a Leica DM5000B fluorescence microscope equipped with filters for FITC, Texas Red and DAPI (Leica). For documentation, images were captured using a Roper Coolsnap Cf digital camera (Cambridge Research Instrumentation, UK) and analysed using Image Pro Plus software version 5.02 (Media Cybernatics, Silver Springs, MD). Hybridisation signals were counted from morphologically intact cells, and overlapping nuclei were avoided by counting in areas where individual cells could be identified.

IHC

IHC analysis was performed as previously described (Levanon et al., 2002). Briefly, paraffin sections were incubated with purified RUNX1 and RUNX3 antibodies (1:100 and 1:1000, respectively) in PBS containing 0.1% Triton X-100 and 3% normal goat serum. Polyclonal anti-human RUNX3 antibodies were raised in rabbits against a 210 amino acid fragment spanning part of the carboxy end of the *RUNX3* coding region. The protein was generated using the prokaryotic expression vector pRSETB-RUNX3. These anti-RUNX3 antibodies specifically detect RUNX3

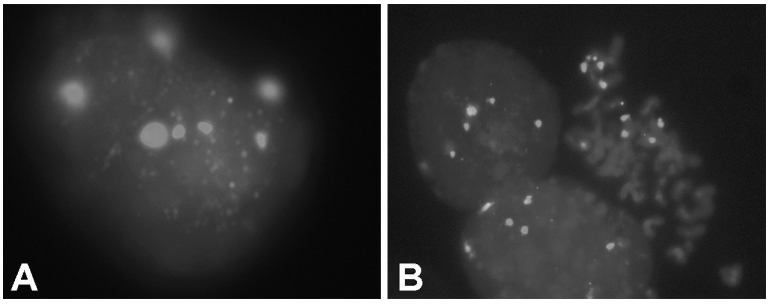

Figure 1 Bicolour FISH analysis of a Y5 gastric tumour cell (A) and HM51 nuclear spreads (B) using a centromere 1-specific probe (red) and a RUNX3-specific probe (green). ***(See page 195 for colour figure)***

Table 3 Results of FISH on patient material

	Y102	**Y5**	**Y11**	**Y4**
Cell count	51	72	67	149
C1:R3 (%)	*2:2 (88)*	*4:3 (64)*	5:5 (8)	*3:2 (29)*
[Cut-off: 5%]		3:3 (19)	5:4 (9)	3:1 (8)
			5:3 (40)	*2:2 (26)*
			5:2 (10)	*2:1 (17)*
			4:3 (9)	
			3:3 (9)	

Most prevalent ratio(s) in italics

Table 4 Results of FISH on cell lines

	TMK1	NUGC4	HSC39	HS746T	ST42	KATOIII	GP220	HM02	HM51	AGS	MKN45	MKN7	MKN74	MKN28
Origin of cell lines (age)	21	35	54	74	ND	55	85	ND	ND	54	62	39	37	70
Cell count	97	118	106	86	77	75	95	80	151	101	80	48	97	99
C1:R3 (%) [Cut-off: 5%]	*6:6 (35)* 6:5 (8) 5:6 (8) *5:5 (16)*	4:4 (9) 3:3 (9) *2:2 (61)*	*4:3 (54)* 3:3 (22)	*3:3 (62)* 2:3 (9)	*2:2 (94)*	*3:3 (60)*	*2:3 (80)*	*3:3 (88)*	6:5 (17) 6:4 (11) *5:4 (46)* 5:3 (11)	3:3 (11) *3:2 (62)*	4:3 (8) *3:3 (70)* 3:2 (8)	3:3 (13) *2:3 (35)* 2:2 (15)	*3:3 (63)* 3:2 (8) 2:3 (5) 2:2 (7)	3:2 (9) *2:2 (79)*

Most prevalent ratio(s) in italics; ND: not determined

proteins of approximately 50 KDa in transfected cells and in human haematopoietic cell lines, both of which show, by Northern blot analysis, expression of *RUNX3* (Le et al., 1999). Biotinylated secondary antibodies and Vectastain ABC complex (Vector Lab, Burlingame, CA) were used for signal detection.

Results

Based on results obtained in a previous study (Carvalho et al., 2004), a panel of 41 EOGCs was used to investigate the role of *RUNX3* in gastric carcinogenesis. Detailed patient information is provided in Table 1. Briefly, all selected cases were microsatellite-stable for the three markers studied (BAT 25, BAT 26 and BAT 40), and no *RUNX3* mutations or infection by EBV were found (Carvalho et al., 2004). The results of the LOH analysis for two markers flanking *RUNX3*

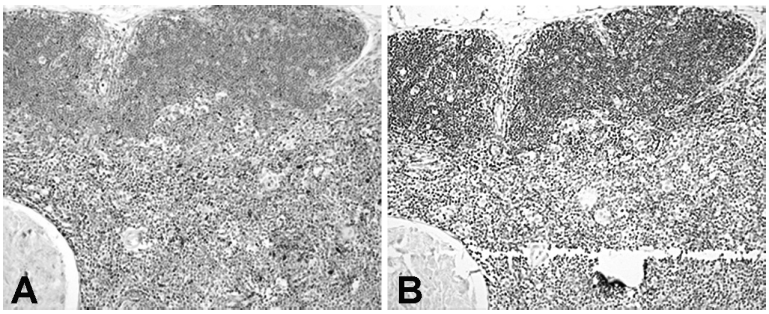

Figure 2 Staining (brown) for RUNX3 (A) and RUNX1 (B) in thymus. RUNX3 expression is seen predominantly in the medulla, whereas RUNX1 expression is mainly observed in the cortex. Nuclei were visualised by counterstaining with haematoxylin-eosin. Visualisation was performed at 10X magnification. ***(See page 195 for colour figure)***

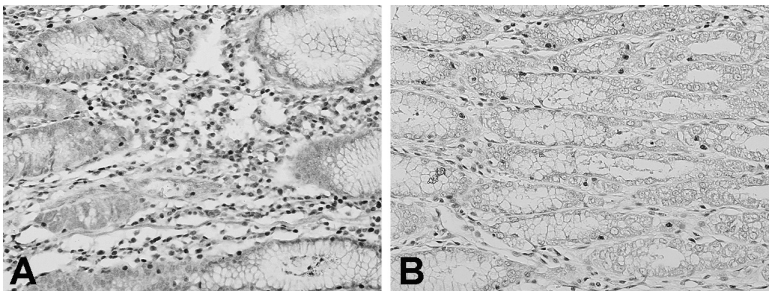

Figure 3 Staining (brown) for RUNX3 in non-neoplastic mucosa of cases Y29 (A) and Y4 (B) showing strong nuclear expression in lymphocytes (A and B) and absence of positivity in non-neoplastic gastric epithelium in the antral and transitional mucosa (A) and in the fundic mucosa (B). Faint cytoplasmatic background staining is observed in both cases. Nuclei were visualised by counterstaining with haematoxylin-eosin. Visualisation was performed at 20X magnification. ***(See page 195 for colour figure)***

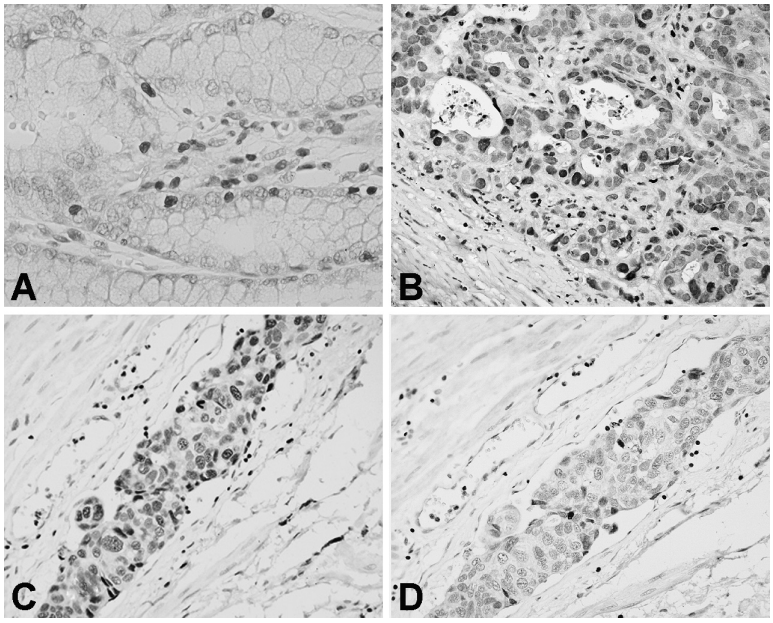

Figure 4 Staining (brown) for RUNX3 in case Y11 (A and B) showing expression in lymphocytes (A and B), absence of positivity in the non-neoplastic epithelial cells of Y11 (A) and positivity in a subpopulation of the tumour cells of Y11 (B). Staining (brown) of the same region of case Y5 for RUNX1 (C) and RUNX3 (D) showing expression in lymphocytes (C and D) and absence of RUNX3 expression in cells that are positive for RUNX1. Nuclei were visualised by counterstaining with haematoxylin-eosin. Visualisation of Y11 (A and B) was performed at 40X magnification, and of Y5 (C and D) at 20X magnification. (***See page 196 for colour figure***)

are depicted in Table 2. Although the panel of EOGCs showed low overall levels of LOH (Carvalho et al., 2004), the highest percentage was seen for the markers flanking *RUNX3*: 25% for D1S234 and 17% for D1S1676.

Given the above observations, FISH was performed on selected EOGC cases, as well as on a panel of cell lines (derived from patients both younger and older than 45 years), in order to understand more clearly how *RUNX3* was affected at the genomic level in gastric tumours. We selected two cases (Y4 and Y11) that were most likely to have true "losses" of *RUNX3* (LOH of both markers flanking the gene), as well as two cases (Y5 and Y102) with retention of both markers, and performed FISH using a *RUNX3*-specific probe. Three of the four cases (Y5, Y11 and Y102) underwent FACS, ensuring that a highly pure epithelial cell population was analysed. A tumour-rich part of the paraffin section of case Y4 was selected for analysis. A summary of the FISH results is depicted in Table 3.

Y102 showed retention of heterozygosity, and FISH analysis confirmed this observation, as most cells presented a centromere 1: *RUNX3* signal ratio (C1:R3) of 2:2. Case Y5 also showed retention of both markers; however, unlike Y102, the C1:R3 values were 3:3 and 4:3 in 83.3% of cells counted (Figure 1A). Both Y4 and Y11 displayed LOH, and FISH analysis revealed ratios of 3:2 and 2:1 for Y4, and ratios of 5:3 for Y11.

In an attempt to analyse the *RUNX3* status on a broader group of tumours, FISH was subsequently performed on a panel of 14 gastric carcinoma-derived cell lines. Four of the 14 cell lines were derived from patients aged 45 years or younger, and seven cell lines were derived from older patients. The age of three patients from which the respective cell lines were derived is unknown. The results are depicted in Table 4. Three of the 14 cells lines (one of which was derived from a young patient) presented a predominant C1:R3 of 2:2. The majority of cell lines, however, showed 2-6 copies of centromere 1, and 2-6 copies of *RUNX3*. In some cases, the number of C1 signals was higher than the number of R3 signals, but at least two copies of *RUNX3* were present in all cells counted (Figure 1B).

We then performed IHC on all cases for which material was available (37/41), in order to investigate the influence of the observed genomic abnormalities on the expression of the RUNX3 protein. Thymus was used as a positive control to validate the RUNX3 antibody (Figure 2A). To exclude cross-reactivity with the Runt domain, a RUNX1-specific antibody was also used. The RUNX3-specific antibody showed expression predominantly in the medulla, whereas RUNX1 expression was mainly observed in the cortex (Figure 2). No RUNX3 expression was seen in the adjacent non-tumorous gastric epithelium of any case (Table 2, Figures 3 and 4A), with the exception of very weak cytoplasmatic positivity in deeper specialised glandular cells of the mucosa (Figure 3). However, in our experience with a wide variety of antibodies, this is an almost universal occurrence, and is interpreted as negative expression. Further, it contrasted with the strong nuclear positivity in lymphocytes, present in all cases analysed and used as an internal control. In contrast to the surrounding non-tumorous epithelium, some weak to moderate nuclear positivity was observed focally in the gastric tumour cell population in 12/37 cases (Table 2, Figure 4B). The remaining 25 cases showed no RUNX3 positivity in the tumour cells analysed (Table 2). Selected cases (13/37, results not shown) were also stained for RUNX1, and expression was detected in both non-neoplastic (notably in the antral pits) and neoplastic gastric epithelium (Figure 4C). Expression was observed in cells negative for RUNX3 (Figure 4D).

Discussion

Runx3 biology has been extensively investigated using several strains of *Runx3*$^{-/-}$ mice (Levanon & Groner, 2004). These studies revealed several cell intrinsic functions of Runx3. In neurogenesis, Runx3 is required for the development and survival of dorsal root ganglia TrkC neurons (Inoue et al., 2002; Levanon et al., 2002). In thymopoiesis, Runx3 is necessary for the silencing of the *CD4* gene during lineage decisions of T cells (Ehlers et al., 2003; Taniuchi et al., 2002; Woolf et al., 2003). In dendritic cells (DCs), Runx3 functions as a component of the TGF-β signalling cascade (Fainaru et al., 2004). When Runx3 is lost, the knock-out DCs do not respond to TGF-β, their maturation is accelerated and accompanied by an increased efficacy to stimulate T cells (Fainaru et al., 2004). The gene has two distinct promoter regions, and it has been demonstrated that their activity is in most cases cell-type specific (Levanon & Groner, 2004).

Conflicting results have recently emerged on the role that *RUNX3* plays in gastric carcinogenesis. Li *et al.* (Li et al., 2002) showed that *Runx3* is expressed in the normal murine gastric mucosa, and that *Runx3*$^{-/-}$ mice (with a homogeneous C57BL/6 genetic background) developed gastric hyperplasia, which the authors attributed to a loss of function of Runx3 in gastrointestinal tract (GIT) epithelium, resulting in promotion of proliferation and suppression of apoptosis in gastric epithelial cells. The authors therefore postulated *Runx3* as a novel TSG in gastric cancer. All *Runx3*$^{-/-}$ mice died within 10

days. These observations were linked with the discovery of *RUNX3* silencing in about 60% of human gastric cancer specimens analysed (Li et al., 2002). These results led to several studies being published where the loss of *RUNX3* appeared an important event in gastric carcinogenesis (Bae & Choi, 2004; Fukamachi & Ito, 2004; Ito & Miyazono, 2003; Osaki et al., 2004).

Brenner and colleagues (Brenner et al., 2004), on the other hand, reported that at ≈ 4 weeks of age, *Runx3*$^{-/-}$ mice (with a heterogeneous ICR and MF1 genetic background) developed colitis and only at an older age (≈ 8 months) went on to develop gastric mucosal hyperplasia. Further, the mice survived for up to 24 months without developing gastric carcinoma (Brenner et al., 2004). As Runx3 could not be detected in GIT epithelium (Brenner et al., 2004; Levanon et al., 2001; Levanon et al., 2003), but was readily detected in the resident leukocytic population of the GIT, the conclusion reached by Brenner *et al.* (Brenner et al., 2004) was that the GIT ailments of the *Runx3*$^{-/-}$ mice were a result of the loss of an intrinsic Runx3 cell function in leukocytes. Notably, gastric mucosal hyperplasia in association with colitis has previously been observed in other mutant mice (Fernandez-Salguero et al., 1997).

In the present study we addressed the role of *RUNX3* in EOGC. LOH results previously obtained by our group pointed to the possibility of *RUNX3* being lost in gastric carcinomas (Carvalho et al., 2004) (Table 2). The LOH frequency observed was somewhat lower than that observed by Li and colleagues (Li et al., 2002). In an attempt to understand the significance of these observations, FISH was performed on two LOH cases (Y4 and Y11) and on two cases showing retention near the *RUNX3* locus (Y5 and Y102) (Table 3), as well as on a panel of 14 gastric cancer-derived cell lines (Table 4). Four out of 14 cell lines were derived from patients younger than 45 years, while seven of the remaining cell lines originated from older patients. The age of three patients from which cell lines were derived is unknown. As it has been previously reported that EOGCs may differ from gastric carcinomas occurring at a later age (Carvalho et al., 2004), we aimed at extrapolating our findings to a wider group of gastric carcinomas.

The retention cases exhibited a normal C1:R3 ratio (Y102) and a predominant C1:R3 ratio of 4:3 and 3:3 (Y5, Figure 1A), indicating chromosome 1 duplications or amplifications in most Y5 cells counted. Noteworthy, a previous study using CGH showed gains in the short arm of chromosome 1 in this case (Varis et al., 2003). Chromosome 1 aberrations were also observed in LOH cases. Various C1:R3 were seen, with most Y11 cells presenting three or more C1 signals and with every cell retaining at least two copies of *RUNX3*. For Y4, most cells presented 2-3 C1 signals and two R3 signals, but a minority (17%) of cells showed a C1:R3 of 2:1, suggestive of an actual deletion of *RUNX3*. This deletion may equally be explained by a gross deletion of the p-arm of chromosome 1.

The FISH results not only explained the observed LOH, but also showed most cells to have at least two copies of *RUNX3*. It appears, therefore, that the results obtained point to a general chromosome 1 instability and a change of the ratio between maternal and paternal alleles, rather than specific deletions of *RUNX3*.

The cell lines showed a variety of C1:R3 ratios, with aneuploidy extensively observed. Although some cell lines show a higher number of C1 signals when compared to R3 signals, no cell line showed counts of fewer than two R3 signals (Figure 1B). It can thus be concluded that the *RUNX3* dosage in tumour cells is at least equal to that of normal cells. No apparent difference was observed between cell lines derived from younger and older patients.

When studying the effect of *RUNX3* aberrations on the level of expression of the RUNX3 protein, it was observed that, in accordance to the findings of Groner and colleagues (Levanon et al., 2001), no RUNX3 expression was detected by IHC in any histologically non-neoplastic

gastric epithelial cells (Figures 3 and 4A). Specifically, no G-cells in the antral mucosa showed any RUNX3 expression (Figure 3A), despite a recent report that RUNX3 is expressed in a subset of normal gastric epithelial cells, notably in chief cells of fundic glands and in G-cells located in the pyloric portion of the normal stomach (Osaki et al., 2004). Furthermore, we observed nuclear RUNX3 staining in some tumour cells (Table 2, Figure 4B). The lack of expression of RUNX3 in histologically non-neoplastic epithelium, together with occasional expression in tumour cells, precludes a role for *RUNX3* as a TSG in EOGC.

It is known that the short arm of chromosome 1 is frequently affected in several types of cancer. Most published results, however, point to the 1p36 region to be amplified, not deleted in gastric carcinomas (Kokkola et al., 1998; Sakakura et al., 1999; Wu et al., 2002). Studies that do report deletions around 1p36 connect this event to an advanced stage of gastric cancer development, not consistent with early events in gastric carcinogenesis (Igarashi et al., 2000; Sano et al., 1991). In this context, it is possible that the methylation found in the CpG island of the P2 *RUNX3* promoter, as described by Li *et al.*, is caused by increased activity of the DNA methyltransferase 1 in advanced gastric tumours (Etoh et al., 2004).

In conclusion, we have shown that *RUNX3* is unlikely to be a TSG for EOGC, as it is not expressed in histologically non-neoplastic gastric epithelium, and at least two copies of the gene are present in the vast majority of cells analysed. We have also demonstrated that the observed LOH may be explained by the frequent copy number changes that we find due to chromosome 1 aberrations. Further research is needed to determine whether the 1p36 region contains genes that play a significant role in gastric cancer.

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5

Early onset gastric cancers have a different molecular expression profile than conventional gastric cancers

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Abstract

Many studies examine the molecular genetics of gastric cancer but few look at young patients in particular and there is no comparison of molecular expression between early onset gastric cancer (≤ 45 years old) and conventional gastric cancers. Expression of COX-2 is elevated in gastric adenocarcinomas compared to non-neoplastic mucosa and in light of studies showing reduced risk of gastric cancer in non-steroidal anti-inflammatory drug users, we have chosen to investigate the expression of COX-2 and related molecules in 113 early onset gastric cancers and compare it with 91 conventional gastric cancers, using tissue microarrays. These markers include molecules known to be important in conventional gastric carcinogenesis such as E-Cadherin, p53, COX-2, TFF1, β-catenin, p16, and c-myc as well as molecules not yet described as being important in gastric cancer such as the transcription factor c-jun, the COX-2 mRNA stabilizer HuR and C/EBP-β, a transcription factor for COX-2.

All markers showed a statistically significant difference between early onset gastric cancers and conventional gastric cancers using a χ^2 test. In particular, early onset gastric cancers displayed a COX-2 Low, TFF1-expressing phenotype whereas COX-2 overexpression and loss of TFF1 was found in conventional cancers, and this difference between early onset gastric cancers and conventional cancers remained statistically significant when adjusted for location and histology ($p<0.0001$, and $p=0.002$ respectively). We found that COX-2 overexpression correlates significantly with loss of TFF1 ($p=0.001$), overexpression of C/EBP-β ($p<0.001$) and cytoplasmic HuR ($p=0.016$). COX-2 was significantly associated with p53 positivity ($p=0.003$). Abnormalities in E-Cadherin correlated significantly with diffuse phenotype whereas high expression of COX-2, loss of TFF1 and overexpression of C/EBP-β correlated with the intestinal phenotype.

Our results provide further evidence that early onset gastric cancer exhibits a distinctive expression profile that may have practical implications.

Introduction

Gastric cancer is the second most common cause of cancer-related death in the world.[1] It exists as two main histological types, diffuse and intestinal, as described by Laurén,[2] and is thought to result from a combination of environmental factors and accumulation of specific genetic alterations, and consequently mainly affects older patients. Here we investigate early onset gastric cancer, which is defined as gastric cancer presenting at the age of 45 or younger. Fewer than 10% of gastric cancer patients fall into the early onset gastric cancer category [3] and it is postulated that genetic factors may be more important in these patients.[4] *Helicobacter pylori*, a known causative agent in gastric carcinogenesis,[5-7] probably still plays a role in the development of gastric cancer in young patients,[8,9] however this is likely to involve a much smaller percentage of patients than in the older age group.

Inherited gastric cancer predisposition syndromes account for approximately 10% of early onset gastric cancers, yet even in these cases the underlying genetic events are not always known. For example, alterations in the E-Cadherin gene have been associated with hereditary diffuse type gastric cancer[10] but so far only approximately 30% of all hereditary diffuse gastric cancer families carry CDH1 germline mutations. Thus only a very small percentage of early onset gastric cancers can be explained by E-Cadherin germline mutations and the occurrence of gastric cancer in young patients remains largely unexplained.

Expression of COX-2 is elevated in gastric adenocarcinomas as compared to the non-neoplastic mucosa[11,12] and in light of studies showing the reduced risk of gastric cancer in non-steroidal anti-inflammatory drug users,[13,14] investigating the difference in COX-2 expression between early onset gastric cancer and conventional gastric cancer may have clinical implications. Thus we have chosen to investigate the expression of COX-2 and related molecules in early onset gastric cancer and conventional gastric cancer using a tissue microarray approach. The COX-2 mRNA stabilizer HuR (Hu-Antigen R) and C/EBP-β (CCAAT/Enhancer-Binding Protein-β), a transcription factor for COX-2, are also examined. Not only is C/EBP-β known to act as a transcription factor for COX-2,[15] but it also acts as a transcription factor in the down regulation of TFF1 (Trefoil Factor 1),[16,17] loss of which has been independently shown to cause gastric adenomas.[18] COX-2 overexpression has been found in these adenomas.[19] Aberrant p53, known to be important in conventional gastric carcinogenesis,[20] is also associated with increased levels of COX-2 expression.[21] In addition, we examine p16, an important cell cycle regulator, which is lost in many gastric cancers.[22]

Wnt signalling may also be targeted through chemoprevention with non-steroidal anti-inflammatory drugs, through a COX-2 independent mechanism[23] and it has been suggested that there is cross talk between COX-2 and Wnt,[24] thus c-myc, β-catenin and c-jun[25] are also investigated in this study due to their involvement in the Wnt pathway. Finally, due to its importance in gastric cancer[10] the expression of E-Cadherin is included.

We aim to ascertain whether discernible differences exist in the expression of these markers, which would lend support to the theory that early onset gastric cancers have distinct molecular characteristics from conventional gastric cancers. By the uncovering of the differences between gastric cancer in old and young patients, we are a step closer to understanding the pathogenesis of gastric cancer and elucidating new genes which may have prompted gastric cancer at a young age.

Methods

Patients/study groups

This research was carried out in accordance with the ethical guidelines of the research review committee of the Academic Medical Centre, Amsterdam. Ninety one conventional gastric cancers (>45 years old), diagnosed between 1993 and 2003, were obtained from the Academic Medical Centre, Amsterdam. One hundred and thirteen cases of gastric carcinoma in patients under 45 years of age, 90% diagnosed between 1994 and 2002 and 10% diagnosed between 1980-1994, were obtained from 24 different institutions throughout the Netherlands through the nationwide database system, and from the Department of Pathology at the Jorvi Hospital (Espoo, Finland). This age cut-off was chosen in order to obtain enough cases to achieve meaningful result. The tumors were classified by an experienced gastrointestinal pathologist (GJAO) according to the Laurén classification as intestinal, diffuse or mixed gastric adenocarcinomas and location was deduced from the pathological report (if available) as seen in Table 1.

Tissue Microarray

Tissue microarrays were constructed from formalin-fixed and paraffin-embedded archive specimens. Three core biopsies (0.6mm cylinders) were taken from histologically representative regions (including heterogeneous areas) of paraffin-embedded gastric tumors and arranged in a new recipient paraffin block (tissue array block) using a custom-built instrument (Beecher Instruments, Silver Spring, MD) as also described previously.[26] Normal gastric mucosa from each case was also included where available (69/204). Cores were arranged in 2 or 3 separate subdivisions together with insertion of liver, lymph node and kidney cores to assist analysis. 189 cases were informative for all markers. In most cases sections were stained immediately after cutting, but if stored, this was done so by wrapping in aluminium foil and freezing at -20°C to prevent loss of antigenicity.

Immunohistochemistry

Sections (4 μm) were deparaffinized and antigen retrieval was carried out by 10 minutes of boiling in 10 mM Tris/1 mM EDTA (pH 9) except for c-myc where no antigen retrieval was used. Subsequently slides were immersed in 0.3% hydrogen peroxide in methanol for 30 minutes and non-specific binding was blocked with 5% normal goat serum for 1 hour at room temperature. The sections were incubated for 1 hour (or overnight in the case of C/EBP-β) at room temperature with the following primary antibodies: p53 (monoclonal antibody combination of DO-7 and BP53-12 Neomarkers, Union City, CA, USA) 1:2000 dilution, E-Cadherin HECD-1 (Thamer) 1:2000 dilution, TFF1/pS2 (Dako,) 1:1200 dilution, c-myc (Santa Cruz) 1:50, NCL c-jun (Nova Castra) 1:200, β-catenin (BD Biosciences, Alphen aan den Rijn, The Netherlands) 1:10000 dilution, p16 ^{INK4A} (Neomarkers) 1:100 dilution, C/EBP-β (H-7):sc-7962 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:50 dilution. The Ultravision antipolyvalent HRP detection system

Table 1 Clinicopathological Characteristics

Age	Histology			Location			Total
	Intestinal	Diffuse	Mixed	Cardia	Non-cardia	Unknown	
≤ 45 yrs old	24	80	9	9	74	30	113
> 45 yrs old	49	31	11	49	42	0	91

(Lab Vision Corp., Fremont, CA, USA) was used to visualize antibody binding sites with 3,3'-diaminobenzidine as a chromogen. DAB Plus was used for c-jun. Sections were counterstained with haematoxylin.

Immunohistochemistry for COX-2 was carried out as above with the following exceptions: Antigen retrieval was carried out in 0.01 M Na-citrate buffer (pH 6.0), followed by immersion in 0.6% hydrogen peroxide in methanol for 30 minutes and then in blocking solution (0.01 M Tris, 0.1 M $MgCl_2$, 0.5% Tween-20, 1% BSA, 5% normal goat serum) for 1 hour. Incubation of the primary antibody was carried out using monoclonal COX-2 antibody at a dilution of 1:100 (Cayman Chemical Co., Ann Arbor, MI, USA) at 4° C overnight.

Immunohistochemistry for HuR was carried out as follows: Specimens were deparaffinized and antigen retrieved using microwave oven (4 x 5 min in 700 W in 0.01M Na-citrate buffer (pH 6.0)). The slides were then immersed in 0.6% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity and in blocking solution (1:66 normal horse serum in PBS) for 15 minutes to block unspecific binding sites. Immunostaining for HuR was performed with monoclonal antibody 19F12 [27], in a dilution of 1:10,000 (1.0 μg/ml) in PBS containing 0.1% sodium azide and 0.5% BSA, overnight at room temperature. The sections were then treated with biotinylated horse anti-mouse immunoglobulin (1:200; Vector Laboratories Inc., Burlingame, CA) and avidin-biotin peroxidase complex (Vectastain ABComplex; Vector Laboratories) and peroxidase staining visualized with 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO). Sections were counterstained with Mayer's hematoxylin.

Scoring

Scoring of immunohistochemistry was carried out for p53,[28] E-Cadherin,[29] TFF,[30] B-catenin,[31] COX-2,[32] p16,[33] HuR,[34] C/EBP-β,[15] c-myc,[35] and c-jun as shown in Table 2.

The overall score of the tumor was the highest score found. No slides showed increased negative staining around the edges. Unequivocal cases were scored by one observer (A.N.A.M.). All other cases (approximately 30 %) were assessed by 2 observers (A.N.A.M. and G.J.A.O.) and agreement reached using a multi-headed microscope. Cases where the staining was not obviously abnormal

Table 2 Scoring of Immunohistochemistry

Molecule	Criteria for Abnormal Scoring
p53	Strong nuclear staining in >30% of cells
E-Cadherin	Absence of membranous staining or presence of clumpy cytoplasmic localization
TFF	Absence of staining in 95% of tumor cells
B-catenin	Nuclear staining accompanied by loss of membranous staining and increased cytoplasmic staining
COX-2	0-no staining; 1- very weak diffuse cytoplasmic staining 2-moderate to strong granular cytoplasmic staining in 10-50% 3- >50% of tumor cells with strong intensity Categories 0 and 1 were COX-2 low, categories 2 and 3 were COX-2 High
p16	<10% nuclear staining
c-myc	Nuclear staining in >5%
c-jun	Nuclear staining in >5%
HuR	The presence of staining was scored separately as positive or negative for the nucleus and cytoplasm of tumor cells.
C/EBP-β	Nuclear staining > 25% of cells

were placed in the normal category. All tissue microarray slides were examined carefully and results documented irrespective of scoring system. Subsequently, various scoring systems in the literature were assessed in order to choose a system that accurately reflected the results obtained and all cases were re-examined based on this scoring system. This was not the case however with more established scoring systems such as with p53, E-Cadherin, COX-2, and B-catenin, which were scored according to well established systems that are familiar to the authors.

Statistical Analysis

The SPSS 11.5 software package was used for statistical analysis. A Chi-squared test was applied to the groups of gastric cancer to determine whether the differences found between antibodies were statistically significant ($p<0.05$). A binary logistic regression model was used to adjust for potential confounding factors such as location, histological type, age of blocks and the hospital from which the block was derived.

Results

Immunohistochemical results for early onset gastric cancer, conventional gastric cancers and the combined results can be seen in Table 3. Variation of staining between cores ranged from no cases in E-Cadherin and β-catenin to 4/204 cases in c-myc, c-jun, p16, TFF1, C/EBP-β. These cases had 1 or 2 cores which fell within the positive category and 1 or 2 that fell within the negative category. The most striking results as seen in Table 3 involve COX-2 and TFF1: the COX-2 High phenotype was present in 66% of conventional gastric carcinomas but in only 10% of early onset gastric cancer; conversely, loss of TFF1 was seen in 73% of conventional gastric cancers, but in only 39% of early onset gastric cancers. In order to critically compare these results obtained for early onset gastric cancer and conventional gastric cancers, we carried out χ^2 analysis. In addition, we compared the groups using a binary logistic regression model to examine whether the χ^2 findings

Table 3 Results of Immunohistochemistry

Antibodies	Immunohistochemical Findings*			Range of Staining	
	Age >45 years old	Age <45 years old	All Ages combined	Negative	Positive
E-Cadherin	37% abnormal	52% abnormal	45% positive	n.r.	n.r.
β-catenin	23% abnormal	35% abnormal	29% abnormal	n.r.	n.r.
p53	48% positive	31% positive	42% positive	0-10%	50-90%
COX-2	66% High	10% High	35% High	n.r.	n.r.
TFF	73% negative	39% negative	55% negative	0-1%	25-99%
HuR cytoplasmic	28% positive	13% positive	34% positive	0%	15%-100%
HuR nuclear	40% positive	28% positive	34% positive	0%	20%-100%
c/ebpβ	80% positive	67% positive	73% positive	0-9%	35-100%
p16	57% negative	34% negative	44% negative	0-5%	20-100%
c-myc	33% positive	52% positive	43% positive	0-5%	6-100%
c-jun	64% positive	74% positive	70% positive	0-5%	6-100%

* to the nearest percent
n.r. – not relevant

Table 4 Statistical Analysis of immunohistochemical results. χ^2 test was carried out for each antibody versus age. The results displayed under the heading Binary Logistic Regression Model are as follows: age, adjusted for location and histology; location, adjusted for age and histology; and histology, (results for diffuse histology given) adjusted for age and location.

Antibodies	Statistical Significance			
	χ^2	Binary Logistic Regression Model		
	Age	Age	Location	Histology
E-Cadherin	p=0.027	none	none	p<0.001 (CI 3.2-16.3)
β-catenin	p=0.049	none	none	none
p53	p=0.009	none	p=0.004 (CI 0.2-0.7)	none
COX-2	p<0.001	p<0.001 (CI 6.3-50.1)	none	p=0.008 (CI 0.1-0.7)
TFF1	p<0.001	p=0.001 (CI 1.7-7.6)	none	p=0.04 (CI 0.2-0.97)
HuR cytoplasmic	p=0.005	none	none	none
HuR nuclear	none	none	none	none
C/EBP-β	p=0.033	none	none	p = 0.001 (CI 0.08-0.52)
p16	p=0.001	none	p=0.014 (CI 1.2-5.6)	none
c-myc	p=0.015	none	none	none
c-jun	p=0.08	none	p=0.012 (CI 1.3-6.1)	none

were significant once adjusted for location and histology. In this way we also compared the cancers with respect to anatomic location (adjusted for age and histology), and histology (adjusted for age and location). In addition, using the binary logistic regression model, we confirmed that neither the age of the blocks, nor the hospital from which they originated (thus possible variation in processing), significantly affected the results for any marker used.

Correlation with age, location and histology

We can see from Table 4 that all markers showed a statistically significant difference between early onset gastric cancers and conventional gastric cancers. COX-2 showed much higher expression levels in conventional gastric cancer than in early onset gastric cancer and loss of TFF1 was much more common in conventional gastric cancer than in early onset gastric cancer. Furthermore, the difference found between the groups with COX-2 ($p<0.001$) and TFF1 ($p<0.001$) remained statistically significant when adjusted for location and histology ($p<0.0001$ and $p=0.002$, respectively).

In addition we found that p53 positivity and p16 negativity correlated significantly with tumors of the cardia ($p=0.007$ and $p=0.016$ respectively), whilst c-jun positivity correlated with non-cardia tumors ($p=0.005$). Abnormalities in E-Cadherin correlated significantly with the diffuse phenotype ($p<0.001$) whereas COX-2 High, loss of TFF1 and overexpression of C/EBP-β correlated with the intestinal phenotype ($p=0.008$, $p=0.04$, and $p=0.001$, respectively).

Correlation between molecular markers

Using a χ^2 test, we found that COX-2 overexpression correlates significantly with loss of TFF1 ($p=0.001$), overexpression of C/EBP-β ($p<0.001$) and cytoplasmic (but not nuclear) HuR ($p=0.016$). This phenotype can be seen in Figure 1. In addition COX-2 was significantly associated with p53 positivity ($p=0.003$). On the other hand, TFF1 did not correlate significantly

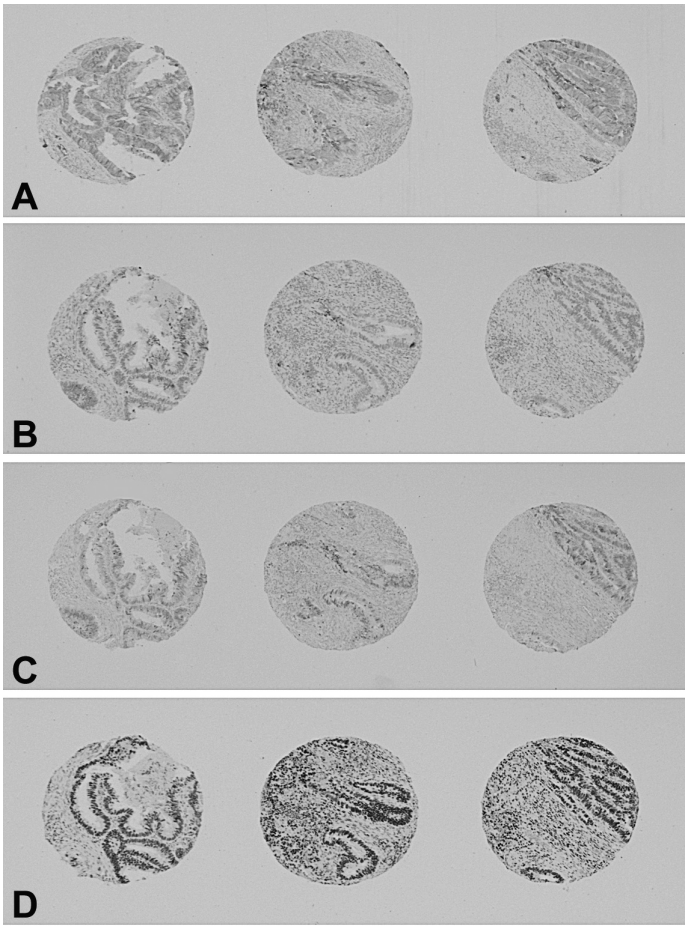

Figure 1 Immunohistochemistry on tissue microarrays for COX-2 (COX-2 High, A), TFF1 (negative, B), HuR (positive cytoplasmic staining, C) and C/EBP-β (positive, D). Magnification 32.5, counterstain haematoxylin. ***(See page 197 for colour figure)***

with cytoplasmic HuR, nuclear HuR, or p16, and neither did the correlation with C/EBP-β reach statistical significance (p=0.059).

Nuclear HuR correlated significantly with c-myc positivity (p<0.001) but did not correlate with C/EBP-β.

No correlation was found between abnormal β-catenin and c-myc or c-jun positivity.

Discussion

Many studies examine the molecular genetics of gastric cancer in general but very few look at young patients in particular and there is no comparison of molecular expression between early onset gastric cancers and conventional gastric cancers. Here we examine a large group of 204 gastric

cancers, approximately half of which are early onset gastric cancers, and compare the expression of ten molecular markers in early onset gastric cancer and conventional gastric cancers.

Expression of COX-2 is elevated in gastric adenocarcinomas as compared to the non-neoplastic mucosa.[11] Interestingly, in this study we find that COX-2 expression varies significantly between early onset gastric cancers and conventional cancers, with COX-2 overexpression occurring rarely in early onset gastric cancers. In light of studies showing the reduced risk of gastric cancer in non-steroidal anti-inflammatory drug users,[13,14] our results may have clinical implications, as they suggest that this reduced risk may apply only to gastric cancers in older patients as COX-2 does not appear to play an important role in early onset gastric cancer. It also implies that genetic changes typical for conventional tumors more readily induce COX-2 expression than those associated with early onset gastric cancer.

HuR is a member of the Hu family of ARE-binding proteins involved in the regulation of mRNA turnover[36] which shuttles between the nucleus and the cytoplasm. In this study we find that cytoplasmic HuR correlates with COX-2 expression in gastric cancer. COX-2 mRNA is known to contain HuR-binding AREs in its 3' untranslated region and HuR has been associated with prognosis and COX-2 expression in human carcinomas.[34,37]

COX-2 is predominantly expressed in intestinal-type gastric carcinomas and its precursor lesions,[38,39] as confirmed in this study. We also find that p53 immunopositivity (as a marker for loss of p53 function) is associated with increased level of COX-2 expression as previously described.[21] These results support the theory that there may be a direct link between the defective p53 pathway and elevated levels of COX-2 expression in cancer cells.

In addition to COX-2, we also find a significant difference in TFF1 expression between the early onset gastric cancers and the conventional cancers, with loss of TFF1 occurring less frequently in the early onset gastric cancer group. Trefoil factor 1 (TFF1) is synthesized and secreted by the normal stomach mucosa and by the gastrointestinal cells of injured tissues. The link between mouse Tff1 inactivation and the fully penetrant antropyloric tumor phenotype[18] prompted the classification of TFF1 as a gastric tumor suppressor gene. Accordingly, altered expression, deletion, and/or mutations of the TFF1 gene have been observed in human gastric carcinomas.[30,40-42] It has been shown that Cox-2 is expressed in Tff1 knockout adenomas,[19] and this inverse relationship of COX-2 and TFF1 expression has been highlighted in our results, where loss of TFF1 and COX-2 High was seen in conventional tumors, whereas retention of TFF1 expression was more likely to pair with a COX-2 Low phenotype as seen in the early onset gastric cancers.

Interestingly, it has been recently suggested that C/EBP-β, a transciption factor for COX-2,[43] plays a role in gastric cancer, through its relationship with COX-2.[15] However, C/EBP-β is also known to act as a transcription factor in the down regulation of TFF1,[16,17] thus providing a fascinating link between COX-2 and TFF1, the two molecules shown here to be expressed differently in early onset gastric cancer and conventional gastric cancer. It raises the possibility that this one molecule could concomitantly downregulate TFF1, whilst upregulating COX-2, thus affecting two different carcinogenic pathways and providing a considerable advantage to tumor growth. In our study the correlation between C/EBP-β and TFF1 was only of borderline significance ($p=0.059$) and thus the precise link between C/EBP-β, COX-2 and TFF1 in gastric cancer needs further exploration.

This study also revealed molecular differences in histological type and anatomic location in gastric cancer. The significant differences found between cardia and body tumors in p53, c-jun and p16 expression, lend further support to the theory that adenocarcinomas arising in the gastric cardia and body occur through different genetic pathways.

Despite the differences seen in COX-2 expression between early onset gastric cancers and conventional cancers, and the possible cross talk between the COX and Wnt pathways,[24] no difference was seen in β-catenin, c-myc or c-jun expression between early onset gastric cancer and conventional gastric cancer when using the binary logistic regression model. The well established Wnt pathway is activated in colorectal cancer, however not much is known about the relative importance of this pathway in gastric cancer and in particular early onset gastric cancer. It has been found previously that immunohistochemical abnormalities of β-catenin are present in 22-27% of gastric cancer.[44,45] Here we confirm that estimate and show that although β-catenin may play an important role in gastric cancer, this role is not specific to early onset gastric cancer. We also found no correlation between β-catenin and c-myc or c-jun suggesting that these particular targets may not depend on activation of the Wnt pathway in gastric cancer.

C-myc is amplified in gastric cancer and its amplification corresponds with c-myc overexpression on immunohistochemistry.[46] The frequency of c-myc positivity found here is supported by previous findings where it was also found to be related to cell proliferation and associated with poor clinical outcome.[35] Interestingly, we have found that nuclear HuR expression correlated with c-myc expression. HuR is known to stabilize c-myc mRNA in vitro,[47] providing an explanation for the correlation between the two molecules, and suggesting for the first time that this interaction may be important in gastric carcinogenesis.

The proto-oncoprotein c-Jun is a component of the AP-1 transcription factor (a dimeric complex of Jun and Fos), the activity of which is augmented in many tumor types. The role of this transcription factor in gastric cancer is largely unknown. An important mechanism in the stimulation of AP-1 function is amino-terminal phosphorylation of c-Jun by the c-Jun N-terminal kinases (JNKs) and it has been shown that the phosphorylation-dependent interaction between c-Jun and TCF4 regulates intestinal tumorigenesis by integrating JNK and APC/beta-catenin, two distinct pathways activated by Wnt signaling.[25] It has been also documented that a COX-2 inhibitor suppresses AP-1 though JNK in gastric cancer.[48] In this study we find c-jun positivity in a high percentage of gastric cancer and find that this positivity occurs more often in cancers of the body of the stomach than the cardia.

Finally, our findings with E-Cadherin, C/EBP-β, TFF1 and COX-2 expression emphasize the fact that diffuse and intestinal cancers differ at a molecular level. It has been suggested that loss of E-cadherin is the fundamental defect in diffuse type gastric carcinoma, and that it provides an explanation for the observed morphological phenotype of non-cohesive cells with loss of polarity and gland architecture.[49,50] In contrast, gland architecture is preserved in the intestinal type of stomach cancer where loss of E-cadherin expression does not occur as commonly.[50] Here we find that abnormal E-Cadherin does not associate directly with early onset gastric cancer if adjusted for histology, but instead correlates significantly with the diffuse phenotype. The converse has also been reported,[51] although in report by Lim *et al* young cases were compared to old without adjusting for histology perhaps explaining this discrepancy.

In summary, we have found further evidence that early onset gastric cancer exhibits a unique expression profile of molecules important in carcinogenesis. This involves a COX-2 Low, TFF1 positive phenotype which appears to occur almost exclusively in early onset gastric cancer. It is interesting to note that these molecules are both involved in inflammation and C/EBP-β and HuR appear to be involved in their regulation in gastric cancer. Our findings add further support to the hypothesis that young patients develop carcinomas with a different molecular genetic profile from that of sporadic carcinomas occurring at a later age.

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6

The *COX-2* promoter polymorphism -765 G>C is associated with early-onset, conventional and stump gastric cancers.

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Abstract

COX-2 overexpression is known to be an important mechanism in gastric carcinogenesis. Previously we have found that early-onset gastric cancer (EOGC) has a unique COX-2 low expressing phenotype that differs significantly from that of the frequent overexpression seen in conventional gastric cancers. In order to investigate whether the COX-2 -765 G>C promoter polymorphism (known to lead to a reduction of the *COX-2* promoter activity in the colon) may explain this difference in expression, we carried out single nucleotide polymorphism (SNP) analysis of 241 gastric cancers including EOGC, conventional gastric cancers and gastric stump cancers as well as in 100 control patients, using real-time PCR technology and sequence analysis, and correlated these findings with COX-2 expression using immunohistochemistry. We found that the C allele was present in 30 % of early onset gastric cancers, 24% of conventional gastric cancer, 23% of stump cancers, in contrast to 41% in the control group. There was a statistically significant difference in the presence of the C allele in patients with gastric cancer compared to the control group ($p=0.007$), with the G allele being associated with gastric cancer. However there was no significant difference between the early-onset, conventional and stump gastric cancer groups. Interestingly, there was no correlation between the presence of the C allele and a difference in COX-2 expression.

In summary, we show that the COX-2 -765 G allele promoter polymorphism is significantly associated with gastric cancer when compared to the normal control group but does not appear to be related directly to COX-2 expression pattern in gastric cancer. Although EOGC appear to have a unique COX-2 expression pattern when compared to conventional gastric cancer, the exact mechanism by which this occurs is yet to be elucidated.

Introduction

Gastric cancer is the second most common cause of cancer-related death in the world.[1] It exists as two main histological types, diffuse and intestinal, as described by Laurén,[2] and is thought to result from a combination of environmental factors and accumulation of specific genetic alterations, and consequently mainly affects older patients. COX-2 is an inducible enzyme and produces prostaglandins in response to various inflammatory stimuli or growth factors. Expression of COX-2 is elevated in gastric adenocarcinomas as compared to the non-neoplastic mucosa[3] and is predominantly expressed in intestinal-type gastric carcinomas and its precursor lesions.[4,5] COX-2 overexpression has been associated with an inhibition of apoptosis,[6] an increased metastatic potential[7] and neoangiogenesis.[8]

Interestingly, we have previously found[9] that COX-2 expression varies significantly between early onset gastric cancers (presenting at ≤ 45 years old, EOGC) and conventional cancers (presenting > 45 years old), with COX-2 overexpression occurring rarely in early onset gastric cancers. In light of studies showing the reduced risk of gastric cancer in non-steroidal anti-inflammatory drug users,[10-12] our results may have clinical implications, as they suggest that this reduced risk may apply only to gastric cancers in older patients, as COX-2 does not appear to play an important role in early onset gastric cancer. It also implies that genetic changes typical for conventional tumors more readily induce COX-2 expression than those associated with early onset gastric cancer. The mechanism behind this difference in COX-2 regulation and expression in these sub-types of gastric cancer is intriguing.

Transcriptional regulation has been shown to be the major mechanism in regulating the expression of COX-2, although posttranscriptional mechanisms such as increased stability of COX-2 mRNA (e.g. via HuR) also seem important.[9] The expression of *COX-2* is regulated by a complex signal transduction pathway in which many nuclear proteins interact with the *COX-2* promoter region and play a decisive role in gene transcription.[13] Naturally occurring single nucleotide polymorphisms (SNPs) in the *COX-2* promoter may therefore have great impact on gene transcriptional activity by altering the binding capability with certain nuclear proteins, resulting in inter-individual variability in susceptibility to cancer and in response to the treatment of patients with COX-2 inhibitors. Indeed, Papafili *et al* [14] have described a polymorphism in the promoter region of *COX-2*, characterized by a guanine (G) to cytosine (C) transition at position -765 (-765G>C). This polymorphism appears to disrupt a *Stimulatory protein 1* (Sp1) binding site, which is considered to be a positive activator of transcription and leads to a 30% reduction of the *COX-2* promoter activity *in vitro*[14] and is also known to be associated with decreased COX-2 expression in the colon.[15]

In this study, we examine the distribution of the *COX-2* -765 G>C polymorphism in 241 gastric cancers (including EOGC, conventional gastric cancer and gastric stumps cancers) and 100 control patients using real-time PCR with MGB fluorescent probes and sequence analysis and we investigate the relationship with COX-2 expression in gastric cancer using immunohistochemistry.

Materials and methods

Patients

Ninety six conventional gastric cancers (>45 years old), diagnosed between 1993 and 2003, were obtained from the Academic Medical Centre, Amsterdam, together with 30 gastric stump

cancers from the Amsterdam post-gastrectomy cohort.[16] One hundred and fifteen cases of gastric carcinoma in patients under 45 years of age, 90% diagnosed between 1994 and 2002 and 10% diagnosed between 1980-1994, were obtained from 24 different institutions throughout the Netherlands through the nationwide database system, and from the Department of Pathology at the Jorvi Hospital (Espoo, Finland). The tumors were classified by an experienced gastrointestinal pathologist (GJAO) according to the Laurén classification as intestinal, diffuse or mixed gastric adenocarcinomas, as can be seen in Table 1. The control group consisted of 100 DNA samples from healthy men, recruited from the department of endocrinology at the Academic Medical Center, Amsterdam as published previously.[17]

DNA isolation

DNA was isolated from formalin fixed tissue using the QIAamp DNA Mini Kit (Qiagen, Venlo, The Netherlands) or the Puregene DNA Isolation Kit (Gentra, Minneapolis, USA) according to the manufacturers instructions. Normal tissue was obtained from a tumor-free lymph node, or where necessary from tissue with a small component of neoplastic cells. DNA concentrations were measured using the NanoDrop spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands).

Real-time PCR

The polymorphism 765G>C in the promoter region of *COX-2* was detected using the LightCycler 2.0 (Roche, Mannheim, Germany) with 5'-cattaactatttacagggtaactgcttagg-3' and 5'-ccccctccttgtttcttgga-3' (Applied Biosystems Foster City, USA) as primers and the MGB fluorescent probes 6-FAM-5'-CTTTCCCGCCTCTCT-3', and TET-5'-CTTTCCCCCCTCTCT-3' (Applied Biosystems Foster City, USA) in a 20µl reaction mixture containing 10µl QuantiTect Probe PCR Kit (Qiagen, Leusden, The Netherlands), 10 pmol forward and reverse primer, 2 pmol of each probe and 50ng genomic DNA. PCR conditions were as follows: 94°C for 15 minutes followed by 45 cycles of 94°C for 15 seconds and 60°C for 30 seconds. In each run three positive control samples (GG, GC and CC allele) as confirmed on sequencing were used together with water as a negative control.

Sequencing

To confirm our result from real-time quantitative PCR, 10% of the samples were sequenced. The promoter region was amplified using the primers 5'-gcatacgttttggacatttag-3' (forward) and

Table 1 Patients characteristics

	No of patients	Age/range	Histology
Early onset gastric cancer (EOGC)	115	≤45 (21- 45 years)	Intestinal-25 (22%) Diffuse-80 (70%) Mixed-10 (9%)
Conventional gastric cancer	96	>45 (47- 86 years)	Intestinal-49 (51%) Diffuse-36 (38%) Mixed-11 (11%)
Gastric stump cancer (GSC)	30	(54-85 years)	Intestinal-26 (87%) Diffuse-2 (7%) Mixed-2 (7%)
Control group	100	(22-52 years)	None

5'-ctaccttcagtgtacatagc-3' (reverse) (Applied Biosystems Foster City, USA). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), according to the manufacturer's instructions. The sequences were analyzed on an ABI 3100 automated sequencer (Applied Biosystems) using the ABI Big Dye Terminator Cycle Sequence Kit (Applied Biosystems, Foster City, USA) and the forward internal primer 5'-gttttggacatttagcgtcc-3'(Applied Biosystems Foster City, USA). Sequences of control samples kindly obtained from Pathology Department at the Johns Hopkins Medical Institute (Baltimore, USA) were also confirmed using this method.

Immunohistochemistry

COX-2 immunohistochemistry was carried out on tissue microarrays (TMA) when available (n=184) and as previously described.[9] In 51 cases it was performed on whole tissue sections and in 6 cases tumor tissue was no longer available or unassessable on the TMA. Sections (4 μm) were deparaffinized and antigen retrieval was carried out by 10 minutes of boiling in 0.01 M Na-citrate buffer (pH 6.0), followed by immersion in 0.03% hydrogen peroxide in methanol for 20 min. Non-specific binding sites were blocked by preincubation with and 0.01 M Tris, 0.1 M $MgCl_2$, 0.5% Tween-20, 1% BSA, 5% normal goat serum for 1h. Incubation of the primary antibody was carried out using monoclonal COX-2 antibody at a dilution of 1:100 (Cayman Chemical Co., Ann Arbor, USA) at 4°C overnight. Antibody binding was visualized using the Powervision+poly-HRP detection system (ImmunoVision Technologies, Daly City, CA, USA) with 3,3 amino-9-EthylCarbazole (Sigma-Aldrich, Zwijndrecht, Netherlands) as chromogen. Sections were counterstained with haematoxylin.

Scoring

The overall score of the tumor was the highest score found. The following scoring criteria of the tumor cells were used: 0, no staining; 1, weak diffuse cytoplasmic staining (may contain stronger intensity in less than 10% of the cancer cells); 2, moderate to strong granular cytoplasmic staining in 10-50% of the cancer cells; 3, >50% of the tumor cells stained with strong intensity. Scores 0 and 1 were categorized as "COX-2 low" and scores 2 and 3 as "COX-2 high" for the statistical analyses.[18] No slides showed increased negative staining around the edges and the age of the block was of no influence on immunohistochemical staining.

Statistics

The SPSS 14.0 software package was used for statistical analysis. A Chi-squared test was applied to the groups of gastric cancer to determine whether there was a statistical difference ($p<0.05$), between the presence of C or G allele as well as to examine the correlation between the presence of the C or G allele and COX-2 overexpression. A binary logistic regression model was used to adjust for potential confounding factors such as location (cardia or body in the case of EOGC and conventional gastric cancer) and histological type.

Results

Distribution of the COX-2 -765 G→C polymorphism

The distribution of the COX-2 -765 promoter polymorphism was examined in 241 cases of gastric cancer, including 96 conventional gastric cancers, 115 EOGCs and 30 gastric stump cancers

as well as in 100 healthy control cases, as can be seen in Table 2. All genotypic distributions were in Hardy-Weinberg equilibrium ($p \geq 0.05$).

Of note the frequencies of the three genotypes (GG, GC, and CC) were similar in EOGC, conventional gastric cancer, and stump cancer, with no statistical difference between groups using a χ^2 test. There was a statistically significant difference in the presence of the C allele in patients

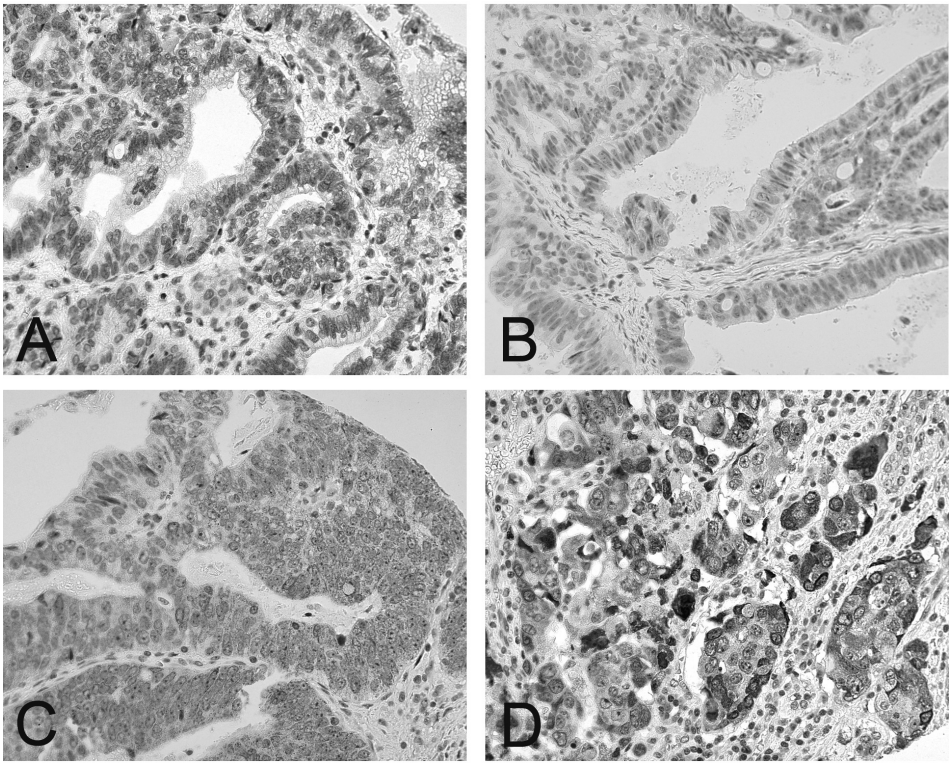

Figure 1 COX-2 Immunohistochemistry. A – category 0, no staining; B – category 1, weak diffuse cytoplasmic staining (may contain stronger intensity in less than 10% of the cancer cells); C- category 2, moderate to strong granular cytoplasmic staining in 10-50% of the cancer cells; D- category 3, >50% of the tumor cells stained with strong intensity. For statistical analysis scores 0 and 1 were categorized as COX-2 low and scores 2 and 3 as COX-2 high. ***(See page 198 for colour figure)***

Table 2 Prevalence of -765G>C COX-2 genotype

COX-2 -765 genotype*	Early-onset Gastric Cancer	Conventional Gastric Cancer	Gastric Stump Cancer	Controls
GG	80/115 (70 %)	73/96 (76 %)	23/30 (77 %)	59 (59%)
GC	33/115 (29 %)	19/96 (20 %)	5/30 (17 %)	32 (32%)
CC	2/115 (2 %)	4/96 (4 %)	2/30 (7 %)	9 (9%)
Presence of C allele	30 %	24 %	23 %	41 %

*All percentages rounded to the nearest digit

Table 3 COX-2 Expression Patterns in Gastric Cancer

Immunohistochemistry	Early-onset Gastric Cancer	Conventional Gastric Cancer	Gastric Stump Cancer
COX-Low	95/110 (86 %)	29/95 (31 %)	12/30 (40 %)
COX-High	15/110 (14 %)	66/95 (69 %)	18/30 (60 %)

with gastric cancer (EOGC, conventional gastric cancer and gastric stump cancer) compared to the control group (p=0.007), with the G allele being associated with gastric cancer.

There was also no association between the presence of any one polymorphism and histological type.

COX-2 expression in gastric cancer

Expression of COX-2 was assessed by immunohistochemistry in 235 of the gastric cancers which were subject to polymorphism analysis including EOGC, conventional gastric cancer and stump cancers as can be seen in Table 3. Of note, 94 of the EOGCs and 90 cases of conventional gastric cancers described in Table 3 have been previously published,[9] whereby a statistically significant difference (p <0.001) was seen between EOGC and conventional gastric cancer, with EOGC having a COX-2 Low expressing phenotype, as well as a statistically significant association with the intestinal phenotype (p <0.001). This statistical difference remained when adjusted for histology and location using a binary logistic regression model (p <0.001). They have been included here for comparison with the gastric stump cancers and for the association with the COX-2 -765 polymorphism.

We found that the COX-2 expression profile of gastric stump cancers bears similarity to that of conventional gastric cancers, with no statistical difference between these two groups (p=0.25) and thus the EOGC group remained significantly different from gastric stump and conventional gastric cancers with respect to COX-2 expression (p<0.001). There was no significant correlation between *COX-2* expression and -765 genotype (p=0.22).

Discussion

The diseases in which a role for COX-2 has been shown are usually characterized by varying individual and even ethnic susceptibility, implying the role of genetic factors.[19] The specific function of COX-2 in the formation of prostaglandins makes it a strong candidate for increasing susceptibility to common cancers. Genetic polymorphisms that alter the level of protein expressed would be anticipated to have a substantial influence on disease activity. Several single nucleotide polymorphisms (SNPs) in *COX-2* have been reported previously, but many of these polymorphisms seem to be functionally insignificant and not associated with susceptibility to cancer.[20-22]

However, it has been shown that the -765G>C *COX-2* polymorphism has been reported to disrupt an Sp1-binding site and displays a lower promoter activity [14] and interestingly, a higher COX-2 expression has been found in the normal mucosa of patients with FAP who carried the -765GG polymorphism,[15] than carriers of the C allele. These findings awaken curiosity within the field of gastric cancer, as to whether it may also play a role here.

In this study, we sought to identify the distribution of the -765 G>C polymorphism in the promoter of the human *COX-2* gene in early-onset gastric cancer, conventional gastric cancer and gastric stump cancers. In addition we looked at the effect of this polymorphism on COX-2 expression. We found that the G allele was associated with gastric cancer including early-onset gastric cancer, conventional gastric cancer and gastric stump cancers, thus the C allele showed a protective effect and there was no significant difference between histological type. In addition we found no correlation between the presence of the G allele and overexpression of the COX-2 protein in either gastric cancer or normal mucosa.

An association between the –765C allele and increased risk to esophageal squamous cell carcinoma has been demonstrated previously [23] and an increased risk of colon cancer in Singapore Chinese who consume high amounts of n-6 polyunsaturated fatty acids has also been associated with this polymorphism. [24] However, a study in Japan showed no association between the -765G>Cpolymorphism and risk of colorectal cancer.[25] The role of this polymorphism in gastric cancer has been variable and the literature is inconclusive. Liu *et al* found that subjects who carried the –1195AA genotype, another *COX-2* promoter polymorphism, had an increased risk of gastric cancer and a significant increase in COX-2 expression,[26] however they failed to show any association with the -765G>C polymorphism and gastric cancer. This may be due the fact that the variant genotypes (CC) of the -765G>Cpolymorphism were rare in the population of their study (<1%), whereas in our study group we saw the CC genotype in 9 % of our control group (which consisted of 100 healthy males as described previously.[17]) Additional controversy in the literature lies with a study by Pereira *et al*, where an association with the C allele and gastric cancer was reported. This is unexpected, as the described decrease in expression has been reported in association with the C allele. [14,15] One would thus expect that the G allele, is responsible for conferring an increased susceptibly to gastric cancer. However, contrary to the study by Brosens *et al* where they found a correlation between COX-2 expression in the normal mucosa and the -765 polymorphism, we find no relationship between the -765 *COX-2* polymorphism and expression in either the tumor or normal tissue, and thus the situation in the stomach does not appear to mirror that of the intestine in this regard. However, despite showing an association with the C allele and gastric cancer, Pereira *et al* also describe that their results revealed a possible protective role for -765C carriers, a finding which is confirmed with statistical significance in our current study. Previous studies have shown that SNPs in the *COX-2* gene vary greatly in different ethnic populations,[14,15] providing a possible explanation for the discrepancy within the literature in relation to gastric cancer.

In summary, we show that the *COX-2* -765 G allele promoter polymorphism is significantly associated with gastric cancer including early-onset gastric cancer, conventional gastric cancers and gastric stump cancer when compared to the normal control group but does not appear to be related directly to COX-2 expression patterns in the stomach. Although EOGCs appear to have a unique COX-2 expression pattern when compared to conventional gastric cancer, the exact mechanism by which this occurs is yet to be elucidated.

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7

Cyclin E Low Molecular Weight Isoforms Occur Commonly in Early-onset Gastric Cancer and Independently Predict Survival

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Abstract

Aim: Post-translational cleavage of full-length cyclin E from the N-terminus can produce low molecular weight (LMW) isoforms of cyclin E containing the C-terminus only. In this study we assess their presence in early-onset gastric cancer (EOGC), stump cancers and conventional gastric cancers and ascertain how they influence survival in EOGC.

Methods: To investigate their presence in gastric cancer, we compared the expression of full-length and LMW isoforms of cyclin E in 330 gastric cancers including early-onset gastric cancer (EOGC), stump cancer and conventional gastric cancer (>45 years old) using antibodies targeted to the N- and C-terminals. In addition we investigated these isoforms further using western blot analysis and RNA interference technology on gastric cell lines.

Results: LMW isoforms were found in 35% of EOGCs, compared to in 8% in conventional gastric cancers and 4% in stump cancers and their presence was visualised and specificity confirmed in cell lines using Western blot analysis and siRNA technology. In addition, C-terminal staining was a positive predictor of survival in EOGC. In contrast, no correlation with survival was found with the N- terminal antibody detecting full-length cyclin E only.

Conclusion: We provide evidence that EOGCs have a unique molecular phenotype and that cyclin E LMW isoforms may independently influence survival in EOGC.

Introduction

Gastric cancer is the second most common cause of cancer-related death in the world.[1] Approximately 5-16% of gastric cancer occurs in patients 45 years old or younger,[2,3] and the clinicopathological and molecular features are believed to be different between gastric cancer occurring in young and old (>45 years of age) patients.[4-6] There are two main histological types of gastric cancer, diffuse and intestinal, as described by Laurén[7] who also noted that diffuse cancers were more likely to occur in younger patients as confirmed in further reports.[8-10] Diffuse histology and young age have previously been considered to have a poor prognosis, but this subject has been widely debated.[2,3,11,12] It is known that regardless of histology or age, patients with small and early cancer lesions who undergo surgical resection have a better chance of survival. However, most patients are diagnosed with advanced stage disease and the five-year survival rate is generally less than 10%.[13]

Gastric carcinogenesis occurs through a combination of environmental and genetic factors although the latter may be more important in young patients.[14] Chronic inflammation caused by *H. pylori* infection is believed to be a critical environmental factor in gastric carcinogenesis.[15-17] However, as the pathogenesis related to *H. pylori* exposure is suspected to take decades, it is more likely to account for cases of gastric cancer in older patients.[16]

Deregulation of the cell cycle is known to be a critical event in the onset of tumorigenesis. Cyclin E is a G_1 type cyclin, which forms complexes with cyclin dependent kinase 2 (CDK2) and subsequently phosphorylates the retinoblastoma protein (Rb), an important tumor suppressor, thereby facilitating S phase entry through the release of E2F transcription factors from Rb. In normal cells, cyclin E accumulates at the G_1/S boundary and is degraded as the cell passes through the S phase. This periodicity is controlled by cell-cycle dependent transcription and post-translational control by ubiquitin-dependent proteolysis (i.e. proteasome degradation).[18] Cyclin E has also been described as having many different functions such as centrosome duplication,[19,20] histone biosynthesis, transcriptional regulation and pre-mRNA splicing.[21] However, as illustrated by several mouse models, where the function of cyclin E was not found to be critical,[22-24] the importance of each of these is not yet fully clear. Cyclin E is amplified and overexpressed in many different tumor types, including gastric cancer.[25,26] In breast cancer LMW isoforms of cyclin E as well as the full length form have been reported to act as predictors of poor survival. These isoforms, described to be tumor specific, are produced at the protein level through unique N-terminal post-translational proteolytic mechanisms [27,28] and it has been found that in breast cancer, they are not subject to cell cycle regulation but instead are constitutively active. [29,30,31]

Accurate predictors of outcome in cancer enable the administration of appropriate treatment for various patient groups thus ultimately helping prolong survival or increase quality of life for these patients. Thus whether cyclin E can help predict survival in gastric cancer is of great interest. LMW isoforms of cyclin E are not detected by all available cyclin E antibodies as an N-terminal directed antibody only detects the full length form whereas a C-terminal antibody will detect both full length and LMW isoforms of cyclin E.[32,33] The presence of LMW isoforms of cyclin E has not yet been investigated in gastric cancer. In this study we used N- and C-terminal directed antibodies to assess the presence of full length and LMW isoforms of cyclin E in 330 different gastric cancers, including 184 EOGCs and we assessed the impact on survival for a subset of these EOGCs where a 12 year follow-up was available. Finally, we visualized these LMW isoforms of cyclin E in gastric cancer, by western blot of gastric cell lines and confirmed that they are not recognized by the N-terminal antibody. Our findings highlight molecular differences between EOGC and conventional

gastric cancer with respect to LMW isoforms of cyclin E and our survival statistics present a role for cyclin E as a positive prognostic indicator in early-onset gastric cancer.

Materials and Methods

Patients/study groups

This research was carried out in accordance with the ethical guidelines of the research review committee of the Academic Medical Centre, Amsterdam. Twenty-eight stump cancers from the Amsterdam post-gastrectomy cohort[34] and 119 conventional gastric cancers from the Academic Medical Centre, Amsterdam (>45 years old) were used. One hundred and eighty four cases of gastric carcinoma in patients under 45 years of age were obtained from institutions throughout the Netherlands through the nationwide search system. Follow-up survival data were available for 44 of these young cases. These 44 cases are recruited from a well characterized large scale study in the Netherlands with long and complete follow-up.[35,36] The original study group consisted of 1,078 patients of whom only 44 were below the age of 45. Only archival paraffin embedded tissue and no frozen material was available from these 44 patients. For the normal control in Western blot analysis, fresh frozen histologically normal non-cancerous gastric mucosa, obtained from a gastrectomy specimen (distant to the site of a perforation) was used.

Construction of Tissue Microarray (TMA)

113 of the EOGCs and 91 of the conventional gastric cancers were assessed immunohistochemically using tissue microarrays constructed from formalin-fixed and paraffin-embedded archival specimens as described previously.[37] Three core biopsies (0.6mm cylinders) were taken from histologically representative regions (including heterogeneous areas) of paraffin-embedded gastric carcinomas and arranged in a new recipient paraffin block (tissue array block) using a trephine apparatus. (Beecher Instruments, Silver Spring, MD, USA). Normal gastric mucosa from each case was also included where available. Cores were placed in 3 separate subdivisions together with insertion of liver, lymph node and kidney cores to assist analysis. Unassessable cores were excluded from the analysis. The highest score of the 3 cores was used for the overall score for a case.

Immunohistochemistry

Paraffin-embedded material: Immunohistochemistry was performed using 4μm sections which were deparaffinised, blocked for endogenous peroxidase activity by immersion in 0.3% H_2O_2 for 20 minutes and heat treated at 100ºC in Tris/EDTA (pH 9) for 10 minutes. Non-specific binding was blocked using 5% normal goat serum for 10 minutes followed by incubation for one hour with the primary antibody. The following antibodies were used: HE12, sc-247, targeting C-terminal[38] (Santa Cruz Biotechnology, Inc, Heidelberg, Germany)1:3200, polyclonal N-terminal antibody H-145, sc-20684 (Santa-Cruz) 1:500. The Powervision Plus poly-HRP detection system (ImmunoVision Technologies, Daly City, CA, USA) in combination with 3,3-diaminobenzadine was used to visualize the antibody binding sites. Sections were counterstained with haematoxylin.

Frozen material: 4μm sections were cut at -20 °C and fixed with acetone. Non-specific binding was blocked using 5% normal goat serum for 10 minutes followed by incubation for one hour with the primary antibody, as above. Endogenous peroxidase activity was blocked using 0.1% natriumazide and 0.3% H_2O_2 in phosphate buffered saline 9 minutes. Peroxidase activity was

then detected using 3,3 amino-9ethyl carbazole (Sigma) in dimethylformamide and sections were counterstained with haematoxylin.

The negative controls for the immunohistochemistry were carried out when optimizing the antibody. Here we performed immunohistochemistry using a variety of antibody dilutions and buffers on both normal gastric mucosa and gastric mucosa with cancer to optimize the antibody and attain minimal background. During this procedure it could be clearly seen that the normal gastric mucosa was negative with occasional nuclear staining (which also confirms that the staining has been successful) whereas the gastric cancers often showed stonger and more widespread nuclear staining. In this way the "cleanness" and accuracy of the staining could be assessed.

All tumors were initially scored using four immunohistochemical categories: 0-4%, 5-10%, 11-50% and 51-100% positivity in the nucleus.[39] Thereafter, cases in the 0-10% categories were deemed negative/normal and cases in the 11-100% category were deemed positive/overexpressing. Of note, as 204 cases were examined using TMA and the remaining 126 on full sections, 63 cases on the TMA were also examined using conventional full section immunohistochemistry which showed a 98.4% concurrence of scoring between these techniques. All cases used for survival analysis were scored on full tumor sections.

Cell Culture and Lysates

Cell lines MB-MDA-157 (breast carcinoma known to produce LMW isoforms of cyclin E [30]), Hs746T and AGS (gastric carcinomas), were grown using RPMI medium supplemented with 10% fetal calf serum, glutamine and antibiotics. To obtain lysates, cells were washed twice with ice-cold PBS and ice-cold modified RIPA buffer (Tris-HCl 50mM, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150mM NaCl, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM Na_3VO_4, 1mM NaF, 0.1% SDS and 1µg/ml each of aprotinin, leupeptin and pepstatin) was added. Adherent cells were scraped with a chilled rubber policeman and the lysate gently rocked for 15 minutes at 4°C to lyse the cells. Lysates from fresh frozen tissue were made by cutting 4µm frozen sections which were immediately incubated in ice-cold RIPA buffer. The lysates were subsequently centrifuged and the pellet discarded. Protein concentrations were measured using Bicinchoninic Acid Solution according to Sigma procedure TPRO-562. (Sigma-Aldrich, Zwijndrecht, The Netherlands)

Western blot analysis.

60µg of protein heated for 10 minutes in sample buffer was used per lane. Proteins were resolved by SDS-PAGE, and electrotransferred to nitrocellulose membranes. The nitrocellulose membranes were blocked overnight in 5% milk in Tris buffered saline with 1% Tween (TBST) and incubated with the primary antibody in 5% milk/TBST (HE-12 at 1:10, H-145 at 1:100) at 4°C for 3 hours. After washing 4 x 5 minutes at room temperature a secondary antibody coupled to HRP (Dako) was added and incubated for 1 hour. Detection of proteins was performed using ECL™ Western Blotting Detection Reagents (Amersham Biosciences, Buckinghamshire, England).

Transfection with siRNA Oligos

The siRNA duplex oligos corresponded to the nucleotides 361-382 (GenBank accession no. XM_049430) of the human cyclin E coding region (with the sequences CACCCUCUUCUGCAGCCAAdTdT and dTdTGUGGGAGAAGACGUCGGUU) and which are known to be specific for cyclin E[40] were synthesized by Dharmacon Research, Inc to include a dTdT 3' overhang. MD-MB-157 cells, which are known to produce LMW isoforms of

cyclin E[30], were transfected when 30-40% confluent with 200nM siRNA oligos in 6 well plates using Oligofectamine reagent (Invitrogen, Breda, The Netherlands) following the manufacturer's protocol and harvested after 21 hours. A control sample was treated in an identical fashion except for the omission of the siRNA duplex oligos.

Statistics

The SPSS 11.5 software package was used for statistical analysis including Chi-squared tests, cumulative survival curves with the univariate Kaplan- Meier method and the stepwise Cox regression approach (proportional hazard model) to identify prognostic significance.[41] All clinical variables were included in this multi-variate analysis. A Chi-squared test was applied to the groups of gastric cancer to determine whether the differences found between antibodies were statistically significant and a binary logistic regression model was used to adjust for diffuse histology.

Results

Overexpression of Cyclin E

The prevalence of cyclin E overexpression was assessed in 184 early-onset gastric cancers, 118 conventional gastric cancers and 28 stump cancers using immunohistochemistry. This was carried out using an antibody directed toward the N-terminus (recognizing full length cyclin E only), and an antibody directed toward the C-terminus (recognizing both full length and LMW isoforms of cyclin E). Thus cases with C-terminal staining but no N-terminal staining contain LMW isoforms of cyclin E only.

IHC results are summarized in Table 1. Overexpression of full-length cyclin E was seen in 26% (48/184) of EOGC cases. This contrasted with conventional gastric carcinomas which showed overexpression of full-length cyclin E in 51% (61/118). Strikingly, when detection of LMW isoforms was included by staining with a C-terminal directed antibody, the overexpression of

Table 1 Results of Immunohistochemistry with N and C terminal cyclin E antibodies

	No. of cases	N-terminal staining	C-terminal staining	Presence of Isoforms*
Early onset gastric carcinomas	184	26% (48/184)	60% (113/184)	35% (65/184)
Conventional gastric carcinomas	119	51% (61/118)	58% (70/119)	8% (9/118)
Stump cancers	28	21% (6/28)	25% (7/28)	4% (1/28)

* as calculated from cases negative for N-terminal staining and positive for C-terminal staining

Table 2 Classification of tumors according to Laurén

	Number of		
	diffuse cancer	intestinal cancers	mixed cancers
Early onset gastric carcinomas	116	46	22
Conventional gastric carcinomas	35	70	14
Stump cancers	2	24	2

cyclin E in EOGC was 2.3 fold higher (113/184) and this difference was statistically significant ($p < 0.001$) using a Chi squared test. This increase in staining due to presence of LMW isoforms was not seen in the conventional gastric cancers, where only an additional 9 positive cases were seen with C-terminal staining i.e. isoforms were present in 35% of EOGCs compared to only 8%

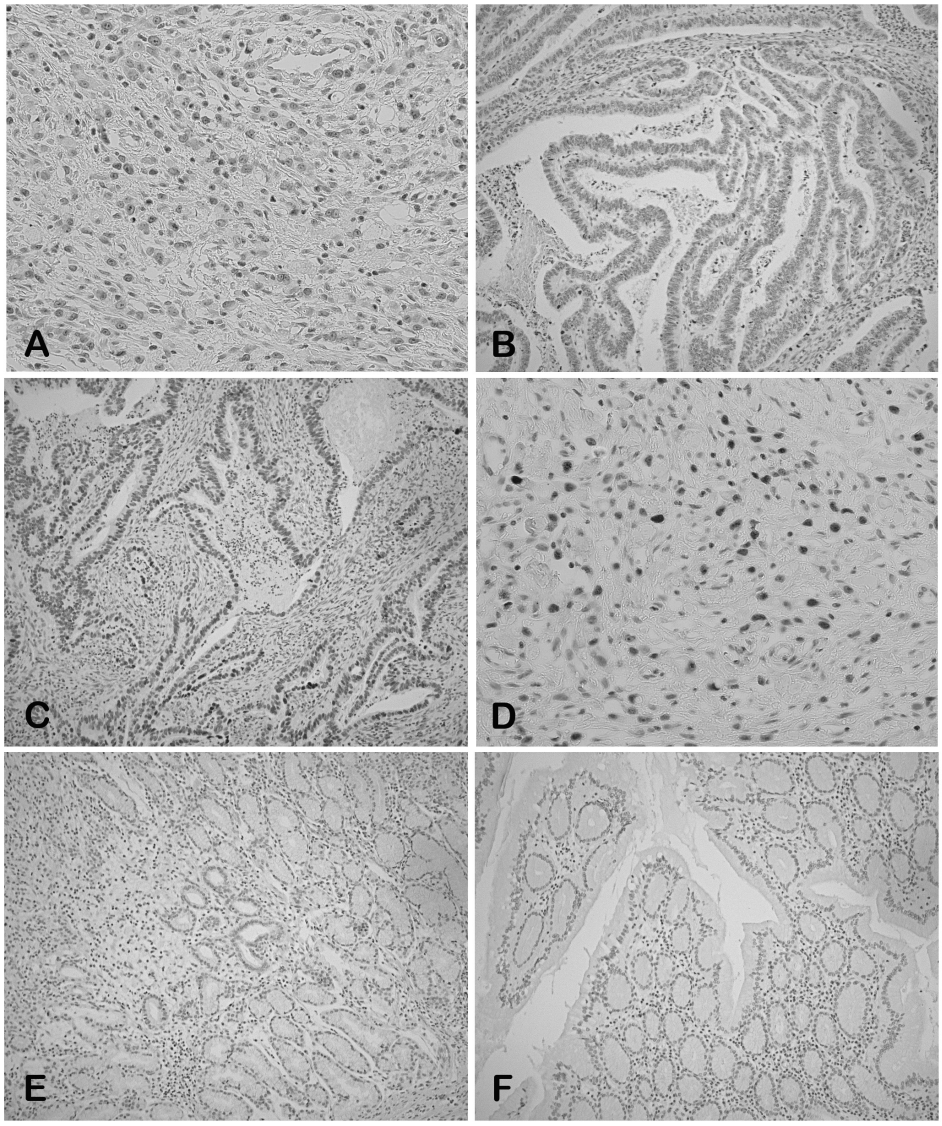

Figure 1 The panel on the left shows immunohistochemical staining (x100) with the N-terminal antibody, negative (A) and positive (C) in tumour tissue and normal gastric mucosa (E). The right panel shows staining with the C-terminal antibody, negative (B) and positive (D) in tumour tissue and in normal gastric mucosa (F). Of note (A) and (D) show staining of the same tumor with different antibodies. ***(See page 199 for colour figure)***

of conventional gastric cancers. A typical immunohistochemical staining with N and C-terminal antibodies in EOGC can be seen in Figure 1.

A larger proportion of the early-onset gastric cancers had a diffuse histology than in the conventional group, which consisted mainly of intestinal cancers, as can be seen in Table 2. Despite this epidemiological overrepresentation of diffuse cancer in young patients, the presence of isoforms of cyclin E (as detected by C-terminal staining only) correlated significantly with age even when adjusted for diffuse histology using a binary logistic regression model ($p < 0.001$, 95% confidence interval 0.095 – 0.382).

Low Molecular Weight Isoforms of Cyclin E

Western blot analysis was carried out in order to visualize the LMW isoforms of cyclin E in gastric cancer and to confirm that they were not recognized by the N-terminal antibody. (Figure 2) Of note, the C-terminal antibody has been widely described in literature as recognizing LMW isoforms of cyclin E.[28-31,33] The breast cell line MD-MBA-157 known to contain isoforms of cyclin E [30] was used as a positive control and normal gastric mucosa from a patient without cancer which

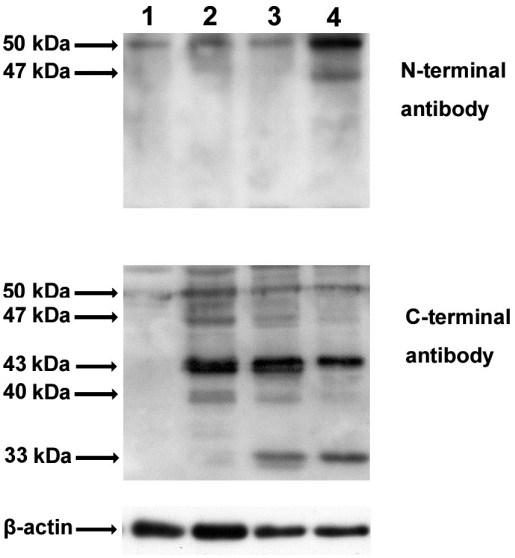

Figure 2 Western Blot for cyclin E using both N-terminal and C-terminal antibodies. 1-normal gastric mucosa, 2-MB-MDA-157 breast cell line, 3-Hs764T gastric cell line, 4-AGS gastric cell line

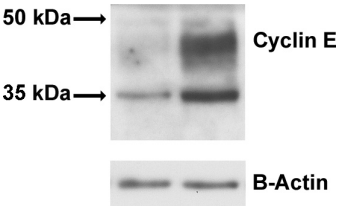

Figure 3 Western Blot for C-terminal cyclin E in the RNA interference treated cell line (left) compared to control (right).

Table 3 Results of the Cox regression (proportional hazard model) analysis

	Correlation with Survival	Significance (p<0.05)	Hazard Ratio	95% Confidence Interval
Multivariate Analysis (inclusion of cyclin E)	Residual disease present at time of surgery	0.001	21.38	6.83-66.96
	Cyclin E negative staining (C-terminal antibody)	0.001	5.63	2.05-15.46
	Lymph node metastases	0.002	12.09	2.44-59.93
	T-stage	none	-	-

was confirmed as negative for cyclin E overexpression on immunohistochemistry, was used as a negative control. As can be seen in Figure 2 the N-terminal antibody (upper panel) recognized the full length form of cyclin E only (50kDa), with the exception of one possible isoform in the AGS cell line at 47 kDa. On the other hand, the C-terminal antibody (lower panel) clearly recognized low molecular weight isoforms of cyclin E in both gastric and breast cell lines, with the predominant protein at 43 kDa and other isoforms seen at 47, 40 and 33 kDa. The normal gastric mucosa showed the presence of full length cyclin E only, using both the N and C-terminal antibodies.

Specificity of Cyclin E LMW Isoforms

In order to confirm that the extra low molecular weight bands detected by the C-terminal antibody are indeed cyclin E, Western blot analysis was repeated using the positive control cell line (MB-MDA-157, reported to contain cyclin E isoforms) transfected with or without siRNA duplex oligos known to specifically downregulate cyclin E.[40] In Figure 3 a knockdown of cyclin E and its LMW isoforms can be seen. The expression of cyclin E is clearly reduced in the cell line exposed to siRNA interference as compared to the control and this downregulation involved not only full length cyclin E, but also the low molecular weight bands, thus confirming that they are indeed also cyclin E. Thus low molecular weight isoforms of cyclin E which have been so widely described in breast cancer, are also present in gastric cancer.

Association of Cyclin E Expression with Survival

In order to assess the possible clinical relevance of cyclin E overexpression in gastric cancer, survival analysis was carried out on 44 EOGCs for which follow-up data were available. In this group survival was correlated with eligibility status (curative/non-curative resection), ($p<0.001$), the presence of residual disease (no residual tumor, positive margins microscopically, or macroscopic/non-curative resection), ($p<0.001$), UICC 1997 lymph node status ($p<0.001$), positive versus negative lymph node status ($p=0.006$) and UICC 1997 TNM stage ($p<0.001$) but not with histological type. Using a univariate analysis, cyclin E staining did not correlate with lymph node status, residual disease status, T stage, TNM stage, or histological type. Notably, positive C-terminal staining (therefore including both full length and LMW isoforms of cyclin E) was found to be an independent predictor of survival using a Cox regression stepwise analysis whereby negative staining had a hazard ratio of 5.63 ($p<0.001$, 95% confidence interval 2.05 – 15.46), (Table 3). This model included the presence of residual disease (no residual tumor, positive margins microscopically, or macroscopic/non-curative resection), positive versus negative lymph node status, T stage and cyclin E staining. It can be seen from Table 3 that residual disease (which

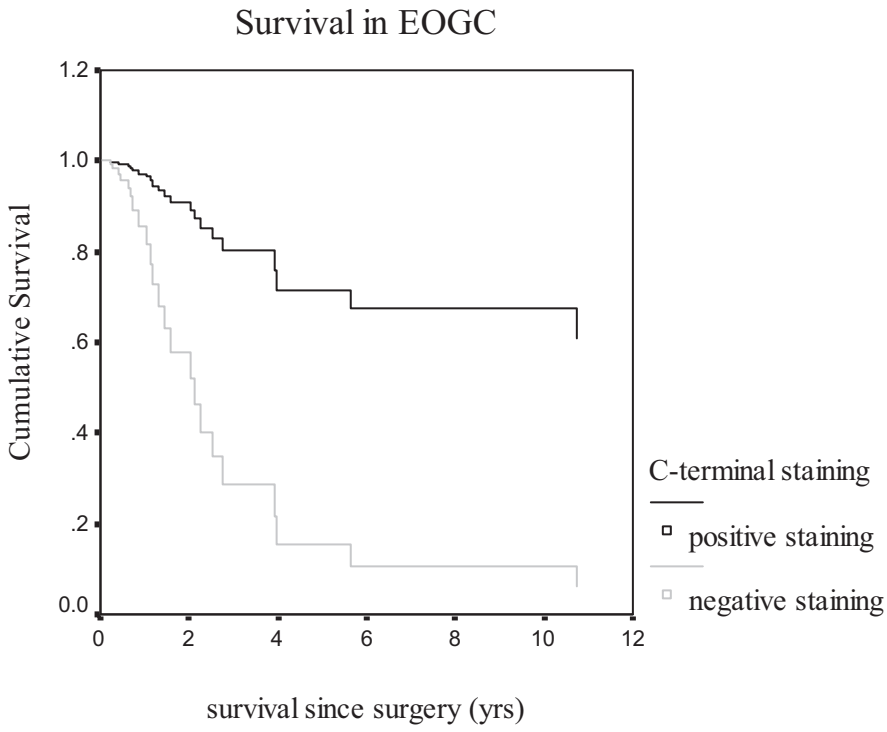

Figure 4 Cox regression Analysis results for cyclin E C-terminal staining. The median survival for the negative group was 2.14 years whereas the median survival for the positive group was greater than 12 years (alive at the time of follow-up).

affected 7 of the 44 patients) was the most important prognostic factor in this group of patients and interestingly the T-stage did not prove to be an independent prognostic indicator of survival in this group.

This association with C-terminal cyclin E staining and survival remained statistically significant with a hazard ratio of 5.166 (p=0.002) when only the cases positive for isoforms were included, (as assessed by negative N-terminal staining and positive C-terminal staining) thus demonstrating that the presence of the LMW isoforms alone is an independent predictor of survival in early-onset gastric cancer. Positive cyclin E staining using the N-terminal antibody staining on the other hand was not a predictor of survival (p=0.71).

Kaplein-Meier analysis showed that the median survival for the cyclin E negative group was 2.14 years whereas the median survival for the cyclin E positive group was greater than 12 years (still alive at the time of follow up) as can be seen in Figure 4. It is interesting to note that this group of young patients had a particularly good survival rate even with the inclusion of those patients with residual disease (7/44).

Discussion

The function and mechanisms of the tumor specific LMW isoforms of cyclin E, which are produced by post-translational cleavage of full length cyclin E by calpain[28] and elastases,[29] are of great interest and it is known that they are not subject to cell cycle regulation.[29,30] In this study we find that, LMW isoforms of cyclin E occur more commonly in EOGC than conventional gastric cancers or stump cancers, thus adding additional evidence that EOGC have a different molecular phenotype than conventional gastric cancer.[6,42] Furthermore, contrary to findings in breast cancer, these isoforms act as a positive predictor of survival in EOGC.

The clinicopathological implications of cyclin E overexpression have been reported to vary with the type of tumor.[33,43-45] Our findings suggest that function of LMW isoforms of cyclin E is different from that of the full length form in EOGC as demonstrated by the increase in survival in patients expressing LMW isoforms, as based on preferential N- and C-terminal staining. This unique role of LMW isoforms in gastric cancer is highlighted by the fact that our findings differ from the classical role of cyclin E as an oncogene, as initially established in carcinoma of the colon [46] and breast [38][47] where it was found to be a negative prognostic factor. In breast cancer deregulation of cyclin E/Cdk2 kinase activity was also shown to induce chromosomal instability and aneuploidy.[48] However, it has also been reported that cyclin E is not significantly correlated with polyploidy or aneuploidy when tumors of similar grade are evaluated unless aberrations in other genes such as *hCDC4* are present.[49] Furthermore, novel functions of cyclin E which conflict with previous dogma have recently been reported [50] whereby cyclin E overexpression in medullary breast cancer was shown to lead to decreased invasive potential. Here, cyclin E overexpression was shown to induce differences in gene expression patterns associated with cell adhesion, as well as rearrangements of the actin cytoskeleton, increased adhesive properties, decreased motility, and decreased invasive potential, indicating an overall abrogated mobility, as a result of cyclin E overexpression and providing a possible molecular basis for our survival findings in gastric cancer.

Our findings are in keeping with previous literature that cyclin E positivity correlates with good prognosis in gastric cancer [51] although in some reports, no correlation was found.[52,53] To our knowledge this is the first study where LMW isoforms of cyclin E have been described in gastric cancer and the association of these isoforms with patient age may provide an explanation for the discrepancy between previous reports.

Our results lend further support to the growing evidence that the role of cyclin E in carcinogenesis is far more complex than first thought and its role appears to vary depending on the cancer type[51] as demonstrated by its role as a positive prognostic indicator in bladder tumors,[45,54] certain lung tumors,[55] skin tumors[56] as well as gastric cancers.[51] This complexity of molecular wiring in carcinogenesis has also been emphasized in recent literature [57,58] with the conclusion that cancer can no longer be viewed purely in terms of a network of oncogenes and tumor suppressor genes. Our findings also add to the increasing literature that EOGCs have a molecular phenotype distinct from that of conventional gastric cancers.[6,42,59]

Gastric carcinoma continues to be a cause of premature death, despite progress in detection and treatment and despite advances in our understanding of the molecular basis of cancer. The need to develop efficient and effective cancer-specific drugs is coupled with the importance of accurate prediction of disease outcome for various patient groups some of whom, due to the biology of their disease, will do better than others and may warrant a different treatment protocol. In this study we found a difference in the survival of EOGC patients between those expressing full-length or LMW isoforms of cyclin E, as based on preferential N- and C-terminal staining. In addition, our findings

highlight that early-onset gastric cancers have a unique molecular phenotype when compared to conventional gastric cancers.

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8

Loss of *CDC4/FBXW7* in early-onset gastric cancer

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Abstract

The *CDC4/FBXW7* gene, which maps to 4q32, encodes a ubiquitin ligase and has been implicated as a tumour suppressor gene in many tumour types. Mutations in colonic adenomas, and the frequent losses on 4q described in gastric cancer prompt speculation about the role of *CDC4/FBXW7* in gastric carcinogenesis.

Here we assessed the role of *CDC4/FBXW7* in gastric cancer, through loss of heterozygosity (LOH) and multiplex ligation-dependent probe analysis (MLPA) on 47 flow-sorted gastric tumours (including early-onset gastric cancers (age ≤ 45 years, EOGC) and xenografted gastric tumours), ploidy analysis on 39 EOGCs, immunohistochemistry of CDC4/FBXW7, its substrates c-myc, c-jun, Notch and cyclin E on 204 gastric cancers using tissue microarrays (TMAs) together with sequence analysis of xenografted gastric cancers and gastric cancer cell lines. Loss of heterozygosity of *CDC4/FBXW7* occurred in 32% early-onset gastric cancer, and was correlated with loss of expression in 26% of cases. Loss of expression was frequent in both early-onset gastric cancers and conventional gastric cancers on tissue microarray analysis suggesting that *CDC4/FBXW7* is an important tumour suppressor gene not only in early onset gastric cancer but in gastric carcinogenesis in general. In addition, a significant correlation was found between loss of CDC4/FBXW7 expression and upregulation of c-myc suggesting that c-myc activation is likely to be an important oncogenic consequence of *CDC4/FBXW7* loss in gastric cancers.

Introduction

Gastric cancer is the fourth most common malignancy in the world and ranks second in terms of cancer-related death.[1] Several classification systems have been proposed, but the most commonly used are those of the World Health Organization (WHO) and of Laurén who describes two main histological types, diffuse and intestinal.[2] Gastric cancer is thought to result from a combination of environmental factors and the accumulation of generalized and specific genetic alterations, and consequently affects mainly older patients often after a long period of atrophic gastritis. The commonest cause of gastritis is infection by *Helicobacter Pylori*, which is the single most common cause of gastric cancer [3, 4].

Research to date has not revealed a specific pathway for gastric cancer, although numerous molecules have been implicated. Aberations of the *CDH1* gene, encoding E-Cadherin, have been shown to be an important initiator of gastric cancer[5] but even in hereditary diffuse gastric cancer mutations of this gene explain only 30-40% of cases.[6] Thus the need to uncover other such critical regulators of gastric cancer is undoubted.

The *CDC4/FBXW7* gene, which maps to 4q32, encodes a ubiquitin ligase and has been implicated as a tumour suppressor gene in many tumour types. The finding of mutations in colonic adenomas, suggest that dysregulation of *CDC4/FBXW7* is a critical step in colon carcinogenesis. [7] This prompts speculation about the role of *CDC4/FBXW7* in the stomach and whether it acts as an important tumour suppressor in gastric cancer, as parallels can often been drawn between gastric cancer and cancer occurring in other parts of the gastrointestinal tract. The finding of frequent losses on 4q in gastric cancers using comparative genomic hybridization [8-12] furthers curiosity about the role of *CDC4/FBXW7* in gastric cancer and to date no studies have been performed on this gene in gastric cancer.

CDC4/FBXW7 forms part of the ubiquitin-proteasome mediated programmed degradation system whereby the addition of ubiquitin polymers to protein substrates directs them to the proteasome for destruction. Ubiquitin ligases called SCF complexes couple to any one of several "F-box" proteins, such as CDC4/FBXW7, which confer specificity of substrate, and over 70 different F-box proteins have been identified in man. [13] FBXW7 targets several oncoproteins, including cyclin E, c-myc, c-jun, Notch 1 and Notch 4 for degradation and its tumour supressor function is thought to be exerted through these substrates. Mutations that disrupt CDC4/FBXW7 are expected to deregulate cyclin E, c-myc, c-jun and Notch signaling thus simultaneously deregulating cell division, cell growth, apoptosis and cell differentiation.[14] Indeed, mutations in ovarian,[15] breast,[16] colorectal,[7] endometrial [17-19] and pancreatic cancer [20] have been described. In addition, mutations in the *CDC4/FBXW7* gene have shown to be associated with aneuploidy in endometrial cancer [19] although this has not been found to be the case in colon cancer.[21]

In order to examine the role of *CDC4/FBXW7* we have chosen to primarily investigate early-onset gastric cancers (EOGC; ≤ 45 years old) as it is more likely that environmental factors are less important in this group when compared to conventional gastric cancer (>45 years old). [22, 23] Indeed, EOGCs are known to have a different molecular and genetic phenotype from that of the older age group.[24-26] In this study we have carried out ploidy analysis and assessed the status of *CDC4/FBXW7* in EOGCs using loss of heterozygosity (LOH) and multiplex ligation-dependent probe analysis (MLPA) on flow-sorted tumour cells and have carried out sequence analysis in gastric cancer cell lines and xenografted gastric cancers. In addition we have assesed the protein expression of the CDC4/FBXW7 substrates cyclin E, c-myc, c-jun and Notch as well as p53 in 113 early-onset and 91 conventional gastric cancers.

Methods

Patients/study groups

Ninety one conventional gastric cancers (>45 years old), diagnosed between 1993 and 2003, were obtained from the Academic Medical Centre, Amsterdam. One hundred and thirteen cases of gastric carcinoma in patients under 45 years of age (coded with the letter Y), 90% diagnosed between 1994 and 2002 and 10% diagnosed between 1980-1994, were obtained from 24 different institutions throughout the Netherlands through the nationwide database system, and from the Department of Pathology at the Jorvi Hospital (Espoo, Finland). This age cut-off was chosen in order to obtain enough cases to achieve meaningful result. The tumours were classified by an experienced gastrointestinal pathologist (GJAO) according to the Laurén classification as intestinal, diffuse or mixed gastric adenocarcinomas (Table 1) and location was deduced from the pathological report. In addition, 8 xenografted gastric carcinomas (coded with the letter X) described by Milne *et al* [27] were included.

Tissue Microarray

Tissue microarrays were constructed from formalin-fixed and paraffin-embedded (FFPE) archive specimens. Three core biopsies (0.6 mm cylinders) were taken from histologically representative regions (including heterogeneous areas) of paraffin-embedded gastric tumours and arranged in a new recipient paraffin block (tissue array block) using a custom-built instrument (Beecher Instruments, Silver Spring, MD) as described previously.[25, 28] Normal gastric mucosa from each case was also included where available (33%, 69/204). Cores were arranged in 2 or 3 separate subdivisions together with insertion of liver, lymph node and kidney cores to assist analysis. 195 cases were informative for all markers. In most cases sections were stained immediately after cutting, but if stored, this was done so by wrapping in aluminium foil and freezing at -20°C to prevent loss of antigenic epitopes. Immunohistochemistry was carried out using full tissue sections for the 8 xenografted gastric cancers.

Immunohistochemistry

Sections (4 μm) were deparaffinized and endogenous peroxidase activity was blocked by immersion in 0.3% H_2O_2 in methanol for 20 minutes. Antigen retrieval was carried out by 10 minutes of boiling in 10 mM Tris/1 mM EDTA (pH 9) except for c-myc and cdc4/Fbw7 where no antigen retrieval was used and for C-jun where a pepsine pre-treatment was performed at a concentration of 0,25% in 0,01M HCl/pH 2.0 for 15 min at 37°C. Non-specific binding was blocked with 5% normal goat serum in PBS (pH 7.4) for 15 minutes at room temperature. The sections were incubated for 1 hour at room temperature with the following primary antibodies: CDC4/FBXW7 (Zymed, South San Francisco, USA) 1:50, p53 (monoclonal antibody combination of DO-7 and BP53-12 Neomarkers, Union City, CA, USA) 1:2000 dilution, NCL c-jun (Nova Castra) 1:200, Cyclin E Ab-5 (clone CYE05), monoclonal antibody, (Neomarkers,

Table 1 Clinicopathological Characteristics

Age	Histology			Location			Total
	Intestinal	Diffuse	Mixed	Cardia	Non-cardia	Unknown	
≤ 45 yrs old	24	80	9	9	74	30	113
> 45 yrs old	49	31	11	49	42	0	91

Fremont, CA, USA) 1:40 dilution, and c-myc (Santa Cruz) 1:50. Notch rabbit polyclonal antibody (Cell Signaling Technologies, Danvers, MA, USA) was incubated overnight at 4 °C, 1:50. The Powervision+poly-HRP detection system (ImmunoVision Technologies, Co, Daly City, CA, USA) was used to visualize antibody binding sites with 3,3'-diaminobenzidine as a chromogen. Sections were counterstained with haematoxylin.

Immunohistochemical Scoring

The overall score of the tumour was the highest score found of the 3 cores.[25] In all cases where the staining was not obviously abnormal, it was placed in the normal category. No slides showed increased negative staining around the edges. The following scoring systems were used: P53 – Cases scored as positive showed strong nuclear staining in > 30% of cells [29]; c-myc and c-jun – Nuclear staining in >5% was scored as positive [25, 30], with lack of staining or presence of cytoplasmic staining only, scored as negative; cyclin E – this was initially graded in 4 categories 0-4%, 5-10%, 11-50% and 51-100% positivity in the nucleus [31]. Thereafter, samples in the 0-10% categories were deemed negative/normal and 11-100% in the positive/overexpression category. CDC4/FBXW7 was scored in 4 categories: 0 – completely negative, 1- weak (nuclear staining in <20% of cells), 2- moderate (21-50%) and 3- strong (>50%). Two categories of negative (0) and positive (1, 2 and 3) were ultimately used in the statistical analysis. Notch scoring was negative if 0% of cells showed nuclear staining, all other staining patterns were scored as positive.

Statistical Analysis

The SPSS 11.5 software package was used for statistical analysis. A Chi-squared test was applied to the groups of gastric cancer to determine whether the differences found between antibodies were statistically significant ($p<0.05$). A binary logistic regression model was used to adjust for potential confounding factors such as location, histological type, age of blocks and the hospital from which the block was derived.

Tissue dissociation and staining

Four 60 μm sections pre-trimmed to exclude non-cancerous epithelial tissue were collected in individual 100 μm-pore gauze bags (Verseidag-Industretextilien GmbH, Kempen, Germany). Sections were deparaffinized, incubated in 10 mM citrate buffer pH 6.0 for 5 mins followed by 60 mins at 80ºC. After cooling and washing with phosphate buffered saline (PBS), enzymatic dissociation (0.1% collagenase I-A (Sigma) and 0.1% dispase) (Gibco BRL, Paisly, Scotland) was carried out as described previously.[32]

Cell concentrations were determined using a Bürcker haemocytometer and cells were incubated with 100 μl of antibody mixture (anti-keratin 5,6,8,17 clone MNF116 (Dako) 1:50, clones AE1,3 (Chemicon)1:100 and V9-2b (anti-vimentin; Department of Pathology, LUMC[33]) 1:5) per million cells and incubated overnight at 4º C. After washing, cells were then incubated for 1 hr at 4ºC with 100 μl of FITC and RPE labeled secondary antibodies (Southern Biotechnology Associates, Birmingham, AL) diluted 1:100 in PBS/0.5% BSA/0.05% Tween 20 (PBATw). Cells were washed and incubated for 30 mins at room temperature with 500 μl DNA staining solution (0.1% DNase free RNase (Sigma), 10 μM propidium iodide (PI), (Calbiochem, San Diego, CA) in PBATw). Samples were stored overnight at 4º C before flow cytometric analysis.

Flow cytometry and cell sorting

Data from 10,000 single keratin-positive tumour cells were collected using a FACSCaliber flow cytometer (BD Biosciences, San Jose, CA). FITC (FL1) and RPE (FL2) fluorescent signals were collected in the logarithmic mode and PI fluorescent signals were collected in the linear mode (FL3). The FL3-A versus FL3-W pulse processor was used to enrich for single cell events during acquisition and analysis. Data were analyzed using the WinList 6.0 and ModFit 3.1 software packages (Verity Software House Inc., Topsham, ME). For DNA-ploidy analysis, the vimentin-positive, keratin-negative cell fraction was used as an intrinsic DNA diploid reference. The DNA-index (DI) was calculated by dividing the mean channel number of the G0G1 fraction of the keratin-positive cells by the G0G1 fraction of the vimentin-positive, keratin-negative cells. DNA-ploidy was defined as follows: DI < 0.95: hypodiploid, 0.95 < DI < 1.05: diploid, 1.05 < DI < 1.95 aneuploid, 1.95 < DI < 2.05 tetraploid and DI > 2.05 aneuploid. N-color compensation was used for post-acquisition spectral cross-talk correction according to the manufacturer's instructions. Keratin-positive tumour cells and vimentin-positive normal stromal cells were flow-sorted using a FACSVantage flow sorter (BD Biosciences, San Jose, CA) using the 488 laser line at 300 mW.

Cell lines

Cell lines TMK1, NUGC4, HSC39, HS746T, ST42, GP220, MKN45, MKN7, MKN74 and MKN28 were cultured in RPMI 1640 (GIBCO BRL, MD, USA) supplemented with 10% fetal calf serum (FCS, GIBCO BRL, MD, USA) and 2% penicillin-streptomycin (GIBCO BRL, MD, USA). Cell line HM51 was cultured in DMEM (GIBCO BRL, MD, USA) supplemented with 10% FCS and 2% Penicillin-Streptomycin.

DNA Extraction

DNA from paraffin embedded and fresh frozen xenografts tissue was isolated using the PUREGENE® DNA Isolation Kit (Gentra Systems, Minneapolis, USA) according to manufacturer's instructions. DNA isolation from cell lines was performed using Proteinase K treatment, followed by phenol/chloroform extractions and precipitation using ammonium acetate and ethanol. DNA from flow-sorted cells were pelleted and treated with proteinase K before use. DNA concentrations were measured using a NanoDrop spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands)

LOH analysis

LOH analysis was carried out using the polymorphic microsatellite repeat markers D4S2934 and D4S2998 which lie 723bp and 7723bp from the telomeric and centromeric side of the *CDC4/FBXW7* gene. The sequences of the primers and their corresponding location were selected through the Genomic Data Base (http://www.gdb.org). PCR amplification was performed using 35 cycles in a 20 µl reaction volume containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1.5 unit of Platinum® Taq DNA polymerase in buffer supplied by the manufacturer, and 0.5 mM of each primer, one of which was labeled with a fluorescent marker with an annealing temperature of 58°C. A minimum of 10 ng of DNA was used for each PCR reaction to prevent induced losses. LOH analysis was carried out using an automated ABI 3100 sequencer with a Genescan™ 350ROX size standard (Applied Biosystems) and the manufacturer's Genescan® 2.1 software. The ratio of peak heights of the two alleles for tumour sample (keratin-positive cells) and normal (diploid, vimentin-positive celles) was calculated and subsequently the normal ratio was divided by the tumour ratio. A value below 0.6 or above 1.6 was interpreted as evidence of LOH whereas values between these

figures were considered retention of heterozygosity. All losses were confirmed by at least one repeat reaction.

Mutation Analysis

All 11 exons of *CDC4/FBXW7* were amplified using primers described previously [20] and shown in Table 2. At least 10 ng of DNA and 10 pmol of primer was used for each reaction and an annealing temperature of 57ºC for all exons except exon 8, where an annealing temperature of 55ºC was used. 35 cycles were carried out using 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 mM forward and reverse primers, at an annealing temperature of 55˚ C and 4 units of Platinum® Taq DNA polymerase (Invitrogen) in a buffer supplied by the manufacturer, with an end volume of 50 µl in a Peltier Thermal Cycler (DYAD™), (Biozyme, Blaenavon, South Wales, UK).

Following purification of PCR products with the QIA quick® PCR purification kit (Qiagen, Leusden, The Netherlands), the sequences were analyzed using the ready reaction Big Dye™ Terminator Cycle Sequence kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3100 automated sequencer (Applied Biosystems).

Multiplex Ligation-dependent Probe Amplification (MLPA)

MLPA was carried out by MRC-Holland, Amsterdam, The Netherlands using *CDC4/FBXW7* probes designed for this study as described by Schouten *et al*. [34] These included 6 probes targeted to each of the possible exon 1 variants (exon1A, exon 1B, exon 1C) as well as exon 2, exon 6 and exon 11 of the CDC4/FBXW7 gene. Amplification products were detected and quantified by capillary electrophoresis using the ABI 3100 automated sequencer (Applied Biosystems) using a ROX-labeled internal size standard (ROX-500 Genescan, Applied Biosystems, Warrington, UK).

Table 2 CDC4/FBXW7 Primers

CDC4/FBXW7	Primers
Exon 1C	FWD atctgatccctctgctgctg REV ctgcagccatctctgatgatac
Exon 2	FWD tcatcacacactgttcttctgg REV tgcttgtgtcctacttcaaaatg
Exon 3	FWD tgtgatctctgggaagaaagg REV gaatcaacatgtggccttttg
Exon 4	FWD gccttcttcaatgccttgtg REV caatttacagccctctttaccac
Exon 5	FWD aaggcaagagcagagacagag REV attcggctcatctgaatgtg
Exon 6	FWD ccaatttcctcacatcttcacc REV tcaagcacaattagttgtcagattag
Exon 7	FWD cgtcagccaggaaaaataatg REV ggggaaaaattgagccaag
Exon 8	FWD catgttttatatcatatgtttgctttc REV acaaaacgctatggctttcc
Exon 9	FWD atgcagcattctaggcttcc REV ttcttcatgccaattttaacg
Exon 10	FWD tcattgccactttatcttagtacctc REV aagtcagggtgaaaatattcataaac
Exon 11	FWD tttcttacatccatgctgatcc REV gaagggcagggagtatatcg

Table 3 Results of LOH, MLPA and IHC on cdc4/FBXW7

Case	D4S2998	D4S2934	MLPA	IHC	Ploidy
Y5	LOH	LOH	not performed	moderate	1.39
Y11	ROH	NI	not performed	not assessable	2.23
Y67	ROH	ROH	retention	moderate	1.15
Y102	ROH	ROH	not performed	weak	diploid
Y103	LOH	LOH	not performed	strong	1.49
Y106	ROH	ROH	not performed	moderate	0.90
Y107	LOH	LOH	not performed	strong	1.33
Y110	ROH	ROH	retention	negative	1.61
Y111	LOH	LOH	loss exon 6-11	strong	1.50
Y112	NI	ROH	retention	weak	diploid + 1.57
Y113	non amplifiable	LOH	not performed	negative	1.4 + 1.65
Y117	LOH	ROH	not performed	moderate	1.49
Y118	ROH	ROH	not performed	weak	1.08
Y121	ROH	ROH	not performed	moderate	1.81
Y126	NI	ROH	retention	moderate	1.33
Y130	ROH	ROH	retention	strong	1.53
Y132*	LOH	ROH	not performed	-	diploid
Y132	LOH	ROH	loss exon 6-11	weak	1.52
Y133	ROH	ROH	retention	moderate	diploid
Y135	ROH	ROH	retention	moderate	diploid
Y137	non amplifiable	ROH	retention	moderate	tetraploid
Y138	ROH	ROH	not performed	strong	1.18
Y139	ROH	non amplifiable	not performed	moderate	diploid
Y140	ROH	LOH	retention	negative	1.75
Y143	ROH	ROH	not performed	moderate	diploid
Y150	ROH	ROH	not performed	moderate	1.73
Y152	LOH	NI	not performed	moderate	0.81
Y156	ROH	ROH	retention	moderate	diploid
Y158	ROH	ROH	not performed	moderate	1.84
Y159	LOH	LOH	not performed	strong	1.15
Y160	LOH	LOH	gain exon 1B	strong	2.23
Y161*	LOH	LOH	not performed	-	0.78
Y161	LOH	LOH	not performed	negative	1.75
Y166	ROH	ROH	retention	weak	diploid + 1.71
Y168	LOH	LOH	gain exon 2	strong	diploid
Y169	ROH	ROH	retention	weak	1.60
Y177	ROH	ROH	retention	negative	1.29 and 1.57
Y180	ROH	ROH	not performed	moderate	diploid
Y182	ROH	ROH	not performed	weak	diploid
Y185	ROH	ROH	retention	moderate	diploid
8.11	NI	LOH	retention	moderate	1.20
X1	ROH	ROH	not performed	moderate	not performed
X2	ROH	ROH	not performed	moderate	not performed
X3	LOH	LOH	not performed	negative	not performed
X4	NI	ROH	not performed	moderate	not performed
X5	NI	ROH	not performed	moderate	not performed
X6	LOH	LOH	not performed	negative	not performed
X7	ROH	ROH	not performed	moderate	not performed
X8	ROH	ROH	not performed	moderate	not performed
Total	**13/40 (32.5 %)**	**13/44 (29.5 %)**			

LOH: loss of hetreozygosity, ROH: retention of heterozygosity, NI: non informative, IHC: immunohistochemistry
* Both keratin-positive clones were subject to LOH analysis in these cases

The data was analyzed using the Coffalyser (MRC-Holland) according to manufacturer's instructions as previously described [35] using the single iteration analysis mode. Reference DNA from normal non-cancerous lymph nodes and non-neoplastic gastric mucosa from three different individuals was used. All MLPA reactions were repeated at least twice. A ratio of less than 0.60 was considered a deletion and a ratio higher than 1.35 considered a gain.

Results

Status of the CDC4/FBXW7 locus (4q32)

LOH analysis at the *CDC4/FBXW7* locus was carried out on 39 early-onset gastric cancers, after tumour tissue dissociation using flow cytometry whereby the tumour cells were separated from the normal cells using vimentin and keratin staining and selective flow-sorting as can be seen in Figure 1. In addition 8 paraffin-embedded xenografted gastric cancers [27] were used. Results of this analysis can be seen in Table 3. Of note, 34 % of cases (16/47) showed LOH of at least one marker, with 13/40 (32.5%) showing loss at D4S2998 and 13/44 (29.5%) showing loss at D4S2934.

Just under half of all cases (19/47, 40 %, according to availability of tumour DNA) were subject to MLPA analysis of the *CDC4/FBXW7* gene, as shown in Table 3. Of note in case Y132, both the diploid keratin-positive cell fraction and the hyperploid keratin-positive cell fraction were subject to LOH analysis and loss was seen in both, suggesting that loss of the *CDC4/FBXW7* locus is an early event. This was also the case with Y161 where sufficient DNA from both the hypoploid and hyperploid keratin-positive cells was available for analysis. MLPA revealed further information about the *CDC4/FBXW7* locus in several cases. Interestingly in one case (Y132) MLPA revealed a partial deletion of the *CDC4/FBXW7* gene on the side where microsatellite loss was also found and this corresponded to weak/reduced expression of *CDC4/FBXW7*. In case Y111 however, despite seeing loss of both microsatellite markers, only a partial deletion of the coding region exon 6-11 was seen with MLPA. Curiously in case Y140, we found loss of D4S2934 on LOH and an intact *CDC4/FBXW7* coding region, yet protein expression was absent, suggesting that the nearby loss on the exon 1 side disrupts the promoter region or affects the binding of molecules important in the transcription of *CDC4/FBXW7*. Interestingly in two cases (Y160 and Y168) an amplification of the *CDC4/FBXW7* gene was found, explaining the LOH found with both microsatellite markers, as LOH measures a change in ratio i.e. measures a relative loss and doesn't necessarily represent a real loss of the region in question, as has been found in other genes in gastric cancer where LOH was observed.[36]

Expression of CDC4/FBXW7 in gastric carcinoma

Normal fundic gastric mucosa showed moderate nuclear expression of CDC4/FBXW7 on immunohistochemistry, which was occasionally more pronounced in the proliferative zone. Weak cytoplasmic staining seen in some cells was deemed non-specific. Lack of CDC4/FBXW7 expression was seen in 5/16 (31%) of the cases with LOH as seen in Table 3, and an additional case Y132, where partial loss of coding sequence was found, showed weak/reduced CDC4/FBXW7 expression.

Expression of CDC4/FBXW7 protein was then examined in 204 gastric carcinomas (of which 195 were assessable) including both EOGCs and conventional gastric cancer using tissue microarrays as summarized in Table 4 and as seen in Figure 2. Negative staining was seen in 17% of gastric cancers, and low or weak staining was seen in 35% of cases. There was no difference

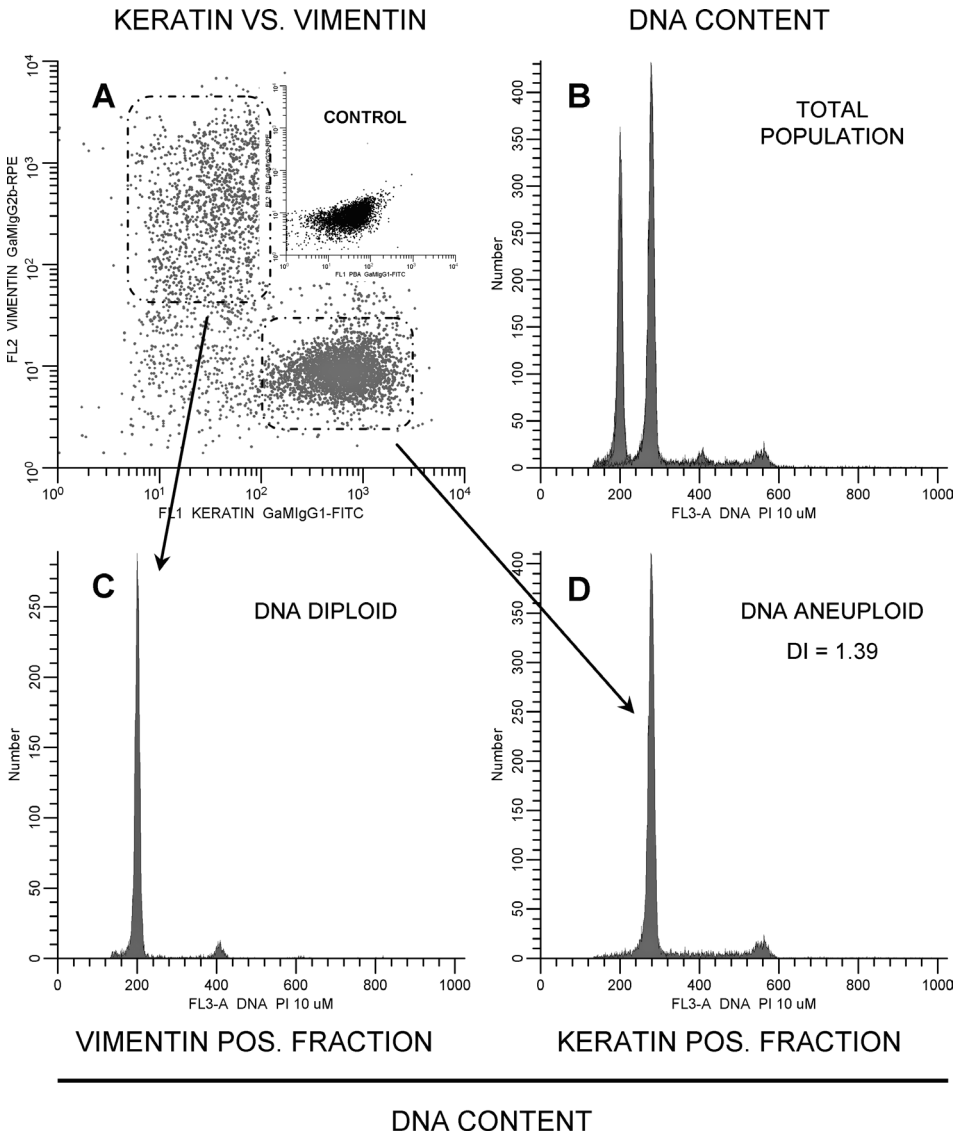

Figure 1 Multiparameter DNA flowcytometry of a FFPE gastric cancer sample. Cells were isolated from a FFPE gastric cancer sample (Y5), stained as described and analyzed on a FACSCalibur flow cytometer. **A***: keratin versus vimentin dot plot. Inlay: negative control only stained for DNA.* **B***: DNA histogram of the total cell suspension showing a bimodal DNA histogram.* **C***: gating on the vimentin-positive cell fraction (green) yields a unimodal DNA histogram representing stromal cells* **D***: gating on the keratin-positive cell fraction also reveals unimodal DNA histogram, with a DI of 1.39, representing gastric carcinoma cells.* ***(See page 200 for colour figure)***

in the expression of CDC4/FBXW7 between EOGCs and conventional gastric cancers using a χ^2 test when assessed on the basis of negative versus positive categories. There was however a statistically significant difference (p=0.007) between CDC4/FBXW7 staining in EOGC and conventional gastric cancers when examining negative or weak versus moderate and strong staining, with negative or weak CDC4/FBXW7 staining occurring more frequently in conventional gastric cancer. This significance remained when correcting for the increased number of diffuse cancers in the EOGC group as well as the variable location within the two groups using a binary logistic regression model (p=0.013). CDC4/FBXW7 expression did not correlate with histological type or location of the tumour, T-stage or the presence of lymph node metastases.

Expression of Cdc4/FBXW7 and its substrates

CDC4/FBXW7 expression and expression of its substrates c-jun, c-myc, cyclin E and Notch was assessed immunohistochemically on 204 gastric cancers using tissue microarrays (Figure 2). A summary of the results can be seen in Table 5. A significant correlation was found between CDC4/FBXW7 expression and the substrate c-myc (p=0.024). CDC4/FBXW7 expression did not correlate with cyclin E (p=0.422), c-jun (p=0.87) or Notch (p=0.174) and correlation with p53 staining was only of borderline significance (p=0.057). In addition, loss of Notch staining occurred more frequently in conventional gastric cancers (35%) than in EOGCs (18%) and this difference was statistically significant using a χ^2 test (p=0.004), as well as on using a binary logistic regression model to correct for histology and location (p=0.034).

Mutation analysis

Mutation analysis of all 11 exons of the *CDC4/FBXW7* gene was carried out on DNA from 11 gastric cancer cell lines as well as on DNA from fresh frozen xenografted tissue of the 2 cases with LOH. No mutations of the *CDC4/FBXW7* gene were found.

Table 4 Results of CDC4/FBXW7 TMA Immunohistochemistry

Type of Staining	negative	weak	moderate	strong
N (nearest percent)	34/195 (17%)	35/195 (17%)	79/195 (41%)	47/195 (24%)

Table 5 TMA Immunohistochemistry together with statistical correlation with CDC4/FBXW7

Antibody	Total Abnormal staining*	Significant Correlation with CDC4/FBXW7 (χ^2)
CDC4/FBXW7	17 % negative	-
Notch#	25 % negative	none
cyclin E	36 % positive	none
c-myc	43 % positive	p = 0.024
c-jun	70 % positive	none
p53	42 % positive	p = 0.057

* to the nearest percent

significant difference between notch staining in EOGC and conventional GC (p=0.004, χ2 test)

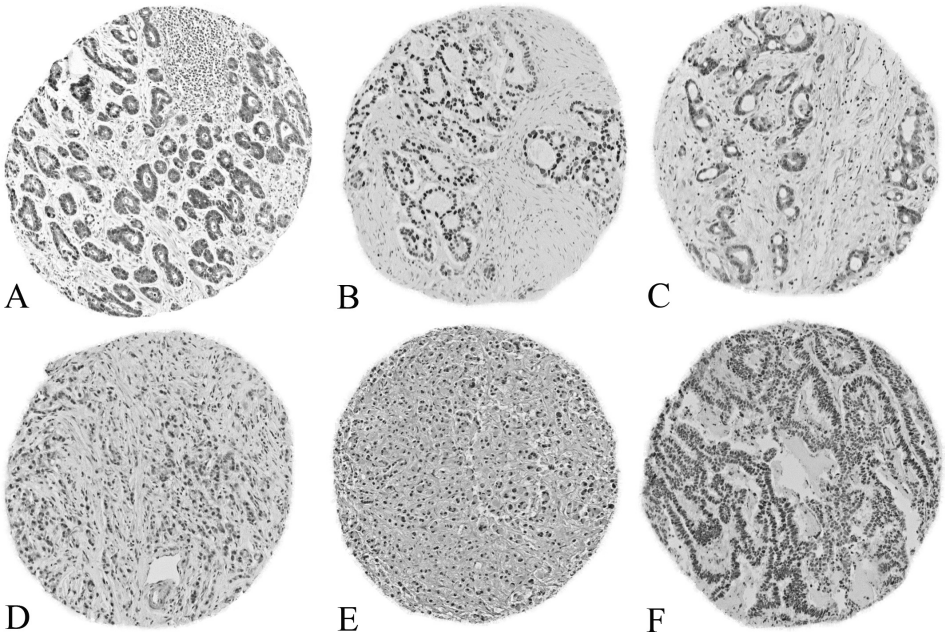

Figure 2 Immunohistochemistry for cdc4 (A), cyclin E (B), c-myc (C), c-jun (D), Notch (E) and p53 (F) (x 100) on tissue microarray cores. ***(See page 201 for colour figure)***

Ploidy Status

In the process of obtaining pure tumour cell populations using vimentin and keratin staining and selective flow-sorting (see Figure 1) ploidy analysis was carried out, the results of which can be seen in Table 3. In 6 cases, bimodal distributions were found after gating on the keratin-positive fraction. In all other cases only unimodal distributions were found in the keratin-positive fraction. Interestingly in Y161, we found both a hypodiploid and hyperdiplod peak, both of which showed loss of both microsatellite markers. In other cases (Y113, Y177), two aneuploid peaks were visualized and in 3 cases (Y112, Y132, Y166) both a diploid and aneuploid subclone of the tumour was observed. Of note, CDC4/FBXW7 expression, LOH of *CDC4/FBXW7* or cyclin E expression did not correlate with ploidy status using Spearmans and Pearson correlation tests. In addition, ploidy did not correlate with histological type.

Discussion

CDC4/FBXW7 is believed to be an important tumour suppressor gene in many cancers, with mutations found in ovarian, breast, pancreatic, colorectal and endometrial tumours. [7, 15-20] In fact loss of chromosome region 4q32 has been reported in 31% of all neoplasms, including 67% of lung cancers, 63% of head and neck cancers, 41% of testicular cancers, and 27% of breast cancers [37] thus raising the possibility that *CDC4/FBXW7* may also be involved in the genesis of many other tumour types. In this study we investigated the role of *CDC4/FBXW7* in gastric cancer with particular attention to early-onset gastric cancer. We found LOH in microsatellite markers

next to the *CDC4/FBXW7* gene in 34 % of cases and nearly half of the cases were also examined using MLPA. In addition, loss of CDC4/FBXW7 expression was seen in 5/16 (31%) of cases with LOH. However, it is known that *CDC4/FBXW7* can lose its tumour suppressor function when haploinsufficient [38] meaning that loss of function of only one allele and a reduction of protein expression rather than complete loss is enough to promote carcinogenesis, and there may still be functional significance of loss of the *CDC4/FBXW7* locus despite presence of the protein immunohistochemically. In addition to loss of expression associated with LOH, we saw loss of expression in 17 % cases overall on tissue microarray analysis of 204 gastric cancers and negative or weak expression in 35%. Interestingly in the group on which LOH was carried out, 15 % of cases showed negative or weak expression in the absence of LOH, suggesting that the loss of CDC4/FBXW7 expression in a subset of gastric cancers may occur through mechanisms other than allelic deletion, such as genetic or epigenetic events in nearby promoters or in molecular regulators of *CDC4/FBXW7*.

In this study we also found that of the reported CDC4/FBXW7 substrates cyclin E, p53, c-jun, c-myc and Notch [14, 39], only the substrate c-myc showed a significant correlation with CDC4/FBXW7 levels in gastric cancer. Expression levels of c-myc are increased in many malignant tumours, and many c-myc mutations affect the stability of the encoded protein [40]. C-myc accumulates in mouse Fbxw7$^{-/-}$ cells [39] as well as in lymphomas from Fbxw7$^{+/-}$ mice [38] and a nucleolar isoform of the FBXW7 ubiquitin ligase is reported to regulate c-myc and cell size. [41] C-myc is known to play a role as an oncogene in gastric cancer [42] and the correlation between CDC4/FBXW7 and c-myc suggests that c-myc activation is likely to be an important oncogenic consequence of CDC4/FBXW7 loss in gastric cancers. Our lack of correlation between CDC4/FBXW7 and cyclin E, Notch or c-jun highlights the complexity of the relationship between CDC4/FBXW7 and its substrates. [38, 43, 44] Although CDC4/FBXW7 has been described to be p53 dependent [38] and it has been reported that cdc4/FBXW7b is a transcriptional target of p53.[45], the relationship of p53 with CDC4/FBXW7 in gastric cancer remains elusive with a correlation of borderline significance. Interestingly, aside from its relationship with CDC4/FBXW7 expression, we found that loss of Notch expression occurred frequently in conventional gastric cancer whereas EOGCs were statistically different in this respect with less frequent loss of Notch expression. These findings add further support to the idea that EOGC have a different molecular phenotype from conventional gastric cancers.[23-25]

Ploidy did not correlate with histological type in this study and this is undoubtedly accounted for by the increased accuracy of the ploidy analysis due to use of the recently developed technique by Corver *et al* [32] where triple staining and dissociation of normal and tumour cells is performed, eliminating the contamination of normal lymphocytes and stromal cells within the diffuse tumour. In the past many comparative genomic hybridization studies/genetic studies have found near normal karyotypes/genomes in diffuse cancer, which may reflect the fact that, due to the diffuse morphology, there is always contamination with normal cells, as even laser microdissection cannot isolate the tumour cells alone in these cases. Although *CDC4/FBXW7* was proposed as a CIN (chromosomal instability) gene for human cancers, particularly in colorectal carcinoma,[7] more recent reports on primary tumours, similar to our findings, do not support this theory finding no association with *CDC4/FBXW7* and ploidy. [21] Our findings also emphasize that the loss we observed does not reflect a more generalized chromosomal instability, but rather is more likely to be a loss critical in gastric carcinogenesis.

In this study we chose to carry out mutation analysis on available fresh/frozen tissue only, due to the high percentage of induced mutations/false positives which can be found on paraffin embedded

material.[46, 47] The lack of *CDC4/FBXW7* mutations found in gastric cancer reflects the fact that *CDC4/FBXW7* mutations in general appear to be less common that originally believed. Where cell lines studies pointed to the mutation occurring commonly in ovarian cancer (3/10 ovarian cancer cell lines),[15] follow-up in primary patient material revealed mutations in only 2 of 95 sporadic cases. [48] Similarly in endometrial cancers, mutations were originally described in 16% (1/51) of endometrial tumours [17] but a subsequent study reports under 3% (1/34).[18]

In conclusion, loss of heterozygosity of *CDC4/FBXW7* occurs commonly in early-onset gastric cancer, and is correlated with loss of expression in 31 % of cases. Loss of expression was seen frequently in both early-onset gastric cancers and conventional gastric cancers on tissue microarray analysis suggesting that *CDC4/FBXW7* is an important tumour suppressor gene not only in early onset gastric cancer but in gastric carcinogenesis in general. In addition, the correlation found between loss of cdc4/FBXW7 expression and upregulation of c-myc suggests that c-myc activation is likely to be an important oncogenic consequence of *CDC4/FBXW7* loss in gastric cancers.

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9

A Novel Region of Amplification at 11p12-13 in Gastric Cancer, Revealed by Representational Difference Analysis, is Associated with Overexpression of CD44v6, Especially in Early-Onset Gastric Carcinomas

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Abstract

Diffuse-type gastric carcinomas (GCs) are often difficult to characterize due to contamination of tumor samples by surrounding normal tissue. As such, information regarding chromosomal aberrations in this subtype of GCs is limited. In this study, we used representational difference analysis to pinpoint genomic amplifications occurring in diffuse-type GCs. We found nine differential products from two novel regions of amplification in two tumors: one product mapped to 19p13.1, and eight mapped to a 1.8 Mb region in chromosomal segment 11p12-13. These amplifications were confirmed using Southern blot analysis and occurred in 3/16 and 6/15 diffuse-type GCs, respectively. CD44, a well characterized cellular adhesion molecule involved in several human malignancies, is encoded by a gene located within 200 kb of the 11p12-13 amplification fragments. We confirmed that overexpression of isoform CD44v6 was correlated with amplification at 11p12-13 in 11/12 diffuse-type GCs. Since diffuse-type GCs occur more frequently in early-onset gastric carcinomas (EOGCs, presented at 45 years of age or younger) than in "conventional" GCs, and the tumors carrying the original amplifications were EOGCs, we investigated overexpression of CD44v6 in 107 EOGCs and 88 "conventional" GCs using tissue microarrays. We found frequent CD44v6 overexpression in both tumor groups (76% and 57% respectively) and, interestingly, significantly more cases with overexpression of CD44v6 in EOGCs than in "conventional" GCs (P=0.005), irrespective of histology. These findings provide further evidence for both the relevance of CD44 in GC, and for distinct molecular characteristics of EOGCs when compared to GCs occurring at a later age.

Introduction

Gastric tumors, despite steady decrease in occurrence, still rank second worldwide in terms of cancer-related deaths (Parkin et al., 2001). According to the Lauren classification, they are divided into two main types, diffuse and intestinal (Lauren, 1965). Due to the nature of diffuse-type gastric tumors, it is often difficult to obtain highly pure tumor populations. Accordingly, the determination of chromosomal aberrations in this subtype of gastric tumors is often undermined by the presence of varying amounts of normal cells, such as stromal cells and lymphocytes.

Representational difference analysis (RDA) is a useful tool in identifying differences at a genomic level between two complex and highly related populations. It combines the technology of subtractive hybridization with kinetic (PCR-based) enrichment of genomic representations of two different genomes. An important advantage of RDA over other commonly used techniques, such as comparative genomic hybridization (CGH), lies in its ability to detect small deletions and amplifications (Wieland et al., 1990; Lisitsyn et al., 1993). RDA (Fig. 1) can be applied to identify genetic aberrations in cancer in two ways: when pure intact tumor DNA is used as a driver and normal DNA from the same patient as a tester, the technique permits the detection of genetic losses, mostly homozygous deletions; conversely, using tumor DNA of sufficient purity (>90%) as a tester will allow detection of genomic amplifications or the presence of DNA pathogens (Lisitsyn et al., 1993; Lisitsyn 1995).

In this study, we employed the RDA technique using the tumor DNA as a tester, and found novel regions of amplification in two diffuse-type gastric tumors. The amplified regions mapped to chromosome locations 11p12-13 and 19p13.1. Several genes can be found in these regions, but *CD44*, located in the 11p13 region, was deemed the most interesting potential target of this amplification.

CD44 is a polymorphic membrane glycoprotein expressed in most human cell types, with reported roles in lymphocyte homing to specific lymph node tissue (Picker et al., 1989), T-cell activation (Haynes et al., 1989) and cellular matrix adhesion (Aruffo et al., 1990). It is known that active migration of tumor cells within the extracellular matrix is a necessary step for invasiveness and metastasis (Marhaba and Zoller 2004). CD44 is thought to be responsible for triggering the cytoskeletal rearrangements and morphological changes necessary for these processes (Thomas et al., 1992; Welsh et al., 1995; Peck and Isacke 1998; Peterson et al., 2000; Ahrens et al., 2001; Akiyama et al., 2001; Bourguignon et al., 2001a,b, 2002; Sohara et al., 2001; Nasreen et al., 2002). CD44 displays extensive size heterogeneity, ranging from the standard 85-95 kDa form to the larger sizes (200 kDa or more) of variant isoforms (CD44v) that result from the presence of variant coding exons v1-v10 in the mRNA (Bates et al., 2001). Expression of these CD44v has been linked to the upregulation of anti-apoptotic genes (Bates et al., 1998; Bates et al., 2001; Fujita et al., 2002; Ghatak et al., 2002; Chen et al., 2004), and it has been shown in different human carcinomas, as well as in non-transformed cells, that activation of MET by hepatocyte growth factor (HGF) depends on the expression of CD44v6 (Orian-Rousseau et al., 2002). Noteworthy, overexpression of CD44v6 in gastric carcinomas (GCs) has been reported in a number of previous studies (Muller et al., 1997; Castella et al., 1998; Saito et al., 1998; Gulmann et al., 2003; Chen et al., 2004; Polkowski et al., 2004; Kim et al., 2005), where it has been linked with tumor progression, invasion, and metastatic behavior.

In our study, we confirmed the presence of the 11p12-13 and 19p13.1 amplifications in the two original tumors through Southern blot analysis, and determined their occurrence in a panel of diffuse-type GCs. Immunohistochemistry (IHC) demonstrated that the amplification at

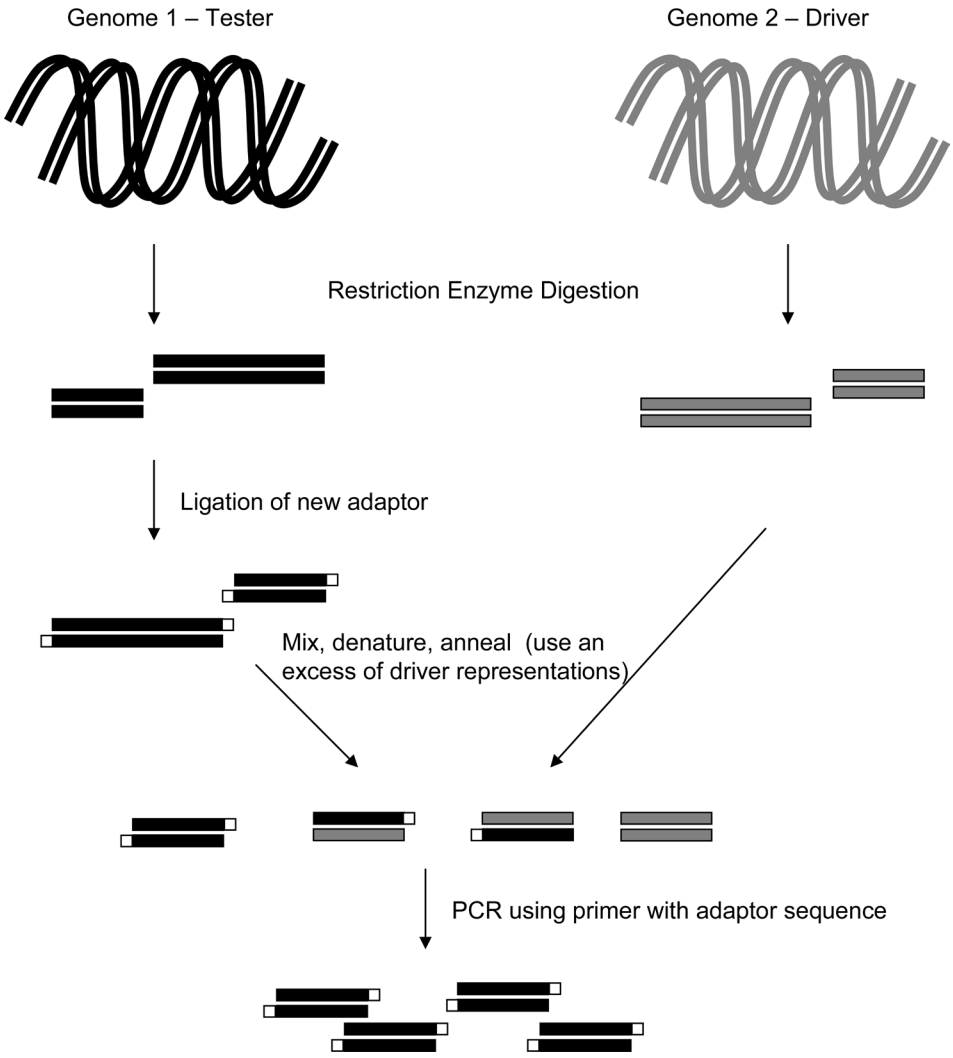

Figure 1 Description of RDA: both tester and driver intact genomic DNAs are digested with the same restriction endonuclease. Following an initial amplification step, adaptors are removed and only the tester DNA representation is religated to a new set of oligonucleotide adaptors. Tester DNA is then mixed with a large excess of driver DNA, and the DNAs are denatured and allowed to reassociate. Reannealed products include homoduplexes of driver DNA (grey, both strands missing adaptors), heteroduplexes of driver and tester DNA (grey and black, adaptor only present in tester strand), and homoduplexes of tester DNA (black, both strands containing adaptors). The tester homoduplexes are exponentially amplified by PCR through primers targeted at the adaptor region. The procedure is repeated twice, through ligation of new adaptors to the enriched DNA fragments

chromosome 11 correlated with CD44v6 overexpression. In addition, using tissue microarray (TMA) technology we found this overexpression to occur commonly in a large GC group. Importantly, overexpression correlated significantly with early-onset gastric carcinomas (EOGC, presented at 45 years of age or younger), providing additional evidence that EOGCs display molecular characteristics different from carcinomas occurring at a later age.

Materials and methods

Patient Material

Ninety-eight GCs from patients over the age of 45 were obtained from the Academic Medical Centre (Amsterdam, The Netherlands) and from the Johns Hopkins Medical Institutions (Baltimore, USA). One hundred and seven cases of GC in patients aged 45 years or younger were obtained from 24 different institutions throughout the Netherlands through the nationwide PALGA search system, and from the Department of Pathology at the Jorvi Hospital (Espoo, Finland). The tumors were classified by an experienced gastrointestinal pathologist (GJAO) according to the Lauren classification (Lauren, 1965) as intestinal-, diffuse- or mixed-type gastric adenocarcinomas and location was deduced from the pathological report (when available). This study was carried out in accordance with the ethical guidelines of the research review committee of the Academic Medical Centre, Amsterdam, as well as in agreement with the Helsinki Declaration.

DNA Isolation

For each case used in RDA, 10-20 μm sections were cut (with a 4 μm section for H&E analysis being cut every sixth section), and tumor-rich areas of the sections were microdissected in order to achieve a highly pure tumor population. The 16 diffuse-type GCs with lower tumor percentages used in the Southern blot analysis were processed without microdissection. DNA isolation was performed by proteinase K treatment, followed by phenol/chloroform extractions, and ethanol precipitation.

Representational Difference Analysis (RDA)

RDA was performed as described previously (Lisitsyn et al., 1993) using two distinct restriction endonucleases. *Hind*III (Invitrogen, Breda, The Netherlands) was initially used but yielded no amplicons. The oligonucleotides used with *Bgl*II were as follows: RBgl24: 5'-AGCACTCTCCAGCCTCTCACCGCA-3'; RBgl12: 5'-GATCTGCGGTGA-3'; JBgl24: 5'-ACCGACGTCGACTATCCATGAACA-3'; JBgl12: 5'-GATCTGTTCATG-3'; NBgl24: 5'-AGGCAACTGTGCTATCCGAGGGAA-3'; NBgl12: 5'-GATCTTCCCTCG-3'. A 1:1 mixture of Recombinant *Taq* polymerase (Invitrogen) and Platinum *Taq* polymerase (Invitrogen) was used in the amplification steps.

Sequence Analysis

The identified differential products after three rounds of amplification were isolated from agarose gels, digested with *Bgl*II and cloned into the pGEM3-ZF+ vector (Promega, Leiden, The Netherlands) following the manufacturer's instructions. Sequencing was performed on an ABI 377 automated sequencer (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands) using the ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and SP6/T7 primers.

Table 1 Genomic Amplifications and CD44v6 Expression Levels

	11A	CD44 IHC	19	% Tumor Cells
X1	1.14	Low	0.94	30-40
X2	0.94	High	1.04	30
X3	1.39	High	0.92	50-60
X4	1.13	N/D	0.95	50-60
X11	1.03	N/D	1.11	<10
X13	1.00	Low	1.10	<20
X14	1.29	High	1.16	<20
X15	1.47	High	1.73	<10
X16	2.53	High	2.30	50
X17	1.20	N/D	0.83	N/D
X18	1.06	Low	1.03	N/D
X19	1.00	Low	0.94	50-60
X20	1.69	High	0.82	<20
Y4	3.29	High	3.41	<20
Y5	1.03	Low	1.05	60
Y6	-	-	0.92	<20

Dark grey boxes: agreement between 11p12-13 amplification and CD44 overexpression; Light grey box: disagreement between 11p12-13 amplification and CD44 overexpression; N/D: not determined; Cut-off for amplification: 1.2

Southern Blot Analysis

Seven to 10 μg of genomic tumor and normal DNA from each case, digested with either *Hind*III or *Bgl*II, was used for Southern blot analysis. The following human probes were used: 11A (from the 11p12-13 region), 19 (from the 19p13.1 region), and E3.9 (Hulsebos et al., 1991) (used as a control probe). Probes were radiolabelled by random priming using the Rediprime II Random Prime Labelling System (Amersham Biosciences, Munich, Germany) according to the manufacturer's instructions. Probes 11A and 19 were preannealed with Hybridime (HT Biotechnology, Cambridge, UK). Hybridization of all probes was then performed at 65°C in 0.5 M sodium phosphate/7% SDS/1 mM EDTA. Washes were performed using phosphate or citrate buffers in decreasing salt concentrations. Visualization of radioactive signals was performed using the Phosphorimager system (FLA-3000, Fuji, Tokyo, Japan), or autoradiography. The blots were scanned and analyzed using the AIDA software (version 3.20.007, Raytest, Straubenhardt, Germany), and the level of amplification was determined by the ratio of tumor to normal signal, after both signals had been standardized with the respective controls. Values above the 1.2 threshold were deemed positive for allelic gains.

Immunohistochemistry (IHC) and Tissue Microarrays (TMAs)

IHC was performed on formalin-fixed paraffin sections using the monoclonal antibody CD44var (v6) (1:300 dilution, Bender MedSystems GmbH, Vienna, Austria) and the polyclonal antibody RFX1 (1:200 dilution, generously provided by Professor Patrick Hearing, SUNY Stony Brook, USA). Briefly, 4 μm sections were deparaffinized and blocked for endogenous peroxidase activity by immersion in 0.3% H_2O_2 in methanol for 20 min. Antigen retrieval was performed in Tris/EDTA buffer (10 mM/1 mM, pH 9.0) for 10 min at 120°C. Non-specific binding sites were blocked in PBS with 10% normal goat serum for 10 min and antibody incubation followed for 1 hr at room temperature. Visualization was performed using the Powervision+poly-HRP detection

system (ImmunoVision Technologies, Daly City, USA) with 3,3-diaminobenzidine (DAB) as a chromogen. Sections were counterstained with hematoxylin. The TMAs used in this study comprised of 107 EOGCs and 88 "conventional" GCs (Table 2), and were prepared as described previously (Milne et al., 2006).

IHC on frozen sections was performed similarly, with the following alterations: frozen sections were fixed for 10 min in acetone; a 1:600 antibody dilution was used; endogenous blocking was performed after staining with antibody, and was carried out using a PBS solution containing 0.1% sodium azide and 0.3% H_2O_2; 3-amino, 9-ethylcarbazole (AEC) was used as a chromogen in place of DAB. No material was available for three cases (Table 1).

IHC Scoring

Abnormal CD44v6 expression in tumor cells was defined as occurring both in the membrane and in the cytoplasm, and was graded as follows: Low – little to no CD44v6 expression; High – moderate to high expression. Normal CD44v6 expression in stomach was seen as membranous staining and present only in the proliferative compartment. Overexpression of RFX1 was defined as nuclear expression of the protein in the tumor cells. No RFX1 expression was observed in the normal gastric epithelium.

Statistical Analysis

The SPSS 11.5 software package (Chicago, USA) was used for statistical analysis. A χ^2 test was applied to determine statistical significance. A binary logistic regression model was used to adjust for location, histological type, age of the block, and the hospital from which the block was derived.

Results

We performed RDA analysis on four EBV-negative diffuse-type GCs which, after microdissection, contained >90% tumor cells (as determined through H&E), in order to identify tumor-specific amplified fragments. Differential products after three rounds of amplification were isolated and cloned into plasmid vectors. Clone inserts were subsequently used for Southern blot analysis on *Hind*III or *Bgl*II digested representations, as well as on genomic DNA, from the tumor and corresponding normal tissue. Tumor Y102 yielded a 310 bp differential product and tumor Y103

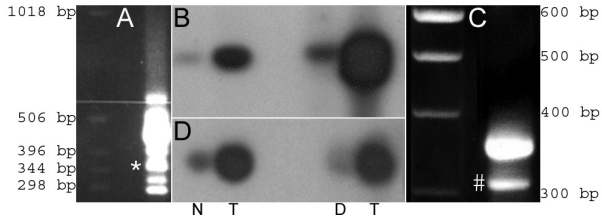

Figure 2 RDA products: the eight differential products obtained from tumor Y103 (A) and the differential product obtained from tumor Y102 (C, marked with "#"). Level of amplification for case Y103 (B, product marked "" used as typical example) and case Y102 (D) seen both in the genomic DNA of the tumor when compared to corresponding normal (N/T) and in the tester representation when compared to driver representation (D/T)*

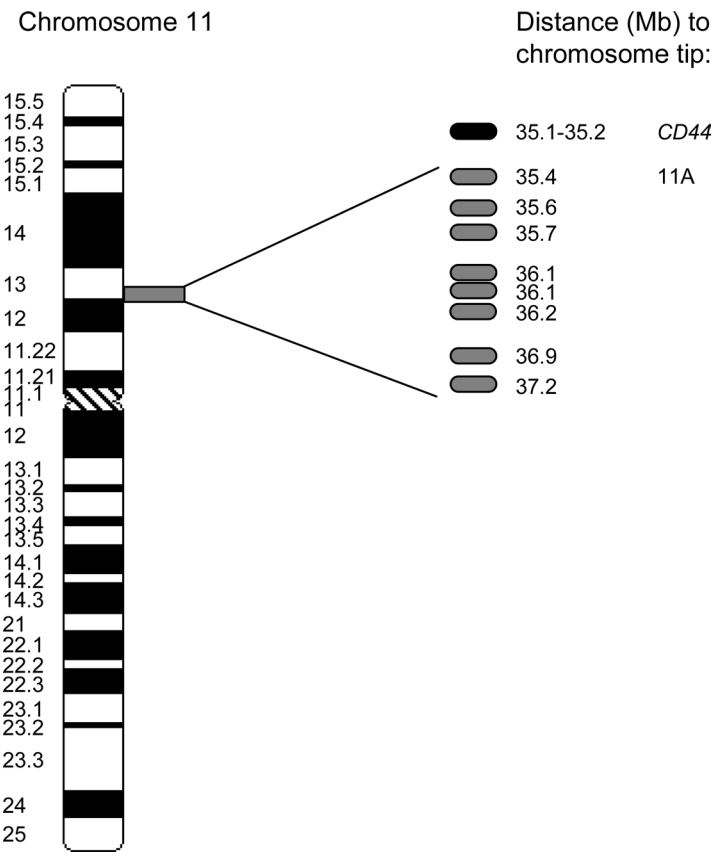

Figure 3 Chromosome 11 amplifications: the grey box represents the chromosome location of the amplification region of 1.8 Mb. Eight amplified fragments are depicted as grey rounded boxes, with the distance to the chromosome 11 tip shown. Amplification fragment 11A is 35.4 Mb from the chromosome tip. The black rounded box located 35.1-35.2 Mb from the chromosome tip represents the location of CD44, approximately 200 kb from 11A

yielded 8 separate fragments (sized 300-600 bp). These nine fragments showed amplification in the representations made of the tumors, when compared to normal DNA, as well as in the genomic DNA of the tumors (again, when compared to the respective normal DNA), thereby excluding PCR artifacts (Fig. 2). Sequence analysis revealed that the 310 bp fragment from Y102 mapped to 19p13.1, whereas all 8 fragments from Y103 mapped to the 11p12-13 region, and spanned 1.8 Mb (Fig. 3).

We then performed Southern blot analysis on a panel of 16 diffuse-type GCs (with varying tumor percentages, Table 1) and respective normal tissue using the fragment from chromosome 19 and one of the chromosome 11 fragments (defined as 11A, Fig. 3) as probes, in order to determine the occurrence of the found amplifications. Using the AIDA software, we determined the signal intensities in both tumor and normal samples for 16 cases using the chromosome 19 probe, and for 15 cases for using probe 11A. Following calculation of the ratio of tumor signal to normal

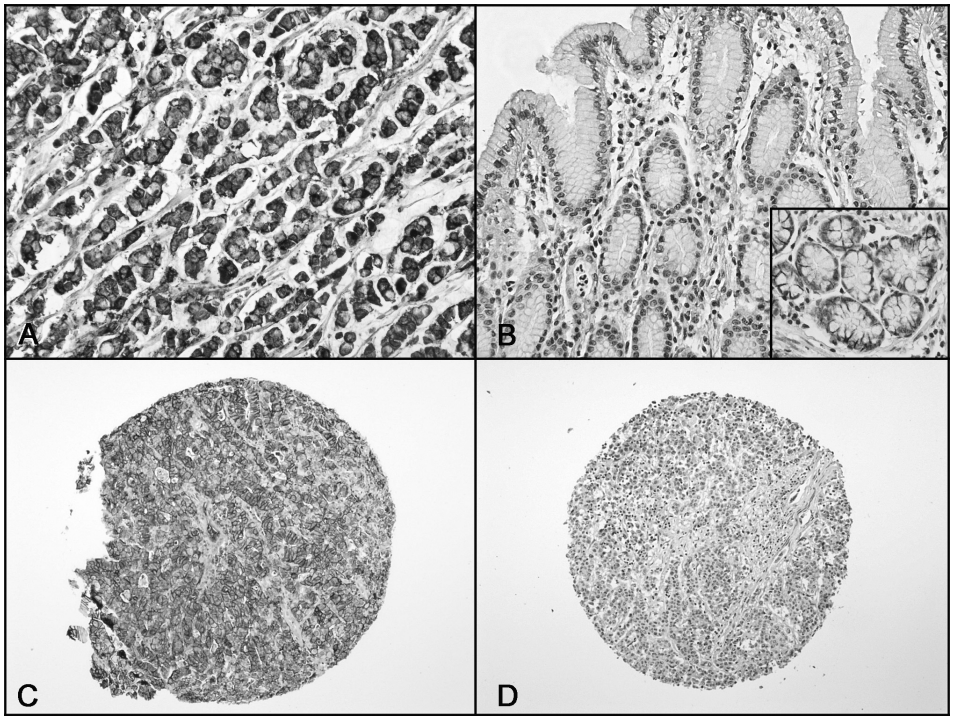

Figure 4 Typical IHC results for CD44v6 on paraffin sections (A and B) and TMAs (C and D): overexpression of CD44v6 can be seen in tumors Y103 (A) and GC25 (C), with membranous and cytoplasmic stain (brown) in tumor cells, and negative staining in surrounding normal tissue; panel B shows absence of expression in the normal epithelium, with the exception of positive stain (brown) in the proliferative region (B, inset); an example of lack of CD44v6 overexpression in a tumor (GC42) is shown in panel D. Nuclei were counterstained with hematoxylin. ***(See page 201 for colour figure)***

signal (after standardization with respective controls), three cases showed amplification for the chromosome 19 probe, and six cases showed amplification for the chromosome 11 probe (Table 1).

To determine whether these amplifications bear functional consequences, we examined the expression level of one of the isoforms encoded by *CD44*, located in the 11p13 region within 200 kb of the 11A probe, and of the product of *RFX1*, located within 200 kb of the chromosome 19 probe. We confirmed CD44v6 overexpression in case Y103 (Fig. 4A), where the original amplification was determined, and stained frozen or paraffin sections from 12 of the other examined cases for this protein isoform. Cases that overexpressed CD44v6 showed membranous and cytoplasmic expression in tumor cells, but no expression in surrounding normal tissue (Fig. 4A and C). CD44v6 expression in normal tissue was restricted to the proliferative compartment (Fig. 4B and inset), with some non-specific staining detected in parietal cells. In all cases but one, there was agreement between genomic amplification in region 11p12-13 and expression level of the CD44v6 protein (Table 1): in all cases with little or no CD44v6 expression, no amplification was observed; in all cases but one with CD44v6 overexpression, there was genomic amplification. Our results show that a large percentage of diffuse tumors have CD44v6 overexpression (7/12, 58%). Noteworthy, all tumors analyzed contained at most 60% of tumor cells as determined

Table 2 Expression of CD44v6 on EOGC and "Conventional" GC TMAs

		Diffuse	Intestinal	Mixed
EOGC	CD44 Low	20	3	3
	CD44 High	57	19	5
"Conventional" GC	CD44 Low	11	22	5
	CD44 High	18	26	6

Table 3 Comparison of CD44v6 expression between EOGCs and "conventional" GCs

	EOGC	"Conventional" GC
CD44 Low	26 (24%)	38 (43%)
CD44 High	81 (76%)	50 (57%)

through H&E sections (Table 1). This means that the level of amplification given in table 1 is an underestimation of the actual amplification. Seven cases were examined for regulatory factor X, 1 (RFX1) expression, and four – including Y102, where the original amplification was determined – displayed overexpression of the protein. However, no correlation was observed between RFX1 overexpression and 19p13.1 amplification.

Given that both cases that gave amplifications through RDA were EOGCs (and knowing that EOGCs are predominantly of the diffuse type), we decided to determine CD44v6 expression levels in a panel of TMAs, consisting of 88 "conventional" GCs (presented at over 45 years of age) and 107 EOGCs (presented at 45 years of age or younger) (Table 2, Fig. 4C and D). The "conventional" group showed CD44v6 overexpression in 50/88 cases (57%), whereas 81/107 EOGC cases (76%) showed overexpression of the protein (Table 3). The much more frequent occurrence of CD44v6 overexpression in young patients as compared old was found to be highly statistically significant (P=0.005). The significance remained strong after adjusting for histological type and location using a binary logistic regression model (P=0.018).

Discussion

RDA is a useful technique for the detection of genomic deletions and amplifications. The advantage of RDA over techniques such as CGH lies in its ability to detect much smaller fragments. In the past it has been used to find deleted areas which included the *DCC* (Lisitsyn et al., 1995) and *BRCA2* (Schutte et al., 1995) genes. The technique also contributed to the discovery of areas of amplification, such as that of the *Mdm2* gene in mouse uterine adenocarcinomas (Risinger et al., 1994).

Due to the inherent difficulty of using subtractive techniques on diffuse-type GCs, our knowledge of chromosomal aberrations specific to this subtype is limited. Therefore we decided to use RDA to search for genomic amplifications in four diffuse-type GCs, selected for high tumor content (>90%). In this study, we were able to locate two novel and distinct regions of amplification, at 19p13.1 and 11p12-13, in two tumors using this technique. Neither region has thus far been shown to be amplified in studies on primary gastric cancer specimens. Previous reports have described loss of 19p (Guan et al., 2000), as well as copy number gains in 19q (Varis

et al., 2003), in the context of gastric tumors, the latter study pertaining specifically to younger GC patients. In a cytogenetic study, recurring breakpoints in 19p13 were observed in GC cases (Ochi et al., 1986). Within 200 kb of the 19p13.1 fragment 12 known genes can be found, including the genes encoding RFX1 and the zinc finger-containing proteins ZSWIM4 and NANOS3. Overexpression of RFX1 in a small group of gastric carcinomas did not appear to be associated with the presence of the chromosome 19 amplification. The 11p13-15 region has been described as being the target of frequent chromosomal rearrangements in a cytogenetic study of nine gastric and esophageal tumors (Rodriguez et al., 1990). Chromosome 11 trisomies have also been reported (Han et al., 1996), and numerical aberrations of this chromosome have been associated with worse prognosis (Kitayama et al., 2003). Within the 11p12-13 region 13 known genes are located, among which *TRIM44* that codes for a protein containing three zinc-binding domains, and *TRAF6*, encoding a protein that associates with, and mediates signal transduction from, members of the TNF receptor superfamily. Of particular interest, however, is *CD44*, located within 200 kb of fragment 11A (Fig. 3). CD44 is a well characterized cellular matrix adhesion molecule, involved in cell-cell interactions, and in cell adhesion and migration. It is a receptor for hyaluronic acid and can also interact with ligands such as osteopontin, collagens, and matrix metalloproteinases (Marhaba and Zoller 2004). CD44 overexpression (especially that of one of its variants, CD44v6) has been reported in the context of gastric cancer (Muller et al., 1997; Polkowski et al., 2004; Kim et al., 2005), either as a late phenomenon in the intestinal metaplasia to gastric cancer progression (Gulmann et al., 2003), or linked to the formation of lymph node metastases in intestinal-type GCs (Castella et al., 1998; Chen et al., 2004), or associated with progression specifically in diffuse-, rather than intestinal-type, GCs (Saito et al., 1998).

Having determined that the chromosome 11 amplification was present relatively frequently in diffuse-type gastric tumors, our aim was twofold: 1) to verify whether this amplification had consequences at the level of CD44 protein expression; 2) given that the two diffuse tumors originally used to search for amplifications were also EOGCs, to determine whether the level of CD44 overexpression was indeed more prevalent in carcinomas derived from young patients than in those derived from "conventional" (older) patients, or whether the overexpression was related to the diffuse histology.

Using an antibody specific to CD44v6, we found that the chromosome 11 amplification levels, as determined by Southern blot analysis, correlated closely with the level of CD44v6 protein expression. In the 12 cases stained for CD44v6, 11 showed agreement between genomic amplification in region 11p12-13 and expression level of CD44v6 protein (Table 1). The low tumor content of the cases analyzed (tumors contained at most 60% tumor cells, Table 1) indicates that the level of amplification is underestimated, and might also provide an explanation for case X2, where CD44v6 overexpression was observed, but no amplification detected (Table 1). In this case, the tumor cell content was 30%, so a possible genomic amplification might have been missed. Alternatively, overexpression of CD44v6 in this case may have resulted from a different mechanism. Interestingly, our finding that genomic amplification might be a mechanism for CD44 overexpression has been previously reported in gastric carcinoma cell lines (Fukuda et al., 2000), where an amplification of the 11p11.2-14 region was correlated with overexpression of CD44E (v8-v10). Fukuda and colleagues found that overexpression of CD44E was associated with transduction of growth and proliferation signals.

When analyzing the expression of CD44v6 on a large panel of GCs, containing both EOGC and "conventional" GCs, we found, in agreement with previous studies, a high percentage of CD44v6 overexpression in gastric cancer, independent, however, of histological type (Table 2, Fig. 4C and

D). It has been shown in different human carcinomas, as well as in non-transformed cells, that activation of MET – with known roles in invasive growth and cellular migration, in proliferation and in differentiation – by its ligand HGF depends strictly on the presence of CD44v6 (Orian-Rousseau et al., 2002). The contribution of CD44v6 to this pathway is twofold: the extracellular domain of this variant forms a complex with HGF and MET, necessary for MET activation; the cytoplasmic tail of this variant is required for the signal transfer from MET to MEK and ERK (Orian-Rousseau et al., 2002). Activation of MET has indeed been reported in GCs (Inoue et al., 2004). Further, it has been demonstrated that a CD44v6 signaling pathway in colon carcinoma cell lines is implicated in anti-apoptotic effects that involve the PI3K/AKT pathway (Bates et al., 1998; Bates et al., 2001), therefore contributing to tumor progression.

Interestingly, CD44v6 overexpression was found to be significantly more prevalent in the panel of EOGCs when compared to the "conventional" GCs panel (P=0.005, Table 3). The significance remained strong when adjusted for histological type and location (P=0.018). It has been shown that the molecular profile of EOGCs differs from that of their "conventional" counterparts (Carvalho et al., 2004; Milne et al., 2006). It is also known that the clinicopathological characteristics of this younger subtype differ from those observed in tumors derived from older patients: it has been claimed that young patients have a poorer prognosis (Theuer et al., 1996), and are more susceptible to develop multifocal tumors than older patients (Furukawa et al., 1989). This more aggressive phenotype might be explained, to some extent, by the increased presence of CD44v6 in EOGCs. To our knowledge, this is the first study to report an age-related difference in CD44v6 expression within the gastric cancer context.

We have demonstrated the use of RDA in determining novel genomic amplifications in a panel of diffuse-type gastric tumors, and have correlated the amplifications at 11p12-13 with a de facto increase in CD44v6 expression. Further, we have linked the overexpression of CD44v6 differentially to EOGCs, rather than to the diffuse histology. This finding further substantiates the different molecular and clinicopathological characteristics of EOGCs, when compared to those of gastric carcinomas arising in older people. It would be interesting to determine whether other genes in or close to the amplified regions show differential expression in different gastric carcinoma subtypes, in order to gain further insight into gastric carcinogenesis and to evaluate their potential role as markers of tumor progression and patient prognosis.

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Molecular Analysis of primary gastric cancer, corresponding xenografts and two novel gastric carcinoma cell lines reveals novel alterations in gastric carcinogenesis.

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Abstract

We report the molecular characterization of eight primary gastric carcinomas, corresponding xenografts, and two novel gastric carcinoma cell lines. We compared the tumors and cell lines, with respect to histology, immunohistochemistry, copy number and hypermethylation of up to 38 genes using MS-MLPA, and *TP53* and *CDH1* mutation analysis where relevant. The primary tumors and xenografts were histologically comparable and shared expression of 11/14 immunohistochemical markers (E-Cadherin, β-catenin, COX-2, p53, p16, TFF1, cyclin E, MLH1, SMAD4, p27, KLK3, CASR, CHFR and DAPK1). Gains of *CASR, DAPK1* and *KLK3* – not yet described in gastric cancer – were present in the primary tumors, xenografts and cell lines. The most prominent losses occurred at *CDKN2A (p16), CDKN2B (p15), CDKN1B (p27/KIP1)* and *ATM*. Except for *ATM*, these losses were found only in the cell line or xenograft, suggesting an association with tumor progression. However, examination of p16 and p27 in 174 gastric cancers using tissue microarrays revealed no significant correlation with tumor stage or lymph node status. Further losses and hypermethylation were detected for *MLH1, CHFR, RASSF1* and *ESR*, and were also seen in primary tumors. Loss of CHFR expression correlated significantly with the diffuse phenotype. Interestingly, we found the highest rate of methylation in primary tumors which gave rise to cell lines. In addition, both cell lines harbored mutations in *CDH1*, encoding E-cadherin. Xenografts and gastric cancer cell lines remain an invaluable research tool in the uncovering of the multistep progression of cancer. The frequent gains, losses and hypermethylation reported in this study indicate that the involved genes or chromosomal regions may be relevant to gastric carcinogenesis.

Introduction

Gastric cancer is the fourth most common malignancy in the world and ranks second in terms of cancer-related death.[1] Several classification systems have been proposed, but the most commonly used are those of the World Health Organization (WHO) and of Laurén who describes two main histological types, diffuse and intestinal.[2] Gastric cancer is thought to result from a combination of environmental factors and the accumulation of genetic alterations, and consequently affects mainly older patients often after a long period of atrophic gastritis. The most common cause of gastritis is infection by *Helicobacter Pylori*, which is the single most relevant environmental factor in gastric cancer.[3]

Research to date has not revealed a specific pathway for gastric cancer, although numerous molecules have been implicated and the need for research in this field is undoubted. Many research techniques require intact DNA, and the search for molecular mechanisms involved in gastric carcinogenesis would be facilitated if a panel of cell lines representing different tumour phenotypes of gastric carcinoma could be developed. Few diffuse gastric cell lines are described in literature and due to the inherent difficulties of making cell lines from primary gastric tumours, most are established from metastatic deposits [4] or ascites fluid.[5],[6] Another approach is to transplant and serially passage primary tumours in nude mice.[7] In addition to facilitating the creation of cell lines, xenograft tissue itself can be an invaluable research aid as it provides more fresh tumour material and reflects the primary tumour more accurately than cell lines.

In this study we report the molecular characterization of eight primary gastric cancers with corresponding xenografts as well as the successful establishment of two novel gastric cancer cell lines, one derived from a human primary tumour of the diffuse type and one from intestinal type gastric cancer. It is assumed that xenografts and cell lines incur genetic and epigenetic changes in addition to those of the primary tumour, but to what extent the genomic alterations in gastric cancer vary between primary tumour, xenograft, and cell line is unknown. For this reason we decided to investigate copy number alterations and CpG island methylation status of a number of tumour suppressor genes and other relevant genes in the primary tumours, xenografts and cell lines using a multiplex ligation-dependent probe amplification (MLPA) technique. MLPA is a quantitative multiplex PCR-based approach that allows the determination of the relative DNA copy number of up to 40 different targets at the resolution of individual genes, in a single experiment.[8] Methylation specific MLPA allows simultaneous detection of CpG methylation and has been successful validated against other methylation techniques. [9] In addition we compared the primary and xenograft tumours histologically, examined the expression profile of fourteen different immunohistochemical markers and carried out *CDH1* and *TP53* mutation analysis in cases with aberrant protein expression.

Methods

Patients and Materials

Sixty primary human gastric cancers were collected from five different hospitals in the Netherlands. A piece of primary tumour was cut into 3 mm cubes, incubated in RPMI medium containing glutamate, fungizone, gentamycin, penicillin and streptomycin for 4hrs or overnight at 4°C and subsequently biologically frozen and stored at -80°C until use or directly washed in PBS prior to subcutaneous transplantation into the flanks of NMRI nude mice (Charles River, Wilmington,

Table 1 Patient details and xenograft growth characteristics

Xenograft	Age of Patient	Histological Type	Time Span between xenograft transplants (months)							
			p0	p1	p2	p3	p4	p5	p6	p7
X1	83	diffuse	4	3 to 5	2	2.5	2.5	2	1.5 to 2	1.5
X2	61	intestinal	6	3	-					
X3	81	intestinal	4.5	-						
X4	68	mixed	4.5 to 7.5	5.5 to 8	7	-				
X5*	51	diffuse	10	-						
X6	65	mixed	5	2.5						
X7	64	intestinal	2 to 5	3	-					
X8	81	intestinal	10 to 14	7	3	-				

* patient with known HNPCC with an MSH2 mutation
p0-p7 indicates xenograft passage number

MA, USA). Mice were cared for in our institute's animal facility with approval of the animal ethics review board. Once the tumour grew to a size of 1-2 cm^3 the mice were sacrificed, the xenografted tumours resected under aseptic conditions and divided into representative portions for paraffin embedding, snap freezing, and direct culturing. In addition, four small pieces were retained for transplantation into subsequent mice as shown in Table 1. Details of the age of the patient and histological type can also be found in this table.

The tissue microarray included 204 gastric carcinomas obtained from 24 different institutions throughout the Netherlands through the nationwide database system, and from the Department of Pathology at the Jorvi Hospital (Espoo, Finland) as published previously (Milne et al 2006). Tumour stage and lymph node status were available in 174 cases.

Cell Culture

Attempts were made to culture all xenografts tissue by cutting the tumours into small pieces and seeding onto 12-well plates in RPMI supplemented with 15% FCS, penicillin/streptomycin and glycine. Murine fibroblasts contaminating the initial culture were removed by differential trypsinization to obtain a pure tumour cell population. Tumour cells from the first passage of X8 were cultured in Dulbecco's modified Eagle's medium, antibiotics and glycine, Ham's F-12 nutrient mix, 15% fetal bovine serum, hydrocortisone, insulin-transferrin-selenium and cholera toxin as shown to assist cell in culture previously.[10] The cells initially grew in clumps and so after 3 weeks they were shaken with liver digestion medium (Gibco) and incubated at 37°C for 30-60 minutes. The supernatant was removed, and fresh liver digestion medium was added to dissociate any remaining clumped cells and the process was repeated until all cells were dissociated. Once the cell line, designated IGC8 was well established, cholera toxin was omitted. Cells from the first and second passage of xenograft 1 were cultured together in RPMI medium with the addition of 15% fetal bovine serum, antibiotics, glutamate, hydrocortisone and insulin-transferrin-selenium as above and named DGC1. Both cell lines were cultured for at least 6 months and at least 24 passages.

Immunohistochemistry

Antigen retrieval (10 minutes of boiling in 10 mM Tris/1 mM EDTA (pH 9)) was carried out on 4 μm deparaffinized sections. Slides were immersed in 0.3% hydrogen peroxide in methanol for 30

minutes and non-specific binding blocked with 5% normal goat serum (1 hour, room temperature). Sections were then incubated for 1 hour at room temperature with one of the following primary antibodies: p53 (DO-7 and BP53-12 Neomarkers, Union City, CA, USA; 1:2000), TFF1/pS2 (Dako, Glostrup, Denmark; 1:1200), p16 ^{INK4A} (Neomarkers, Fremont, CA, USA; 1:100), SMAD4 (sc-7966 Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200), COX-2 (Cayman Chemical Co., Ann Arbor, MI, USA; 1:800), cyclin E monoclonal antibody (Neomarkers;1:40), p27 clone: 1B4 (Novocastra, Newcastle upon Tyne, United Kingdom;1:250), or overnight at 4°C for the following primary antibodies: E-Cadherin HECD-1 (Thamer, Uithoorn, The Netherlands; 1:2000), MLH1 (Pharmingen, San Diego, CA, USA; 1:50), β-catenin (BD Biosciences, Alphen aan den Rijn, The Netherlands;1:10,000 dilution), KLK3/PSA (Dako, 1:400), CHFR (Abnova, Taipei City, Taiwan, 1H3-A12, 1:200), CASR (Abcam, Cambridge, United Kingdom, 1:200) and DAPK1 (Lifespan Biosciences, Seattle, Washington, U.S.A. 1:50). The Ultravision antipolyvalent HRP detection system (Lab Vision Corp., Fremont, CA, USA) was used to visualize antibody binding sites with 3,3'-diaminobenzidine as a chromogen. Sections were counterstained with haematoxylin.

Immunohistochemistry on cytospins was carried out with preparations fixed with acetone with an additional fixation step using zamboni for p53. Non-specific binding was blocked using 5% normal goat serum for 10 minutes followed by incubation with the primary antibody as above, except for the following dilutions: β-catenin 1:2000, COX-2 1:50 and p53 1:400. Endogenous peroxidase activity was blocked using 0.1% natriumazide and 0.3% H_2O_2 in phosphate buffered saline for 9 minutes. Peroxidase activity was then detected using 3,3 amino-9ethyl carbazole (Sigma-Aldrich, Zwijndrecht, The Netherlands) in dimethylformamide.

Scoring of immunohistochemistry was carried out as follows: P53 was positive if strong nuclear staining was present in > 30% of cells; E-Cadherin was considered abnormal in the absence of membranous staining or presence of clumpy cytoplasmic localization; TFF1 was deemed positive if cytoplasmic staining was present in >5% of tumour cells; B-catenin was scored as abnormal if there was nuclear staining accompanied by loss of membranous staining; COX-2 was scored into the categories 0-4 follows: 0 – no staining, 1 – weak diffuse cytoplasmic staining (may contain stronger intensity in <10% of cells), 2- moderate to strong granular cytoplasmic staining in 10-50% of tumour cells, 3 – strong intensity in >50% of tumour cells; p16 was positive if there was nuclear staining in >10% of tumour cells; SMAD4 and MLH1 were scored as negative if there was absence of nuclear staining in the tumour cells, in the presence of positive staining in an internal control and staining of p27 was scored semi-quantitatively by estimating the percentage of tumour cell nuclei staining (0-5%, 6-25%, 26-50%, 51-75%, 76-100%) with all immunoreactive nuclei regarded as positive, irrespective of the intensity of staining and thereafter categorized as p27 low (0-25%) or p27 high (26%-75%) for statistical analysis.

Tissue Microarray and Statistical Analysis

Tissue microarrays were constructed from formalin-fixed and paraffin-embedded archive specimens as described previously [11] and stained and scored as above. The SPSS 11.5 software package was used for statistical analysis. A Chi-squared test was applied to determine whether there was a statistically significant correlation ($p<0.05$) between p16, p27 staining and tumour stage or lymph node metastases.

Table 2 Results of the Methylation Specific Mulitplex Ligation-dependent Probe Analysis.

Gene	Chr pos	P1		X1p0		X1p2		C1		P2		X2p0		X2p1		P3		X3p0		P4		X4p0		X4p2		P5		X5p0		P6		X6p0		P7		X7p0		X7p1		P8		X8p0		X8p2		C8	
		C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M
CREM	10p12.1																																															
TIMP3	22q12.3						M		M																											M		M									
APC	5q22		M		M		M		M		M		M		M		M		M		M		M		M		M		M		M		M		M		M		M		M		M		M	
PARK2	6q26														G																																	
CDKN2A	9p21								G										L						L				L				L														
MLH1	3p22.1		M		M		M		M										L																												
TNFRS1A	12p13								G																																							
ATM	11q23																		L						L				L																		
RARB	3p24																																															
MLH3	14q24.3																																															
CDKN2B	9p21																		L										L				L														
HIC1	17p13.3																																															
PAH	12q23																												G																		
CHFR	12q24.33		M		M		M		M																L																						
BRCA1	17q21																																L														
BCL2	18q21.3																																															
CASP8	2q33.2																																															
CDKN1B	12p13.2																		L						L																				L		L	
TSC2	16p13.3																																															
PTEN	10q23.3																		L																													
BRCA2	13q12.3														G				G				L		L																							
CDK6	7q21.3																																															
CD44	11p12																																															
RASSF1	3p21.3		M		M		M		M																								L														
CDH1	16q22.1																																G														
DAPK1	9q22								G																						G		G													
VHL	03p25.3																																													L		
AI651963	10p14											G		G								G		G																							
ESR1	6q25.1		M		M		M		M						M	L		L																													
KLK3	19q13						G											G																	G		G		G				G		G		
TP73	1p36																							G																							
FHIT	3p14.2															L		L										L				L						G								
IGSF4	11q23																					M		M																							
CDH13	16q24.2				M		M		M									G	M																		M		M		M		M		M		M	
TNFRSF7	12p13																																															
GSTP1	11q13																																															
CTNNB1	3p22																					G		G																							
CASR	3q21	G		G		G		G				G		G						G		G		G														G		G				G		G		

M- methylated; C – copy number; L- loss; G- gain. Grey shading where methylation analysis not performed.

DNA Extraction and Microdissection

Serial 8-9 μm deparaffinized haematoxylin stained sections were carefully microdissected if necessary (to achieve >85% tumour cells) using an inverted microscope and a sterile needle. DNA from paraffin material and xenograft material was isolated using the PUREGENE® DNA Isolation Kit (Gentra Systems, Minneapolis, USA) according to manufacturer's instructions. Similarly, xenograft DNA was extracted from the initially transplanted primary tumours (passage 0) from all tumours. In addition DNA was isolated from the 1st passage of X2 and X7 and the second passage of X1, X2 and X7. DNA isolation from cell lines was performed using proteinase K treatment, followed by phenol/chloroform extractions. DNA concentrations were measured using the NanoDrop spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands)

Methylation Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA)

MLPA reagents were obtained from MRC-Holland, Amsterdam, The Netherlands (ME001 tumour suppressor kit; and the P083 CDH1 kit; www.mlpa.com) and used according to the manufacturer's instruction.[8] Amplification products were detected and quantified by capillary electrophoresis on an ABI 3100 automated sequencer (Applied Biosystems) using a ROX-labeled internal size standard (ROX-500 Genescan, Applied Biosystems, Warrington, UK).

The data was analyzed as previously described [9] using the Coffalyser software(MRC-Holland). Briefly, the total of all peak areas was used for normalization of each sample after which each probe was compared to reference normal DNA and a tumour to normal DNA copy number ratio was determined. As suggested by the manufacturers, a global normalization mode was employed for the ME-001 kit thereby treating every probe as a control probe, and a single iteration mode was used on the Coffalyzer software, whereas for the P083 kit normalization with a gene subset (i.e. designated control probes) was appropriate. Reference DNA from normal non-cancerous lymph nodes and non-neoplastic gastric mucosa from three different individuals was used. All MLPA reactions were repeated at least twice. A ratio less than 0.60 was considered a deletion and a ratio higher than 1.35 considered a gain. Of note, the ME 001 kit contains 41 probes targeted to 38 genes. Identical results were obtained for the 2 different *MLH1*, *BRCA2* and *RASSF1* probes thus results for one of the probes have been presented in Table 2.

Mutation Analysis

The sequence of the TP53 coding region was assessed using RT-PCR. RNA from cell pellets of DGC1 was extracted using Trizol (Invitrogen, Breda, The Netherlands) and the quality assessed by agarose gel electrophoresis. Subsequently, complementary DNA was synthesized using the M-MLV RT enzyme kit (Invitrogen), pd (N)6 random primers (Amersham Biosciences, Roosendaal, The Netherlands) and RNA inhibitor (Roche, Basel, Switzerland), according to the manufacturer's instructions. The *TP53* coding sequence was amplified by PCR of three overlapping segments, using the following primers: 5'-GCTTTCCACGACGGTGACA-3'(A forward), 5'-ACTCCCCTGCCCTCAACAA-3'(A reverse), 5'-TCTGTCCCTTCC-3'(B forward), 5'-GCTCTGACTGTACCACCATC-3'(B reverse), 5'-TTGCGTGTGGAGTATTTGGA-3'(C forward) and 5'-GGTCTTTGAACCCTTGCTTGC-3' (C reverse). 35 cycles were carried out using 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 μM forward and reverse primers, at an annealing temperature of 55° C and 4 units of Platinum® Taq DNA polymerase (Invitrogen) in a buffer supplied by the manufacturer, with an end volume of 50μl in an MJ Research Thermal Cycler (DYAD™), (Bio-Rad Laboratories, Inc., Waltham, MA, USA).

Following purification of PCR products with the QIA quick® PCR purification kit (Qiagen, Leusden, The Netherlands), the sequences were analyzed using the ready reaction Big Dye™ Terminator Cycle Sequence kit (Applied Biosystems) and an ABI 3100 automated sequencer (Applied Biosystems) and visualised using CodonCode Aligner 1.5.1 software (CodonCode Corporation, Dedham, MA, USA).

All 16 exons of CDH1 were amplified from genomic DNA isolated from the cell lines. Conditions and primers are available from the author on request. Following purification of PCR products with the MSNU03050 multiscreen HTS 96 wells filtration system (Millipore, Etten Leur, The Netherlands), the sequences were analyzed using the ready reaction Big Dye™ Terminator Cycle Sequence kit version 1.1 (Applied Biosystems) and an ABI 3730 automated sequencer (Applied Biosystems) and analyzed using PolyPhred (http://chum.gs.washington.edu/PolyPhred.html). For CDH1, Genbank Accession Number NM_004360.2 was used as a reference sequence the start codon defined as position 1 and the presence of a new splice sites were assessed using the splice site prediction programmes www.cbs.dtu.dk/services/NetGene2/, www.genet.sickkids.on.ca/~ali/splicesitefinder and www.fruitfly.org/seq_tools/splice).

Results

Establishment of Xenografts and Cell Lines

Direct culture of primary human gastric tumors was attempted with twenty nine gastric carcinomas. None of these attempts led to the establishment of a cell line, but yielded at most short-term outgrowths of epithelial islands. Subsequently, sixty gastric cancers collected between 2002 and 2005 were transplanted into NMRI nude mice. Eight of these gave rise to tumors which grew to a visible size in the mouse and six of these continued to grow on transplantation into another mouse. The length of time taken to reach transplantable size (1-2 cm^3) for each tumor

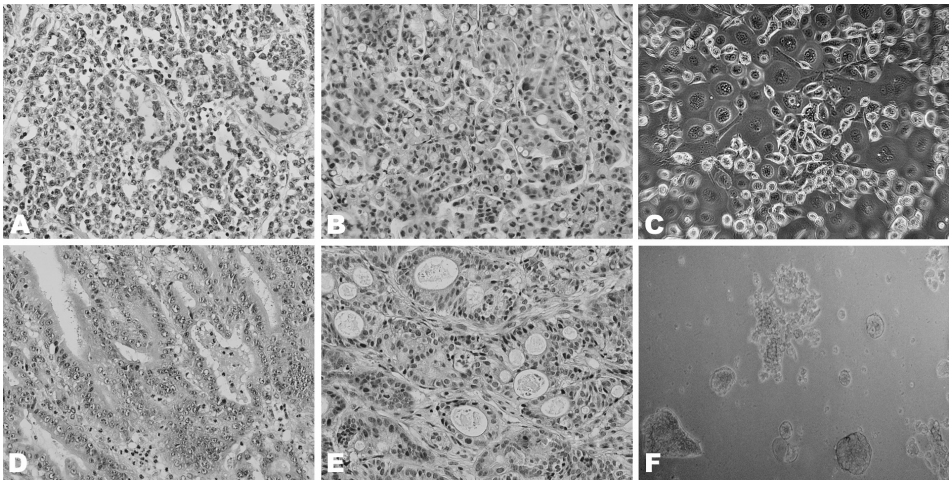

Figure 1 showing the H+E stain (x 40) of P1 (A), M1(B), P8 (D) and M8 (E) together with the cell lines DGC1 (C) and IGC8 (F) in culture, which were established from the corresponding xenografts. ***(See page 202 for colour figure)***

Table 3 Results of immunohistochemistry

	E-Cadherin	B-catenin	TFF1	COX-2	p53	cyclin E	p16	MLH1*	SMAD4*	P27	CASR	CHFR	DAPK	KLK3
P1	negative	negative	negative	2	positive	negative	negative	negative	positive	76-100%	positive	negative	positive	negative
X1	negative	negative	negative	1	positive	negative	negative	negative	positive	76-100%	positive	negative	positive	negative
C1	negative	negative	negative	1	positive	negative	negative	negative	positive	n.a.	n.a.	positive	n.a.	negative
P2	normal	normal	negative	2	normal	positive	positive	positive	positive	6-25%	positive	positive	positive	negative
X2	normal	normal	negative	1	normal	positive	positive	positive	positive	6-25%	positive	positive	positive	negative
P3	normal	nuclear	negative	3	normal	positive	positive	positive	positive	6-25%	positive	positive	positive	negative
X3	normal	nuclear	negative	1	normal	positive	positive	positive	positive	6-25%	positive	positive	positive	negative
P4	normal	normal	positive	4	normal	positive	positive	positive	positive	6-25%	positive	positive	positive	negative
X4	normal	normal	positive	3	normal	positive	positive	positive	positive	51-75%	positive	positive	positive	negative
P5	normal	normal	positive	3	normal	positive	negative	positive	positive	26-50%	positive	positive	positive	negative
X5	normal	normal	positive	2	normal	positive	negative	positive	positive	26-50%	positive	positive	positive	negative
P6	normal	normal	positive	3	normal	positive	positive	positive	positive	26-50%	positive	positive	positive	negative
X6	normal	normal	positive	1	normal	positive	positive	positive	positive	76-100%	positive	positive	positive	negative
P7	normal	normal	negative	3	normal	positive	positive	positive	positive	0-5%	positive	positive	positive	negative
X7	normal	normal	negative	3	normal	positive	positive	positive	positive	51-75%	positive	positive	positive	negative
P8	abnormal	normal	negative	3	normal	positive	positive	negative	positive	6-25%	positive	positive	positive	negative
X8	abnormal	normal	negative	3	normal	positive	positive	negative	positive	26-50%	positive	positive	positive	negative
C2	abnormal	normal	negative	3	normal	positive	positive	negative	positive	n.a.	n.a.	positive	n.a.	negative

grey shading indicates abnormal staining
*xenografts stained with these antibodies were difficult to accurately interpret due to high background staining of mouse tissue
n.a. – not assessable (poor quality of staining on cytospin)

Table 4 Results of Tissue Microarray Immunohistochemistry

Gene of interest	Immunohistochemical findings	significance of result
p16	44% negative staining	no correlation with tumor stage or metastases
p27	52% p27 high phenotype	no correlation with tumor stage or metastases
CASR	100 % positive	does not appear to act as TSG in gastric cancer
CHFR	33% negative	loss correlates with diffuse histology (p=0.001)
DAPK	6% negative	loss correlated with older age (p=0.002)
KLK3	100 % negative	KLK3 is not amplified in gastric cancer

can be seen in Table 1 and the selection for more aggressive tumor cells was demonstrated by the decreased time between transplantations. Histological assessment of the xenograft tumors revealed tumors of the same grade as the original primary tumor in all cases. Attempts were made to culture all 8 xenografts and passages thereof. Cell line DGC1, derived from a human primary tumor of the diffuse type was established from the first and second xenograft passages and cell line IGC8, derived from a human primary tumor of the intestinal type was established from the first xenograft passage. The morphology of the primary tumor, corresponding xenograft and cell lines can be seen in Figure 1. DCG1 consisted of a flat monolayer in culture and had a population doubling time of seven days. IGC8, derived from an intestinal gastric tumor grew in three dimensional structures composed of strings of cells, and never exceeded 80% confluency. It had a population doubling time of 10 days. Both cell lines were cultured for six months and at least 24 passages. Further characterization of the cell lines was done by the assessment of copy number changes and determination of the methylation status of a number of tumor suppressor genes and other relevant genes as described below, as well as by immunohistochemistry and mutation analysis of TP53 and *CDH1*. DGC1 harbored a mutation in TP53 c.125G>C p(Arg72Pro). Both cell lines revealed the same frame shift mutation (c.377del) in the *CDH1*/E-cadherin gene. In addition DGC1 contained a c.1901 C>T(p.Ala634Val) mutation whereby a new splice donor site is predicted resulting in a frameshift most probably leading to nonsense mediated decay. In IGC8, the variation c.2253C>T, a silent alteration that does not seem to affect the splicing was found and considered as an infrequent polymorphism. MLPA analysis of the CDH1 gene revealed no exon deletions or duplications.

CpG Island Methylation Phenotype and Copy Number

To determine genetic and epigenetic changes incurred by the primary tumor, xenografts and subsequent cell lines, we carried out methylation specific multiplex ligation-dependent probe amplification (MS-MLPA) with probes targeted to many known tumor suppressor genes, genes involved in cell signaling, adhesion and apoptosis. Results of this analysis can be seen in Table 2, where the primary tumors, labeled P1-P8 can be compared to the corresponding xenograft X1-X8, and the two novel cell lines C1 and C8, now designated DGC1 and IGC8. Interestingly, 3 out of the 8 primary tumors had a normal gene copy number for all the 38 genes examined.

Gains were most prominently seen for *CASR*, encoding a calcium-sensing receptor (5/8 cases), *KLK3/PSA*, encoding the prostate specific antigen kallikrein 3 (4/8 cases) located at chromosomal region 19q13 and *DAPK1*, that codes for a death-associated protein-kinase (3/8 cases). Single gains were observed for *TNFRS1A*, *PARK2*, *PAH1*, E-cadherin/*CDH1*, and *TP73*, in the xenografted lesions and cell line. Also in one case, one of the cell lines showed a gain for p16/*CDKN2A* however, losses of this gene were mostly seen (4 cases). Further losses were detected for the cell cycle regulators p15/*CDKN2B*, located at chromosomal region 9p21 (3/8 cases; all

with loss of p16 as well), p27 (3/8 cases) and *ATM* (3/8 cases). No homozygous deletions were found for any of the genes. *FHIT* and *BRCA2* showed both losses and gains, indicating potential genomic instability at these sites.

Losses in combination with methylation were seen for the DNA repair gene *MLH1*, the checkpoint gene *CHFR* (1/8 loss, 2/8 methylation), *RASSF1* (1/8 loss, 1/8 methylated) and *ESR1* (1/8 loss, 2/8 methylated). Methylation occurred in 4 cases at the locus for H-cadherin/*CDH13*, however in one instance this occurred in combination with a gain in copy number. The *APC* gene was methylated in all cases as was also seen in normal non-neoplastic gastric mucosa. Of note, the highest rate of gene methylation was found in the cases P1 and P8 from which cell lines were derived.

Protein Expression and Mutation Analysis

In order to compare the protein expression of the primary gastric tumor, xenograft and cell line, we carried out immunohistochemistry for E-Cadherin, β-catenin, COX-2, p53, TFF1, cyclin E, p16, MLH1, SMAD4, p27, CHRF, KLK3/PSA, CASR and DAPK1 on paraffin embedded material of the xenografts and primary tumors and cytospins of the cell lines. These results are summarized in Table 3 where abnormal staining is highlighted with grey shading.

The primary tumor, xenograft and cell lines shared the same expression pattern for all markers examined except for p16/CDKN2A in X3 (where MLPA analysis showed loss of one copy of this gene), p27 and COX-2. In cases P2 to P6, the primary tumor had a stronger expression of COX-2 than in the xenograft. Interestingly, no expression of KLK3 was found on immunohistochemistry despite amplifications being found at this gene locus.

Case 3 displayed nuclear localization of β-catenin and complete absence of β-catenin was found in case 1. In addition, cases 1 and 8, showed a complete lack of MLH1 protein expression in the tumor cells which corresponded to the methylation of *MLH1* found through MS-MLPA.

Cases 1 and 8 with abnormal IHC for either p53 and/or E-Cadherin were subjected to mutation analysis of the respective genes. These cases were also the ones from which cell lines were derived and therefore mutation analysis was performed on the cell lines, revealing mutations in the respective genes. MLPA analysis of the *CDH1* gene for these two cases revealed no exon deletions or amplifications.

Correlation of immunohistochemical staining with clinicopathological characteristics

In order to investigate the biological relevance of some of the genetic changes found using MS-MLPA, we carried out immunohistochemistry for CDKN2A (p16), CDKN1B (p27/KIP1), KLK3/PSA, CASR, CHRF and DAPK1 on 174 gastric cancers including early-onset gastric cancer and conventional gastric cancers using tissue microarrays and correlated the expression of these markers with clinicopathological parameters. Expression rates of these markers together with a summary of the relevant clinical correlations and significance of the result can be found in Table 4.

P27 high immunohistochemistry (nuclear staining in ≥ 26% of cells) was present in 52% of cases and there was no statistical difference between diffuse and intestinal cancers. Absence of p16 expression was found in 44% of cases with no difference in histological type as reported previously.[11] No correlation was found between either p16 or p27 and T-stage ($p=0.194$ and $p=0.435$ respectively) or lymph node status ($p=0.48$ and $p=0.38$ respectively) despite the fact that loss of CDKN2A (p16) and CDKN1B (p27/KIP1) appeared to occur exclusively in the xenografted tumor, and occurred especially in later passages in the xenografts (X4 p2,X8 p2).

CHFR staining was lost in 33 % of gastric cancers and there was a significant difference between staining in diffuse and intestinal histology, with loss found more commonly in the diffuse type gastric cancer (p=0.001). There was no correlation with age, location (cardia versus fundus) or tumor stage. CASR was expressed in all cases but a clear distinction between normal expression or possible overexpression could not be reproducibly made. On the other hand, KLK3/PSA was negative in all cases with no expression found, despite amplification at this gene locus. Loss of DAPK expression, a gene that was found to be amplified using MLPA, was seen in 6%. Interestingly, loss of DAPK1 expression correlated significantly with older age (p=0.003) with 91% (10 of 11 cases of loss) occurring in conventional cancers. There was no correlation with histology, location or tumor stage.

Discussion

Cell lines and xenograft materials are widely used by researchers to document characteristics of a particular human cancer. In the process of the establishment of these model systems, additional genetic and epigenetic changes may occur or alternatively, specific clones with these changes may be selected for. In this study we characterized a group of eight xenograft tumors with their corresponding primary tumors and we report the establishment of two novel gastric cancer cell lines derived by passaging in nude mice. To study the alterations in primary tumors and derived xenografts and cell lines we compared these lesions with respect to their histology, expression of relevant proteins using immunohistochemistry, changes in copy number of a number of tumor suppressor genes as well as genes important in processes such as cellular growth and signaling, DNA repair, and apoptosis. In addition, methylation of many of these genes was studied by MS-MLPA analyses and where relevant, *TP53* and *CDH1* mutation analysis was performed. Furthermore we extrapolated some of the genetic changes found by examining protein expression of 174 gastric cancers using tissue microarrays.

The histology of primary tumors and their xenografts was comparable as was the expression pattern as studied by immunohistochemistry that was shared in the primary tumor, xenograft and cell lines for all markers examined, (E-Cadherin, β-catenin, COX-2, p53, TFF1, cyclin E, p16, MLH1, SMAD4 and p27) except for p16 and COX-2. In one case, in contrast to the primary tumour, the xenograft showed a loss of p16 expression accompanied by allelic loss. In five cases, the primary tumor had a stronger expression of COX-2 than in the xenograft, possibly due to the change in the growth factor environment.

MLPA analysis provided us with information regarding the copy number of 38 genes, with primary gastric tumors as well as their derived xenografted lesions and cell line found to have copy gain of *CASR*, *DAPK1* and *KLK3*. In addition, *CASR* gains were also detected in xenograft passages and the corresponding cell line of three additional cases where it was not observed in the primary tumor. Similarly, *KLK3* and *DAPK* gains were also detected in xenografts and cell lines which had no corresponding aberrations in the primary tumor.

CASR maps to 3q13-21 and is a calcium-sensing receptor, potentially of influence on gastric acid secretion, fluid transport in the colon, and possibly involved in calcium-handling and differentiation in the gastrointestinal tract. [12],[13],[14],[15] Interestingly this gene has been described as having a possible role in abnormal differentiation or malignant progression in the colon,[16] thus the uncovering or excluding of a role for CASR in gastric cancer is of great interest. However in 174 gastric cancers we found no loss of CASR, out ruling its role as tumor suppressor

gene in gastric cancer. Pituitary adenomas [17] are so far the only neoplasms documented to have overexpression of CASR (Calcium-sensing receptor) and the functional relevance of this gain in gastric cancer, which was found in 2 of our 8 primary tumors, has yet to be established.

Another novel finding was amplification of *KLK3* (Kallikrein 3/Prostate Specific Antigen), a kallikrein-like protease with androgen-response elements that is widely amplified and overexpressed in prostate carcinoma.[18] Interestingly overexpression of PSA has been described in gastric cancers,[19] however in a further examination of 174 gastric cancer, we found no expression of KLK3, suggesting that another target at this locus is of importance in gastric cancer. *KLK3* is located at chromosomal region 19q13, a region that where amplifications have been described in early-onset gastric cancer [20] where it is associated with overexpression of cyclin E. Indeed in this study we found overexpression of cyclin E in all cases that showed gain of *KLK3* except for the cell line of case 1, suggesting that this is in fact the critical gene amplified at this locus.

The third most frequently detected gain, found in 2/8 primary tumors, was for *DAPK1* (death-associated protein kinase), a positive regulator of programmed cell death and recently described as a novel p53 target.[21] However, DNA methylation and histone deacetylation of *DAPK1* has been found in colorectal and gastric cancers in association with silencing of this gene.[22],[23],[24] This discrepancy may reflect genetic instability at this site, changes in the balance of apoptosis, or the detected change in copy number may not lead to an increase in expression of the protein. To investigate this we carried out IHC on an additional 174 gastric cancers and found a loss of expression in 6% of cases which correlated significantly with conventional gastric cancer (age > 45 years). This means that DAPK does not appear to play a role in EOGC as was previously thought [23]. In addition it appears to play a less important role in gastric cancer than previously published [22],[24]. The finding of a gain at this locus still remains unexplained, although it is possible that the amplification may involve another critical gene at this locus. Gains in genes other that DAPK1 were detected only once or twice and were absent in the primary tumors.

The most prominent losses were detected for *CDKN2A (p16)*, *CDKN2B (p15)*, both located at 9p21, *CDKN1B (p27/KIP1)* and *ATM*. Further losses in conjunction with hypermethylation in other cases were detected for *MLH1*, *CHFR*, *RASSF1* and *ESR1*. The loss of copy numbers for the genes encoding cyclin dependent kinase inhibitors such as *CDKN2A (p16)*, *CDKN2B (p15)* and *CDKN1B (p27/KIP1)* reported to be important in tumor progression, was found only in the cell line, the xenografts or the last passage of the xenografts studied, suggesting indeed that these changes might be associated with tumor progression. Therefore, protein expression of two of these markers markers, p16 and p27 was examined in a further 174 gastric cancer cases using tissue microarrays. However, the expression data did not show any significant correlation with tumor stage or lymph node status. There are conflicting reports in the literature regarding p16 and its association with lymph node metastases [25],[26],[27]. Similarly, although gastric cancer with low expression of p27 has been reported to be associated with aggressive characteristics and a worse outcome [28] another report shows that it has prognostic value only though its correlation with p53 status.[29]

Interestingly, two genes involved in DNA repair and checkpoint control, *MLH1* and *CHFR* (checkpoint with FHA and ring finger) also showed loss or methylation. Methylation of these genes was observed in the primary tumors as well as in all derived xenograft lesions and cell lines and was accompanied by lack of expression of the protein as assessed by immunohistochemistry for MLH1. Furthermore, in an additional 174 gastric cancer cases, loss CHFR expression was seen in 33% and correlated significantly with diffuse histology. Hypermethylation of *CHFR* has been

reported to occur concurrently with *hMLH1* hypermethylation in gastric neoplasia, [30] as has been confirmed by our findings

Hypermethylation was also detected for *RASSF1A* (Ras association domain family protein 1), *ESR1*(estrogen receptor 1), and *CDH13* (T-cadherin, H-cadherin) and this has been reported previously in gastric cancer [31],[32],[33] albeit that hypermethylation of *ESR*1 was previously described only in gastric cancer cell lines.[34] *APC* promoter 1A was found to be hypermethylated in all samples. This is in accordance with previous literature showing that hypermethylation in one of the two described *APC* promoters, promoter 1A, occurs in gastric cancers as well as in non-cancerous gastric mucosa [35] and is considered both age related and tissue-specific.

It is known that hypermethylation is a powerful mechanism for the silencing of tumor suppressor genes, DNA repair genes and metastasis inhibitor genes in cancer. There is a need for sensitive and robust multiplex methods for the detection of aberrant methylation of promoter regions in paraffin embedded material. Here we report the use of MS-MLPA for the simultaneous detection of methylation status in 26 genes, including tumor suppressor genes, in gastric cancer. Interestingly, we found the highest rate of methylation in the primary tumors which eventually gave rise to two novel gastric carcinoma cell lines. In addition, both cell lines were found to harbor mutations in the gene encoding E-cadherin (*CDH1*), a gene that is found mutated in hereditary gastric cancer of the diffuse type [36] although they were derived from primary gastric cancers of different histology. This resulted in respectively abnormal or a lack of expression of the protein.

Overall, although more gains and losses were found in the xenograft tumors and cell lines than in the primary tumors, the majority of changes could also be detected in primary tumors. Moreover, some of these changes may indeed represent alterations present in more aggressive subclones or be induced by the establishment of the xenografts as may happen during tumor progression. We report here several novel findings in regard to (epi)genetic alterations during gastric cancer progression. In particular we demonstrate gains in *KLK3* (19q13), *DAPK1* (9q22) and *CASR* (3q21). We describe that loss of CHFR occurs frequently in gastric cancer and correlates with diffuse histology. Xenografts and gastric cancer cell lines therefore remain an invaluable research tool that may assist us in the uncovering of the multistep progression of cancer.

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Do Collision Tumors of the Gastro-esophageal Junction Exist? A Molecular Analysis

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Abstract

Collision tumors are thought to arise from the accidental meeting of two independent tumors. Here we present five gastro-esophageal junction tumors consisting of two collision tumors and three composite tumors (characterized by two divergent lineages originating from the same neoplastic clonal proliferation), as diagnosed on histology. In an attempt to prove this distinction at a genetic level we performed *TP53* sequence analysis and p53 immunohistochemistry. In addition, loss of heterozygosity (LOH) analysis using ten microsatellite markers was carried out. An identical *TP53* mutation and a similar pattern of retention and LOH were found in both neoplastic components of the presumed collision tumors, suggesting that both components are derived from a single precursor cell that undergoes divergent differentiation in the evolution of the tumor. In the composite group, one case had a genetic basis for the possible diagnosis of a collision tumor, with a *TP53* mutation in the adenocarcinoma component only, and a different pattern of retention and loss of heterozygosity. These findings imply that it is not possible to recognize true collision tumors from immunohistological appearance alone and suggest that the longstanding histological criteria for the diagnosis of these neoplasms have no molecular basis.

Introduction

Gastric and esophageal carcinomas remain a leading cause of cancer mortality worldwide. Moreover, the incidence of adenocarcinoma arising at the gastro-esophageal junction (GEJ) appears to be increasing.[17] These junctional adenocarcinomas may develop from a Barrett esophagus and Barrett carcinoma is currently the most rapidly increasing cancer in the Western world.[22]

Since 1919 collision tumors were thought to arise from the accidental meeting and interpenetration of two independent tumors.[20] In 1961, the first case of a cardia collision carcinoma was described and the following three criteria were proposed:[5] 1) the two components should show at least a partial topographical separation; 2) the squamous component should lie on the esophageal side of the tumor and the adenocarcinoma component on the gastric side; 3) there should be little or no evidence of intermediate histological structure. Others believed that an intermediate pattern at the point of collision was still acceptable for the diagnosis of a collision tumor.[24,31] Collision tumors need to be distinguished from composite tumors (characterized by two divergent lineages originating from the same neoplastic clonal proliferation), as it is possible that a different treatment is warranted, depending on the type of collision tumor encountered.

Collision tumors occur at a higher frequency than would be expected statistically if their combined presence were merely coincidental.[11] A possible explanation for this is the presence of field cancerization, which occurs due to long term exposure to carcinogens, whereby multiple transforming events give rise to genetically unrelated secondary primary tumors with independent mutations[2,6,23,25] and thus the chance that these tumors may in some instances "collide" is increased. In some situations environmental triggers explain the collision phenomenon, such as the association of *Helicobacter pylori* with the synchronous occurrence of MALT (mucosa-associated lymphoid tissue) lymphoma and adenocarcinoma of the stomach,[21,32] where a single carcinogenic agent interacts with the two neighboring tissues, inducing the development of tumors of different histogenesis in the same organ.[18] The collision of carcinomas with carcinoid tumors has also been reported frequently and it has been suggested that carcinoids can produce substances with a growth promoting effect accounting for the occurrence of a second tumor in the vicinity.[3]

However, many collision tumors have no explanation for their occurrence, and as most diagnoses have been made on histology alone, the question exists as to whether histological classifications accurately reflect the molecular findings in these tumors. Genetic analysis would provide evidence regarding whether the tumors originate from the same or different clones. Here we present two tumors of the gastro-esophageal junction, which had the histological appearance of true collision tumors (Figure 1). In case A an adenocarcinoma collided with a squamous cell carcinoma, and in case B an adenocarcinoma (with a signet ring cell appearance) collided with a squamous cell carcinoma. We compared these two cases with three cases (C, D, and E) that had a histological appearance of an adenosquamous (composite) tumor. The finding of a tumor with glandular and squamous elements (adenosquamous carcinoma) is a well-recognized entity, included in the WHO classification[12] and has been characterized at a molecular level,[30] thus providing a reference point from which to compare the two collision tumors. In an effort to determine the clonality of the two separate components in each case, we characterized the molecular-genetic alterations of each tumor component including *TP53* sequence analysis, loss of heterozygosity at ten chromosomal loci, and p53 immunohistochemistry.

Patients and Methods

This research was carried out in accordance with the ethical guidelines of the research review committee of the Academic Medical Center, Amsterdam.

Description of Cases

Case A: Macroscopically, a 9 cm diameter ulcerated tumor was present at the cardia, extending into the esophagus. Microscopically the tumor was composed of an adenocarcinoma adjacent to a squamous cell carcinoma with no intermingling at their interface (Figure 1A). A fibrous band separated the two components.

Case B: Macroscopically a poorly circumscribed tumor 5 cm in maximum dimension was present in the distal esophagus extending through the gastro-esophageal junction into the cardia. Microscopically it consisted of a squamous cell carcinoma proximally and a diffuse signet ring cell carcinoma distally (Figure 1B). A thin fibrous band separated these components and no intermingling was observed. These findings of a tumor with diffuse signet ring cell and squamous cell carcinoma components is an entity that to our knowledge has never been described at this location.

Case C: Macroscopically, a 3 cm diameter tumor was present at the gastro-esophageal junction, which was microscopically composed of distinct adenocarcinoma and squamous cell carcinoma components. There was widespread intermingling of components.

Case D: A polypoid tumor 10 cm in length was present at the gastro-esophageal junction, which consisted of a squamous cell carcinoma extending through the muscularis propria. Near the surface a well-differentiated adenocarcinoma component was present with some intermingling with the adjacent squamous cell component.

Case E: A 1.5 cm diameter tumor was present at the gastro-esophageal junction. Microscopically the majority of the tumor consisted of a poorly differentiated tumor with distinct separate adenocarcinoma and squamous cell carcinoma areas intermingled with the poorly differentiated areas.

Based on histology, a collision tumor was diagnosed in cases A and B, and an adenosquamous (composite) tumor in cases C, D, and E.

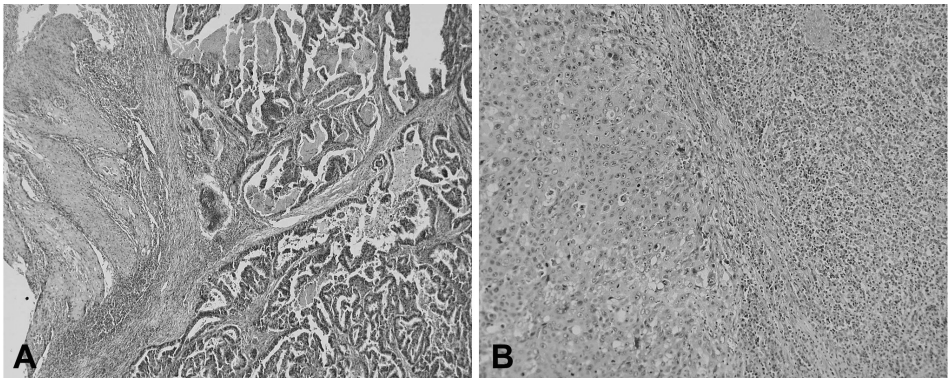

Figure 1 Haematoxylin and eosin staining of cases A (x50) and B (x100) showing the interface of the adenocarcinoma and squamous cell carcinoma components. ***(See page 202 for colour figure)***

Table 1 Microsatellite markers used for loss of heterozygosity analysis

Microsatellite Marker	Chromosomal Region	Tumour Suppressor Gene
D3S1478	3p21	*FHIT*; *hMLH1*
D5S346	5q21	*APC*
D9S171	9p21	*$p14^{ARF}$*; *$p16^{INK4A}$/CDKN2A*; *$p15^{INK4B}$/CDKN2B*
D10S2491	10q23	*PTEN*
D14S68	14q24.3	frequently deleted region in Barrett carcinomas
D16S2624	16q22.1	*CDH1*
D18S64	18q21.32	*DCC*; *DPC4*/*SMAD4*; *SMAD2*
TP53	17p13.1	*TP53*
P53Alu	17p13.1	*TP53*
Bat26	2p16	MSI consensus marker

Immunohistochemistry

Immunohistochemistry for p53 was performed using the monoclonal antibody combination of DO-7 and BP53-12 (Neomarkers, Union City, CA, USA). Sections were deparaffinized and antigen retrieval was carried out by 10 minutes of boiling in 10 mMTris/1 mM EDTA (pH 9). Subsequently slides were immersed in 0.3% hydrogen peroxide in methanol for 30 minutes and non-specific binding was blocked with 5% normal goat serum for 1 hour at room temperature. The sections were incubated with the primary antibody at a 1:2000 dilution for 1 hour at room temperature. The Ultravision antipolyvalent HRP detection system (Lab Vision Corp., Fremont, CA, USA) was used to visualize antibody binding sites with 3,3'-diaminobenzidine as a chromogen. Sections were counterstained with haematoxylin and p53 immunostaining was considered positive when staining was present in >30% of tumor cells.

TP53 Mutation Analysis

The *TP53* gene was initially amplified from RT-PCR material derived from fresh frozen tissue of one neoplastic component of each tumor. Mutations were subsequently confirmed in both tumor components using DNA derived from paraffin-embedded material. Due to unavailability of frozen material in case C all exons were amplified using DNA isolated from paraffin embedded material.

DNA Extraction and Microdissection

In case A, the squamous cell carcinoma component was composed of squamous islands surrounded by a dense lymphocytic infiltrate, thus requiring microdissection with the PALM® Laser Microbeam Microdissection system (Microlaser Technologies, Bernried, Germany). Slides for this method were prepared by fixing PALM® LPC membrane onto specialized thin glass slides, applying 0.1% poly-L-lysine and mounting a 6µm tissue section. This was subsequently stretched on a hot plate at 50°C and left at 60°C for 1 hour.

For the remaining 4 cases, serial 6 µm sections were cut with a microtome, deparaffinized using a standard xylene and serial ethanol protocol, stained with haematoxylin and carefully microdissected using an inverted microscope and a sterile needle. Both cases had discrete areas of each tumor thus facilitating accurate microdissection without risk of contamination with normal tissue. Normal control tissue was dissected from lymph nodes or, in the case where lymph node metastases occurred, from distant normal gastric mucosa. DNA was isolated using the PUREGENE® DNA Isolation Kit (Gentra Systems, Minneapolis, USA) according to manufacturer's instructions. DNA

concentrations were measured using PicoGreen® dsDNA Quantitation Kit (Molecular Probes, Leiden, The Netherlands).

RT-PCR

Twenty 4-5 μm thick sections of frozen material containing >90% tumor cells were used for RNA extraction using Trizol (Invitrogen, Breda, The Netherlands) and the quality of RNA was assessed by agarose gel electrophoresis. Subsequently, complementary DNA was synthesized using the M-MLV RT enzyme kit (Invitrogen), pd (N)6 random primers (Amersham Biosciences, Roosendaal, The Netherlands) and RNA inhibitor (Roche, Basel, Switzerland), according to the manufacturer's instructions.

PCR amplification was performed to include the entire *TP53* coding sequence by amplification of three overlapping segments, using 1 μl of cDNA (total 30 μl) and the following primers: 5'-GCTTTCCACGACGGTGACA-3'(A forward), 5'-ACTCCCCTGCCCTCAACAA-3'(A reverse), 5'-TCTGTCCCTTCC-3'(B forward), 5'-GCTCTGACTGTACCACCATC-3'(B reverse), 5'-TTGCGTGTGGAGTATTTGGA-3'(C forward) and 5'-GGTCTTTGAACCCTTGCTTGC-3' (C reverse). 35 cycles were carried out using 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 mM forward and reverse primers, at an annealing temperature of 55° C and 4 units of Platinum® Taq DNA polymerase (Invitrogen) in a buffer supplied by the manufacturer, with an end volume of 50μl in a Peltier Thermal Cycler (DYAD™), (Biozyme, Blaenavon, South Wales, UK).

PCR on Paraffin Material

The following primers were used for amplification of paraffin embedded material: exon 2 and 3 forward: 5'-GCTTGGGTTGTGGTGAAACA-3', reverse: 5'-TCCAGGTCCCCAGCCCAAC-3'; exon 4 fragment 1 forward: 5'- ACCTGGTCCTCTGACTGC-3', reverse: 5'-TTTTCTGGGAAGGGACAG-3'; fragment 2 forward: 5'-AAGCTCCCAGAATGCCAG-3', reverse: 5'-AAGCCTAAGGGTGAAGAG-3'; exon 5 forward: 5'-TGCTGCCGTGTTCCAGTTGC-3', reverse: 5'-AGGCCTGGGGACCCTGGGCAACC-3'; exon 6 forward: 5'-CTCTGATTCCTCACTGATTGCTCT-3', reverse 5'-GGAGGGCCACTGACAACCA-3'; exon 7 forward: 5'-CTTGCCACAGGTCTCCCCAA-3', reverse: 5'-AGGGGTCAGCGGCAAGCAGA-3'; exon 8 forward: 5'-TGCCTCTTGCTTCTCTTTTC-3', reverse: 5'-TCCTCCACCGCTTCTTGTC-3'; exon 9 forward: 5'-AGACCAAGGGTGCAGTTATG-3', reverse: 5'-CGGCATTTTGAGTGTTAGA-3'; exon 10 forward: 5'-GTTGCTTTTGATCCGTCA-3', reverse: 5'-GAATCCTATGGCTTTCCAAC-3'; exon 11 forward:

5'-GGGCACAGACCCTCTCAC-3', reverse: 5'-GTGGGAGGCTGTCAGTGG-3'. At least 10 ng of DNA and 10 pmol of primer was used for each reaction and an annealing temperature between 55 and 60° was used. Other conditions were identical to the PCR amplification described above.

Sequence Analysis

Following purification of PCR products with the QIA quick® PCR purification kit (Qiagen, Leusden, The Netherlands), the sequences were analyzed using the ready reaction Big Dye™ Terminator Cycle Sequence kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3100 automated sequencer (Applied Biosystems).

Loss of Heterozygosity

LOH analysis was carried out using ten polymorphic microsatellite repeat markers. The sequences of the primers and their corresponding location were selected through the Genomic Data Base (http://www.gdb.org) (Table 1). The primers used for the p53ALU marker were 5'-GAATCCGGGAGGAGGTTG-3' (forward) and 5'-TTCTGCTCCTTTTTTGGCTG-3' (reverse) and those used for the *TP53* marker were 5'-AGGGATACTATTCAGCCCGAGGTG-3' (forward) and 5'- ACTGCCACTCCTTGCCCCATTC-3' (reverse). PCR amplification was performed using 35 cycles in a 20 µl reaction volume containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1.5 unit of Platinum® Taq DNA polymerase in buffer supplied by the manufacturer, and 0.5mM of each primer, one of which was labeled with a fluorescent marker. Annealing temperatures ranging from 50ºC to 64ºC were used. A minimum of 10 ng of DNA was used for each PCR reaction to prevent induced losses. LOH analysis was carried out using an automated ABI 3100 sequencer with a Genescan™ 350ROX size standard (Applied Biosystems) and the manufacturer's Genescan® 2.1 software. The ratio of peak heights of the two alleles for tumor sample and normal was calculated and subsequently the normal ratio was divided by the tumor ratio. A value below 0.6 or above 1.6 was interpreted as evidence of LOH whereas values between these figures were considered retention of heterozygosity. All losses were confirmed by at least one repeat reaction.

Results

P53 Abnormalities

Due to their frequent and early occurrence in gastro-esophageal tumors, *TP53* mutations provide a powerful molecular tool for assessment of clonality. Thus direct *TP53* sequence analysis and immunohistochemistry for p53 was carried out for all cases (A-E). The results are presented in Table 2. All cases but one (case C) had a *TP53* mutation.

Both tumor components of the two presumed collision tumor cases (A and B) shared the same *TP53* mutation. The group of composite tumors as diagnosed on histology consisted of cases C, D, and E. In case C a known polymorphism was found in both tumor components. Case D however had a *TP53* mutation in the adenocarcinoma component only, with no mutation found in the squamous component. In case E the same *TP53* mutation was found in both tumor components.

Table 2 Results of TP53 sequence analysis and immunohistochemistry

Case	Histology	Mutation Ad	P53 IHC	Mutation SC	P53 IHC	Position	Amino Acid Change
A	collision tumor	G to A	positive	G to A	positive	14466 exon 8, codon 266	Gly to Glu
B	collision tumor	G to A	positive	G to A	positive	14487 exon 8, codon 273	Arg to Hist
C	composite tumor	polymorphism	negative	polymorphism	negative	12139 exon 4, codon 72	Arg to Pro
D	composite tumor	C to G	positive	none	negative	14502 exon 8, codon 278	Pro to Arg
E	composite tumor	C to T	positive	C to T	positive	14752 exon 9, codon 331	Gln to stop codon

Table 3 Results of Loss of Heterozygosity Analysis

Microsatellite Marker	Case A		Case B		Case C		Case D		Case E	
	SC	AC	SC	AC	SC	AC	SC	AC	SC	AC
D3S1478	LL	R	LL	LL	R	R	R	R	R	R
D5S346	LS	LS	LS	LS	LL	LL	LL	R	LS	LS
D9S171	R	R	R	R	R	R	LS	R	R	R
D10S2491	LS	LL	LL	LL	R	R	R	LS	R	R
D14S68	R	R	R	R	R	R	LL	LL	R	R
D16S2624	R	R	N.I.	N.I.	R	R	LL	R	R	R
D18S64	R	LS	LL	LL	LL	LL	N.I.	N.I.	LS	LS
TP53	LS	LS	LL	LL	LL	LL	LS	LS	LS	LS
P53Alu	N.I.	N.I.	N.I.	N.I.	N.I.	N.I.	LL	LL	LS	LS
Bat26	No MSI	No MSI	No MSI	No MSI	No MSI	No MSI	No MSI	No MSI	No MSI	No MSI

LS – Loss of small Allele; LL – Loss of large Allele; AC – Adenocarcinoma; SC – Squamous Cell Carcinoma

Immunohistochemistry for p53 corresponded with the molecular genetic findings and showed overexpression in both tumor components in cases A, B, and E. Case C was negative for both components and Case D showed positivity in the adenocarcinoma component only (figure 2).

Detection of Loss of Heterozygosity

Further evidence regarding the clonality of these tumors was obtained through LOH analysis of each component using a panel of ten polymorphic markers (Table 1), at sites near known or suspected tumor suppressor genes considered potentially important in gastro-esophageal carcinogenesis. The results are shown in Table 3.

Both neoplastic components of the two presumed collision tumor cases (A and B) had a similar pattern of loss and retention. In Case A the results were the same for five loci and three markers revealed a difference between the neoplastic components. The results for Case B were identical in both components for all the markers studied. In all cases where LOH for both components was observed, this involved the same allele. One exception was at 10q23 in case A, where loss of the large allele was found in the adenocarcinoma component and a loss of the small allele in the squamous component.

The results for the group of three presumed composite tumors revealed an identical pattern of loss and retention for cases C and E. Case D however showed a different pattern of retention and loss at four of the eight informative loci.

Discussion

The pathogenesis of human cancers is thought to be the result of a stepwise accumulation of genetic alterations involving oncogenes and tumor suppressor genes. A tumor can be considered clonal with respect to crucial early genetic events[7,15] whereas it is often heterogeneous for genetic changes occurring late in tumor progression. Molecular genetic analysis provides stronger evidence for a multi- or monoclonal origin than phenotypic appearance, as important genetic alterations will be retained during carcinogenesis whereas phenotypic features may change. If two tumors arise

independently and are associated by coincidence only, the genetic alterations present are expected to be different from each other because of the different tumor origins.

Mutational analysis of a molecule critical to carcinogenesis is central in providing clues regarding clonality.[2,6] In gastro-esophageal tumors, the prevalence, early occurrence and wide variety of TP53 mutations distributed throughout the gene, make sequence analysis of *TP53* a useful tool for differentiating between biclonal and monoclonal origins. Of note, even in the situation of field cancerization of the esophagus, where G to T transitions of *TP53* can occur throughout the esophagus due to environmental factors such as tobacco, the resulting mutations are not specific but are dispersed throughout numerous codons.[13] Thus in field cancerization a wide range of different *TP53* mutations occurring at different times are present within the affected field which give rise to the second primary tumors characteristic of field cancerization. This is in contrast to the role of aflotoxin in hepatocellular carcinoma where it induces a specific *TP53* mutation at codon 249. Aflotoxin is one of the only known carcinogens to induce a specific *TP53* mutation in human cancer, with no such specific mutations described in the esophagus.[13] It is also possible to follow the clonal evolution of cancer with markers that identify LOH.[4,10] In this study, a molecular genetic approach using *TP53* sequence analysis with supporting LOH analysis was applied to investigate five gastro-esophageal junction tumors, each of which consisted of two histologically separate components. Two of these tumors (A and B) had the histological appearance of collision tumors and the other three cases (C, D and E) were deemed composite tumors on histology.

Surprisingly, the molecular data provided here showed no evidence that case A and B were collision tumors, despite their characteristic histological appearance. In both cases, the tumor components had an identical *TP53* mutation and so the likelihood that they are collision tumors is virtually zero. This conclusion is supported by the concordant LOH at multiple loci, again providing evidence that the two neoplastic components initially arose from a common precursor.

The results of the group of presumed composite tumors also revealed interesting results. Cases C and E had an identical pattern of loss and retention on LOH analysis thus confirming their composite nature. In case D however, a *TP53* mutation was found in the adenocarcinoma component only, with no *TP53* mutation found in the squamous component. The LOH findings of the two components were also disparate with a different pattern of results at four of the eight markers. These data provide evidence suggesting that this case may be a true collision tumor. However, it can be argued that this *TP53* mutation could have occurred as a late event and that the two tumor components may share early genetic events and thus have the same clonal origin. The difficulty of distinguishing a clonal origin with subsequent sub-clone formation from genetically unrelated tumors with widespread instability is highlighted in this instance.

The results show that despite a striking difference in morphology and relative separation of tumor components, a collision tumor cannot be assumed on appearance alone. There is no definitive explanation for the unusual appearance of these neoplasms but it is likely that growth factors have a strong influence on morphology and that the different microenvironments of the esophagus and cardia exert different effects on the two tumor components. Heterogeneity within a tumor often occurs as a result of increasing genetic instability during tumor progression and the accumulation of different molecular and genetic aberrations may also contribute to a difference in morphology. An example of tumor heterogeneity can be seen in case A at loci 3p21 and 18q21.32 where the differences in LOH may represent a diversion in tumor progression and formation of sub-clones. At 10q23 in case A, the pattern of LOH was unusual in that the small allele was lost in one component and the large allele in the other component. It is possible that these findings do not actually represent a real physical loss of this marker but rather have occurred due to an

aberrant mitotic crossing over event resulting in two homozygous daughter cells, thus giving the appearance of a loss of heterozygosity. In many of the cases studied (B, C, E) the pattern of LOH was identical for both components. However, genetic differences accounting for the appearance of these components may exist at loci other than those examined here.

To our knowledge, this is the first study seeking molecular genetic evidence for the histological diagnosis of collision tumors through both mutation analysis and LOH analysis. The literature regarding collision tumors consists mainly of case reports, most of which are based on histological appearance and immunohistochemistry. Given the results of cases A and B in this study, many of the "collision tumors" reported in the literature may represent clonal neoplasms with two divergent phenotypes. In fact in examples where genetic analysis has been performed, such as in carcinosarcomas of the uterus, most cases have been found to be clonal, thus representing carcinomas with sarcomatous areas, i.e. composite tumors.[8,19,26] Similar findings have been found for other putative collision tumors: gynecological tumors,[1,16] tumors in the pancreas,[27] and some tumors with glandular and neuroendocrine components.[9] This suggests that collisions tumors have been widely over-diagnosed in the past.

Despite this possible over-diagnosis of collision tumors there are reports where genetic techniques have been used to show genetic differences in collision tumors[14,29,10] demonstrating that collision tumors do indeed occur in various parts of the body. In the case of female patients (this study consists of males only) a clonality X-inactivation analysis based on methylation pattern is another useful method to demonstrate clonality.[28]

Although histological examination is the cornerstone of our practice of pathology, the molecular-genetic era has provided the tools to resolve issues that until now have been theoretical debates. From this study we can surmise that it is not possible to recognize true collision tumors from histological appearance alone, and that the criteria for the diagnosis of these neoplasms does not always hold true. Indeed the occurrence of a collision tumor is probably far rarer than what is reflected in the literature. The finding of a genetic basis for clonality in the cases of presumed collision tumors and the finding of a possible collision tumor among presumed composite tumors challenges our methods of diagnosis of these tumors and suggests that the longstanding histological criteria for the diagnosis of these neoplasms have no molecular basis.

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Inflammatory myofibroblastic tumor with ALK/TPM3 fusion presenting as ileocolic intussusception: an unusual presentation of an unusual neoplasm.

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Abstract

Inflammatory myofibroblastic tumor is a rare spindle cell lesion of indeterminate malignant potential occurring in both pulmonary and extrapulmonary tissues. This report describes an unusual presentation of an unusual tumor at an unusual location: an intramural ileal case of inflammatory myofibroblastic tumor presenting with intussusception in a twenty-nine year old woman. We characterize this tumor through microscopic and ultrastructural analysis, extensive immunohistochemical analysis, ploidy analysis, EBV in-situ hybridization, and we report the finding of an *ALK/TPM3* fusion using FISH.

Introduction

First described in the lung, IMT represents a clinicopathological distinctive group of tumors, which affects predominantly children and young adults. It can however occur at any age and has been reported in a wide variety of extrapulmonary body sites [1].

Originally considered to represent an aberrant inflammatory response to tissue injury with the myofibroblast as the primary cell type and an associated mixed inflammatory cell infiltrate, IMT is now classified as a neoplasm on the basis of its potential for local recurrence, infiltrative growth, vascular invasion and malignant transformation. Sarcomatous transformation of IMT, reports of aneuploidy in addition to demonstration of clonal characteristics and rearrangements of chromosome 2p23 and the *ALK-1* gene [2] provide further support for the concept that IMT and inflammatory fibrosarcoma are neoplastic processes and part of a continuously expanding spectrum of benign and malignant myofibroblastic proliferations.

IMT seldom presents in the ileum and intussusception is a rare complication of IMT [3], thus we document an unusual presentation of IMT in an unusual site: an intramural ileal case of IMT presenting with intussusception in a twenty-nine year old woman. Microscopic, immunohistochemical and ultrastructural findings as well as EBV and ploidy status of this case are outlined together with our findings of an *ALK* gene rearrangement and are discussed in relation to current knowledge of IMT.

Methods

The tissue specimens were routinely fixed in 10% buffered formalin and then were embedded in paraffin. Consecutive 3-μm-thick sections were cut from a selected tissue block and stained with haematoxylin and eosin.

Immunohistochemistry: Antigen retrieval was carried out on deparaffinized sections by 15 minutes of boiling in Trilogy™, Declere® reagent (Cell Marque, Hot Springs, AR) or DakoCytomation TRS pH 6 (Glostrup, Denmark), except for the SMA antibody where no antigen retrieval was carried out. Immunostaining was performed using Optimax® Plus 2.0 Immunostainer (BioGenex Corporation, San Ramon, CA). The following primary antibodies were used: Vimentin (Dako, 1: 20,000), SMA (Sigma, 1:2,000), CD68 (Dako, 1:400), Desmin (Dako, 1:100), AE 1+3 (Biogenex, 1:50), Cam 5.2 (Becton Dickenson, 1:100), S100 (Dako, 1:2000), CD31 (Dako, 1:200), CD34 (Cellmark Diagnostics, Abingdon, UK, 1:100), Neurofilament (Dako, 1:1000), CD117 (Dako,, 1:400), Alk-1 (Dako, 1:25), Kappa (Dako, 1:8,000), Lambda (Dako, 1:8,000), P53 (Dako, 1:20). Envision™/HRP (Dako High, Wycombe, Bucks, UK) was used to visualize antibody binding sites with 3,3'-diaminobenzidine as a chromogen. Sections were counterstained with haematoxylin.

Ploidy Analysis: Five 50 um sections were deparaffinized in xylene, rehydrated, minced using the Dako Medimachine and digested for 30 minutes at 37°C with 0.5% pepsin. Cells were washed twice with PBS and filtered through nylon mesh. Yield was assessed on stained cells cytocentrifuged onto sialinised slides. Subsequent washing was followed by incubation for 30 minutes at 37° C with 500µl DNA staining solution (0.1% DNase free RNase (Sigma), 10µM propidium iodide (PI), (Calbiochem, San Diego, CA) in PBS). A FACScaliber flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) was used to determine ploidy status.

EBV Detection: Epstein-Barr Virus (Lytic) PNA Probe/Floresein (Dako) was used according to manufacturer's instruction and subsequently visualized using DakoCytomation PNA ISH Detection Kit.

FISH Analysis: Dual-color interphase FISH was performed on 5 μm paraffin sections of formalin fixed tumor specimen using LSI®ALK Dual Color Rearrangement Probe (Vysis, Inc., Downer´s Grove, IL), containing two labelled DNAs that mapped to either 3´ telomeric (SpectrumOrange) or 5´centromeric (SpectrumGreen) *ALK*. Additional FISH experiments were carried out using the digoxigenin-labeled *ALK* P1 [PAC RP11-1111H1 (specific for 3'end of *ALK* catalytic domain)], and biotin-labeled BAC RP11-205M9 (that contains *TPM3*/1q25 gene) and BAC RP11-121I1 (that contains *TPM4*/19p13.12 gene) DNAs. FISH was performed according to Zymed protocol and data were obtained on a Leica DMRB (Leica, Wetzlar, Germany) fluorescence microscope equipped with a cooled black and white charged couple device camera (Photometrics, Tuscon, AZ), run by Quips SmartCapture™ FISH Imaging Software (Vysis, Bergisch-Gladbach, Germany).

Case Report

A 29 year old woman presented with a six week history of crampy abdominal pain which became constant and acutely severe over an 18 hour period. The pain radiated to her back and was associated with alternating constipation and diarrhea, vomiting and weight loss. She remained apyrexial throughout the course of her illness. On examination she had a palpable, mildly tender mass in her right iliac fossa, and faeces were positive for occult blood. Laboratory investigations revealed anemia, raised CRP and a normal white cell count.

Using colonoscopy a mass in the proximal ascending colon was seen and computerized tomography (Fig 1) together with magnetic resonance imaging demonstrated evidence of ileocolic intussusception with proximal small bowel obstruction. At laparotomy a polypoid mass was palpated at the lead point of the intussusception and she underwent a right hemicolectomy.

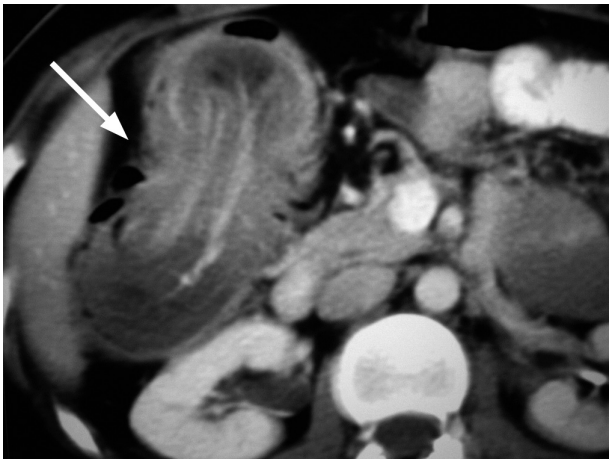

Figure 1 Computerized Tomography (CT) findings showing a typical sausage shaped mass (arrow) at the site of intussusception.

Following surgery, recovery was complete and the patient was relieved of her ongoing abdominal pain.

The resected specimen consisted of ileum and caecum with a 20 cm length of ileum intussuscepted into the caecum. A 2.5cm firm, yellow ill-defined mass was identified within the wall of the ileum at the lead point of the intussusception.

Microscopy revealed a tumor infiltrating the muscularis propria and extending into the submucosa, composed of spindle cells with large round/oval nuclei, prominent nucleoli and plentiful cytoplasm, admixed with an inflammatory infiltrate of numerous lymphocytes, histiocytes, plasma cells and eosinophils (Fig 2a). Many large "ganglion like" cells were also present (Fig 2b). The tumor was confined to the intestinal wall and there was no evidence of serosal involvement.

Immunohistochemistry was positive in tumor cells for vimentin, actin (SMA) and CD68, and was negative for desmin, cytokeratin, S100, CD31, CD34, neurofilament protein and CD117. Staining with Alk-1 (Anaplastic Lymphoma Kinase) revealed diffuse smooth cytoplasmic staining (Fig 2c). Immunohistochemistry for kappa and lambda light chains revealed a polyclonal cell population. Moderate p53 staining was present in a minority of tumor cells and in-situ hybridization with Epstein Barr virus was negative. Ploidy analysis by flow cytometry demonstrated a diploid pattern with no evidence of aneuploidy.

Ultrastructural examination revealed cells with well developed junctions, subplasmalemmal densities and rich in rough endoplasmic reticulum with well developed golgi apparata. Two populations of intracytoplasmic filaments were present: intermediate filaments (vimentin) and actin filaments with small fusiform densities. Some cells with prominent rough ER and circumferential arrays of actin filaments were also present

Cytogenetic analysis of this tumor using fluorescent in-situ hybridization revealed rearrangements of the *ALK* gene and characterized the fusion partner of this rearrangement. Using dual-color Split-Apart LSI®ALK probe, two juxtaposed (red/green) signals were seen in 15% of tumor cells, whereas the remaining 85% of tumor cells were abnormal, showing both split apart and fused red and green signals indicating an *ALK* rearrangement (Fig. 3a). The translocation of the ALK catalytic domain in a subset of cells was confirmed by FISH analysis with the ALKP1 probe specific for *ALK* 3′ end. Co-hybridization of the ALKP1 probe with a probe containing the *TPM3*/1q25 gene, yielded fused red and green signals in 73% of cells (Fig. 3b), whereas co-hybridization of the ALKP1 probe and a probe containing the *TPM4*/19p13.12 gene showed two

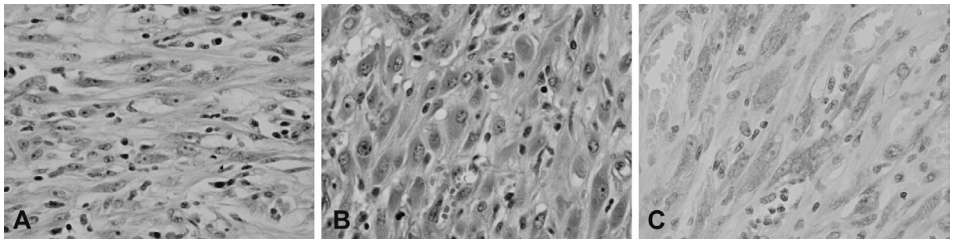

Figure 2 (A) Haemoatoxylin and eosin staining (x250) showing a tumor composed of spindle cells with large round/oval nuclei, prominent nucleoli and plentiful cytoplasm, admixed with an inflammatory infiltrate of numerous lymphocytes, histiocytes, plasma cells and eosinophils (B) Haemoatoxylin and eosin staining (x250) showing ganglion-like cells in the tumor. (C) Immunohistochemistry (x250) for Alk-1 showing diffuse smooth cytoplasmic staining. ***(See page 203 for colour figure)***

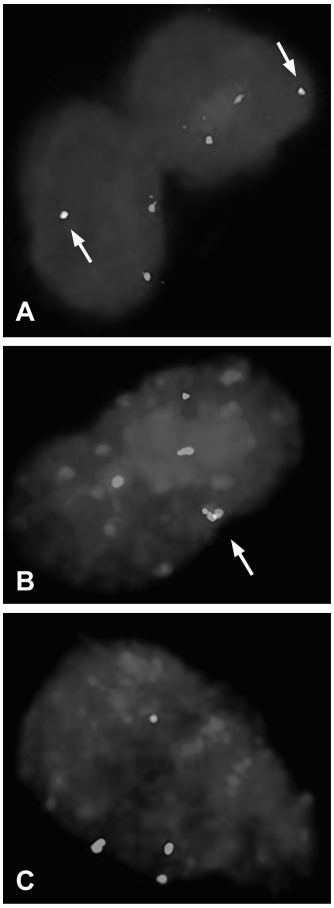

Figure 3 Dual-colour FISH on interphase nuclei from tumor specimen. Using Split-Apart LSI®ALK probe, one overlapping red/green and split apart single red and green signals were detected (arrows), indicating ALK rearrangement (A). Co-hybridization of digoxigenin labelled ALKP1 and biotin labelled RP11-205M9 (that contains TPM3/1q25 gene) probes revealed a single red, a double green and an overlapping red/green signal (arrow), indicating the presence of ALK-TPM3 fusion (B). Co-hybridization of digoxigenin labelled ALKP1 and biotin labelled BAC RP11-121I1 (that contains TPM4/19p13.12 gene) probes showed two normal green and red signals (C). ***(See page 204 for colour figure)***

separate red and green signals (Fig. 3c). These results confirmed the rearrangement of *ALK* gene and the presence of *ALK-TPM3* fusion in tumor cells.

Discussion

The morphology, immunophenotype and ultrastructure of this case established a diagnosis of inflammatory myofibroblastic tumour (IMT) with a myxoid/vascular pattern [4]. The presentation

of this unusual neoplasm differed from the classical presentation of fever, weight loss, microcytic anaemia, thrombocytosis, hyperglobulinaemia and a raised erythrocyte sedimentation rate.

Various predictors of biological behavior have been proposed including round cell transformation, a predominance of "ganglion-like cells," nuclear pleomorphism, aneuploidy and p53 expression [5]. The tendency for local recurrence appears to be related to the initial site of the tumor, and occurs more commonly in abdominal and sinonasal tumors [1]. Surgical resection is the treatment of choice [4] and both chemotherapy and radiotherapy have not proven effective to date [6].

The etiology of IMT is unclear. Human Herpes Virus-8 as well as overexpression of IL-6 and cyclin D1 in the myofibroblasts, has been implicated in the tumourigenesis of IMT [7]. Other infections postulated to induce IMT include mycobacterium avium intracellulare, corynebacterium equi, campylobacter jejuni, bacillus sphaericus, coxiella burnetti, echerichia coli [8] and Epstein-Barr [9] virus although none are conclusive.

In recent years, the genetic mechanisms underlying IMT pathogenesis are being unraveled. Cytogenetic analyses have disclosed chromosomal translocations involving chromosome band 2p23, associated with constitutive activation of the anaplastic lymphoma kinase (*ALK*) gene (a member of the insulin receptor family of receptor tyrosine kinases normally expressed in the nervous system) in approximately half of the examined cases [2] and resulting in aberrant expression of ALK protein. Three patterns of immunohistochemical staining in for ALK-1 are seen in IMT depending on the fusion partner [10],[11],[12]. At present, a number of *ALK* fusion partners have been described in IMTs, including *TMP3*/1p23-*ALK*, *TMP4*/19p13.1-*ALK*, *CLTC*/17q23-*ALK*, *CARS*/11p15-*ALK*, *RANBP2*/2q13-*ALK* [10],[11],[12],[13] and *ALK-ATIC* [14]. As *TPM3* or *TPM4* involvement is the most frequent fusion partners, we screened this case for the possible fusion of ALK with these genes. The hybridization pattern proved that TPM3-ALK fusion is present and the diffuse smooth cytoplasmic staining on immunohistochemistry is consist with this finding [10],[11],[12]. Clonal abnormalities of *ALK* are also typically associated with anaplastic large cell lymphomas (ALCLs), which present a characteristic translocation t(2;5)(p23;q35) or variant translocations involving the *ALK* gene in about 60% of cases [12],[15] and this translocation is the first example of an identical receptor tyrosine kinase fusion found in both a lymphoid and mesenchymal tumour. However, the preferred *ALK* fusion partners differ in ALCLs and IMTs such that *NPM-ALK* fusions, found in 80% of ALCLs, have not yet been identified in IMTs, and *TMP3-ALK* fusions are rare in ALCL.

The relatively high frequency of ALK staining in IMT and the demonstration of *ALK* rearrangements suggests that ALK dysregulation is an important mechanism of tumorigenesis, similar to ALCL and these molecular findings further support the neoplastic nature of at least a subset of inflammatory myofibroblastic tumors. ALK positivity is more frequent in younger patients whereas tumors in patients over forty years of age are usually negative. In ALCL, ALK positivity is associated with a better prognosis, but the prognostic significance of ALK involvement in IMT is unclear and whether ALK positive and ALK negative IMTs represent distinct clinicopathological entities with different therapeutic and prognostic implications, is still unknown.

This is a neoplasm of uncertain malignant potential and clear communication to the clinician of the lesion's potentially aggressive behavior and the need for long-term follow up are vital. Early recognition of possible signs of intussusception, which may also be revealed from the clinical history, may prevent a delay in diagnosis. Complete surgical resection is needed for prompt treatment, to avoid recurrences and prevent unnecessary and potentially harmful chemotherapy or radiotherapy. Close follow-up should be carried out to recognize the recurrences earlier and the

optimal management of locally aggressive and recurrent disease should be decided individually for each patient. The designation of tumors with overlapping morphological features and unpredictable clinical behavior remains problematic but with an increase in knowledge regarding the molecular findings in the pathogenesis of these lesions, such as the importance of *ALK* rearrangements and the relevance of the fusion partners, we can hope to aid the clinical diagnosis and predict prognosis.

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Summary and Discussion

Gastric cancer is the fourth most common malignancy in the world and ranks second in terms of cancer-related death.[1] Gastric cancer is thought to result from a combination of environmental factors and the accumulation of specific genetic alterations due to increasing genetic instability, and consequently affects mainly older patients, often after a long period of atrophic gastritis. The incidence of adenocarcinoma of the stomach is declining worldwide and this is mainly accounted for by the decline in the intestinal type. There has also been a change in the anatomical distribution of this malignancy over recent decades, with a fall in the incidence of mid and distal gastric cancer and a progressive increase in adenocarcinoma of the proximal stomach and cardia.

Tumorigenesis is considered a multistep process involving generalized and specific genetic alterations that drive the progressive transformation of cells into cancer. Central to this transformation are genetic or epigenetic changes in the genome which specifically activate oncogenes with a dominant gain of function, and produce alterations in tumor suppressor genes which cause loss of function. There is however by no means a clear-cut pattern of mutations in gastric cancers, and the genetic research can often be hampered by the diversity of changes that are induced by *Helicobacter pylori* infection, diet, ageing and other environmental factors. Tumors are unquestionably riddled with genetic changes yet we are faced with an unsolvable puzzle with respect to a temporal relationship. In order to solve this problem, one approach is to investigate tumors that are less influenced by these environmental factors. Gastric cancers occurring in young patients, known as early-onset gastric cancers, the main subject of this thesis, provide an ideal background on which to try and uncover the initiating stages in gastric carcinogenesis, as the role of genetics is presumably greater than in older patients, with less of an impact from environmental carcinogens. Ultimately they may provide vital information about molecular genetic pathways in sporadic cancers and may aid in the unraveling of gastric carcinogenesis as discussed in **chapter 2**.

Gastric Cancer is rare below the age of 30; thereafter it increases rapidly and steadily to reach the highest rates in the oldest age groups, both in males and females. The intestinal type rises faster with age than the diffuse type and is more frequent in males than in females. Early onset gastric cancer (EOGC) is defined as gastric cancer presenting at the age of 45 or younger. Approximately 10% of gastric cancer patients fall into the EOGC category, although rates vary between 2.7%[2] and 15%[3] depending on the population studied. Young patients more frequently develop diffuse lesions which often arise on the background of histologically "normal" gastric mucosa. It is postulated that genetic factors may be more important in EOGC than in older patients as they have less exposure to environmental carcinogens[4]. *Helicobacter pylori* may still play a role in the development of gastric cancer in young patients, [5, 6] although this is likely to involve a much smaller percentage of patients than in the older age group.

Approximately 10% of young gastric cancer patients have a positive family history,[7] some of which are accounted for by inherited gastric cancer predisposition syndromes, where the underlying genetic events are not always known but can involve *CDH1* germline mutations. The 90% without a family history emphasizes that the occurrence of gastric cancer in young patients remains largely unexplained.

The clinicopathological features of gastric carcinoma are said to differ between the young and elderly patients and it has been claimed that young patients have a poorer prognosis.[8] Others report that tumor staging and prognosis for young patients is similar to older patients and depends on whether the patients undergo a curative resection.[3, 7, 9] Young patients with gastric cancer in the United States are more likely to be black, Asian or Hispanic.[10] Relative to older patients, young patients have a female preponderance, a more frequent occurrence of diffuse cancer and less intestinal metaplasia.[7, 10, 11] This predominance of females is considered by some to be due to

hormonal factors.[12] Cancers in young patients are more often multifocal than in older patients [13] as is also seen in hereditary diffuse gastric cancer.[14]

Thus early onset gastric cancers are known to have a different clinicopathological profile than conventional gastric carcinomas. This suggests that they represent a separate entity within gastric carcinogenesis and indeed the evidence at a molecular genetic level provided in this thesis supports this. It is known that microsatellite instability which usually occurs at a frequency of 15% in older gastric carcinomas is consistently absent in young patients as found in **chapter 3** and described elsewhere in the literature, and this is despite analysis of distal tumors (where MSI is usually commoner) and inclusion of mixed and intestinal type tumors (diffuse tumors generally have less MSI).[15] However, it may be possible that geographical factors play a role.[16] A lack of microsatellite instability excludes the mutator phenotype as an important predisposing factor in the development of early-onset gastric cancer. This contrasts with the situation in colorectal cancer where 58% of patients without HNPCC aged under 35 years showed evidence of microsatellite instability.[17] Interestingly, the LOH findings in chapter 3 also show that losses are infrequent in EOGC. [18] In **chapter 4**, we look more closely at the loss of *RUNX3* that was found in chapter 3, and here we exclude it from having a tumor suppressor function in EOGC,[19] although as some of the cell lines used in this study were from conventional gastric cancers, the implications may be more far-reaching and include conventional gastric cancer.

In **chapter 5** we found that the molecular expression profile of EOGC and conventional gastric cancers differ with EOGCs having a COX-2 Low, TFF-1 expressing phenotype.[20] Here we also find that aberrant expression of E-Cadherin correlated significantly with diffuse type [20] thus refuting the finding that a higher incidence of aberrant E-Cadherin expression occurs in EOGC regardless of histological type [11].

In order to ascertain a mechanism for the lack of COX-2 overexpression in EOGC, as found in chapter 5, we examine the -765 G/C promoter polymorphism in COX-2 in **chapter 6**. Interestingly we find that this polymorphism is significantly associated with gastric cancer, although its prevalence did not vary between the gastric cancer groups and did not correlate with COX-2 protein expression. An explanation for the abberant COX-2 regulation in EOGC is still of great interest.

The expression of low molecular weight isoforms of cyclin E are also known to differ between EOGC and conventional cancers, being present in 35% of EOGCs, compared to in 8% of conventional gastric cancers and 4% of stump cancers, as found in **chapter 7**. In addition, we also found in this chapter that immunohistochemical staining of low molecular weight isoforms of cyclin E were found to be an independent positive prognostic indicator in early-onset gastric cancer.

In **chapter 8**, we look into the *cdc4/FBXW7* gene, a ubiquitin ligase specific to cyclin E that has been implicated as a tumor suppressor gene in many tumor types. In this chapter we find frequent LOH at the cdc4 locus and allelic loss was found to correlate with lack of protein expression in 26 % of early-onset gastric cancer. In addition, it appears that cdc4 regulation of c-myc occurs in gastric cancers although functional studies of hcdc4 and its substrates in primary tumors require further investigation.

In **chapter 9**, using representational difference analysis to characterize genetic changes particular to diffuse gastric cancer, a novel amplification at 11p 12-13 was found and was associated with overexpression of CD44v6 especially in EOGC when compared to conventional gastric cancer [21] lending further support to the hypothesis that young patients develop carcinomas with a different molecular genetic profile from that of sporadic carcinomas occurring at a later age.

In **chapter 10**,we report the molecular characterization of eight primary gastric carcinomas, corresponding xenografts, and two novel gastric carcinoma cell lines using MS-MLPA, IHC for 14 markers as well as *TP53* and *CDH1* mutation analysis. We find novel gains of *CASR, DAPK1* and *KLK3* as well as losses and hypermethylation of *MLH1, CHFR, RASSF1* and *ESR* in primary tumors. Of particular interest was that loss of CHFR expression correlated significantly with the diffuse phenotype. Xenografts and gastric cancer cell lines remain an invaluable research tool in the uncovering of the multistep progression of cancer.

In **chapter 11** we step aside to look at the phenomenon of collision tumors of the gastro-esophageal junction from the molecular genetic prospective and we find that actually what histologically appears as a collision tumor may not be so when analysed using LOH and *p53* mutation analysis. [22] This result challenges our expectations of pathology and highlights the need for incorporation of molecular studies into our diagnostic practice. In **chapter 12** we take a step in the other direction, towards the small intestine and we describe an inflammatory myofibroblastic tumor with an *ALK/TPM3* fusion,[23] a rare tumor which is also found in young people and children.

In summary, observations of human cancers and animal models implicate numerous genetic changes in gastric cancer. However, the multistep pathway of carcinogenesis which occurs in some epithelial cancers and which has allowed accurate clinical and pathologic characterization is not yet elucidated in gastric cancer. Gastric cancer exhibits heterogeneity in histopathology and molecular changes that have impeded the uncovering of a temporal molecular pathway. Gastric cancers often occur without any consistent mutational abnormality and with a considerable variation in pathogenesis ranging from a stepwise progression of changes (gastritis -> metaplasia ->dysplasia -> invasive carcinoma) to tumors arising in the absence of a precursor lesion. As we can see from the evidence presented in this thesis, EOGCs differ from conventional gastric cancers, not only at a clinicopathological level, but also at a molecular genetic level. If this is indeed due to the fact that the environment plays a smaller role in the triggering of the carcinogenic pathway, the investigation of this group of cancers may reveal genetic changes which assist in the task of proposing a multistep pathway for gastric cancer. Further study of hereditary gastric cancers and early onset gastric cancer as unique subsets of gastric cancer may aid us in the search for a gastric cancer pathway. The rarity of hereditary gastric cancer often hampers research in this field. On the other hand, early-onset gastric cancers, although relatively scarce, provide an ample number of cancers if they can be collected at a nationwide level. Recent developments of techniques adapted to paraffin material will maximize the number of cancers available for research and the use of new technologies, combined with EOGC material may set us well on the road to unraveling gastric carcinogenesis.

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14

Nederlandse Samenvatting

Maagkanker is de vierde meest voorkomende kankersoort ter wereld en nummer twee wat betreft kanker-gerelateerde sterfte.[1] Maagkanker wordt veroorzaakt door een combinatie van omgevingsfactoren en een accumulatie van gegeneraliseerde en specifieke genetische afwijkingen. Het komt meestal bij oudere patiënten voor, vaak na een lange periode van atrophische gastritis. Wereldwijd nam het aantal gevallen van maagkanker de afgelopen decennia af, voornamelijk door een vermindering van het aantal gevallen van intestinaal type maagcarcinoom. De locatie van de tumoren is ook veranderd, met minder maagkanker in het midden en het distale deel van de maag en een toename van adenocarcinomen in de proximale maag en cardia.

Tumorigenese wordt gezien als een proces bestaande uit meedere stappen van gegeneraliseerde en specifieke genetische veranderingen die de transformatie van een gewone cel tot een kanker cel bevorderen. Veranderingen die leiden tot de activatie van oncogenen of tot de inactivatie van een tumor suppressie gen spelen hierbij een centrale rol. Er bestaat echter geen duidelijke inzicht in welke mutaties in welke volgorde belangrijk zijn, en genetisch onderzoek naar het ontstaan van maag kanker wordt bemoeilijkt door de veelheid van veranderingen die geïnduceerd worden door zowel *H. Pylori*, als ook dieet, vergrijzing en andere omgevingsfactoren. Ondanks veel verschillende studies naar het ontstaan van maag kanker blijft ons begrip van de moleculaire achtergrond en vooral van de verantwoordelijke moleculaire signaleringsroutes beperkt. Een manier om hierin een beter inzicht te krijgen is om tumoren te onderzoeken die mogelijk minder belast zijn door omgevingsfactoren. In maagtumoren die optreden in patiënten jonger dan 45 jaar – de zogenaamde "early onset gastric cancers" (EOGCs), de groep patiënten waaraan het onderzoek in dit proefschrift gewijd is, – worden omgevingsfactoren van minder belang geacht in de carcinogenese dan in oudere patiënten (de groep "conventionele maagcarcinomen"). Om die reden lijkt de EOGC groep bij uitstek geschiktom de belangrijke genetische afwijkingen van maagtumoren in kaart te brengen. Misschien zal dit uiteindelijk belangrijke informatie opleveren over de moleculaire genetische route naar maag kanker en zal het bijdragen inzicht te krijgen in maag carcinogenese in het algemeen (**Hoofdstuk 2**).

Maag kanker is zeldzaam bij mensen jonger dan 30 jaar; daarna neemt het langzamerhand toe en in de oudste leeftijdscategorieën komt het het vaakste voor, bij zowel mannen als vrouwen. Maag kanker van het intestinale type komt vaker bij oudere mensen en vrouwen voor. Ongeveer 10% van maagtumoren behoort tot de EOGC groep, variërend van 2.7% [2] tot 15%, [3] afhankelijk van de bevolkingsgroep die wordt bestudeerd. In jongere patiënten worden vaker diffuse maagcarcinomen gevonden, vaak met een histologisch normale achtergrond van het overige maagslijmvlies. Er is gepostuleerd dat genetische factoren belangrijker zijn in EOGC dan in oudere patiënten omdat de jonge patiënten minder hebben bloot gestaan aan omgevingsfactoren.[4] *H. Pylori* kan nog steeds een rol spelen in het ontstaan van maag kanker in jonge patiënten,[5, 6] maar de bijdrage zal waarschijnlijk kleiner zijn dan in de conventionele groep van maagtumoren. Rond 10% van jonge patiënten met maag kanker heeft een positieve familie anamnese [7]. Een deel hiervan is ontstaan in de context van een erfelijk maagkanker predispositie syndroom. Sommige gevallen kunnen genetisch verklaard worden door het vinden van een *CDH1* kiembaan mutatie maar meestal zijn de onderliggende genetische afwijkingen onbekend. De 90 % van EOGC zonder familie anamnese laat zien hoe beperkt het inzicht is in het ontstaan van deze tumoren.

De klinische en pathologische kenmerken van maag kanker verschillen tussen oude en jonge patiënten en beweerd wordt dat jonge patiënten een slechtere prognose hebben.[8] Maar, andere publicaties laten zien dat stagering en prognose hetzelfde zijn bij jonge en oude patiënten en dat het afhangt van een al dan niet curatieve resectie.[3, 7, 9] In de Verenigde Staten hebben jonge patiënten met maag kanker vaker een Afrikaanse, Aziatische of Latijns-Amerikaanse

achtergrond.[10] In vergelijking met oudere patiënten, komen er meer vrouwen, meer diffuse type maagcarcinomen en minder intestinale metaplasie voor in de EOGC populatie.[7, 10, 11] Deze vrouwelijke meerderheid wordt volgens sommige mensen veroorzaakt door hormonale factoren.[12] Maagtumoren in jonge patiënten zijn vaker multifocaal dan in oudere patiënten,[13] zoals ook bij het erfelijke diffuse maagcarcinoom wordt gezien.[14]

Het is dus bekend dat EOGC een ander klinisch en pathologisch profiel heeft dan de conventionele maagtumoren. Dit suggereert dat ze een aparte entiteit vormen binnen de maag carcinogenese en het moleculaire bewijs in dit proefschrift ondersteunt deze theorie. Microsatelliet instabiliteit (MSI) wordt normaal in ongeveer 15 % van conventionele maagtumoren gevonden en is afwezig in jonge patiënten, zoals beschreven in **Hoofdstuk 3** en elders in de literatuur. Dit ondanks het feit dat zowel distale tumoren (waar MSI vaker wordt gezien) als ook intestinale en gemengde typen tumoren (diffuse tumoren hebben minder MSI) werden geanalyseerd. [15] Het is echter mogelijk dat geografische factoren een rol spelen.[16] Het ontbreken van MSI sluit het "mutator" fenotype uit als belangrijke oorzaak in de ontwikkeling van EOGC. Dit in tegenstelling tot in de dikke darm carcinogenese, waarbij 58% van de patiënten jonger dan 35 jaar, zonder HNPCC, MSI heeft.[17] Interessant is dat de LOH bevingen in Hoofdstuk 3 laten zien dat dit niet vaak voorkomt in EOGC. [18] In **Hoofdstuk 4**, kijken wij nauwkeurig naar het verlies van het *RUNX3* allel dat in hoofdstuk 3 gevonden was, en we sluiten uit dat het een rol als tumor suppressor gen heeft in EOGC.[19] Verder, gezien het feit dat sommige cellijnen in deze studie uit conventionele maagtumoren gemaakt waren, suggereren onze resultaten dat dit ook het geval is in conventionele maagtumoren.

In **Hoofdstuk 5** hebben wij aangetoond dat het profiel van moleculaire expressie in EOGC anders is dan in de conventionele maagtumoren en dat EOGCs een fenotype hebben waarin TFF-1 tot expressie komt in combinatie met een lage COX-2 expressie.[20] Wij laten hier ook zien dat afwijkende E-Cadherine expressie is gecorreleerd met het diffuse fenotype, [20] dit in tegenstelling tot het idee dat afwijkende E-Cadherine expressie vaker voor komt in jonge patiënten onafhankelijk van het histologische type. [11]

Om het mechanisme van de in hoofdstuk 5 genoemde lage COX-2 expressie in EOGC te onderzoeken, kijken wij in **Hoofstuk 6** naar het -765 G/C promoter polymorfisme van *COX-2*. Interessant is de bevinding dat dit polymorfisme geassocieerd is met maagtumoren. Er was echter geen verschil tussen de EOGC en conventionele carcinomen en er was geen correlatie met COX-2 eiwit expressie. Een verklaring voor de afwijkende COX-2 expressie in EOGC blijft een interessante vraag.

In **Hoofdstuk 7** is gebleken dat de expressie van low molecular weight isovormen van cycline E anders is in EOGC dan in conventionele maagtumoren, met aanwezigheid van isovormen in 35% van EOGC en in maar 8% van conventionele maagtumoren en 4 % van maag stomp tumoren. Wij vinden ook in dit hoofdstuk dat positieve immunokleuring van de low molecular weight isovormen van cycline E een onafhankelijke marker voor overleving in EOGC is.

In **Hoofdstuk 8**, kijken wij naar het *hcdc4/FBXW7* gen, een ubiquitine ligase die specifiek is voor cycline E en die wordt gezien als een tumor suppressor gen in meerdere tumor typen. In dit hoofdstuk vinden wij dat LOH van het *cdc4* locus vaak voorkomt en er was een correlatie was met eiwit expressie in EOGC. Mogelijk reguleert cdc4 c-myc in maag kanker, alhoewel verder functioneel onderzoek van hcdc4 en zijn substraten nodig is.

In **Hoofdstuk 9**, gebruiken wij "representational difference analysis" om de specifieke genetische veranderingen van diffuse maagtumoren te karakteriseren en wij vinden een tot nu toe onbekende amplificatie op 11p 12-13 geassocieerd met overexpressie van CD44v6 in met name EOGC [21].

Deze bevinding levert dus nog meer bewijs voor de hypothese dat jonge patiënten een carcinoom met andere moleculaire genetische kenmerken ontwikkelen dan die op latere leeftijd.

In **Hoofdstuk 10**, hebben wij acht primaire maagtumoren, hun corresponderende xenografts en twee nieuwe maag cellijnen gekarakteriseerd door middel van MS-MLPA, immunohistochemie van 14 markers en *TP53* en *CDH1* mutatie analyse. Wij vonden amplificatie van *CASR*, *DAPK1* and *KLK3* samen met verlies en hypermethylatie van *MLH1*, *CHFR*, *RASSF1* and *ESR* in primaire tumoren. Verlies van CHFR expressie correleerde met het diffuse fenotype. Xenografts en maagkanker cellijnen blijven van groot belang voor het onderzoek naar de stapsgewijze tumor progressie van maagkanker.

In **Hoofdstuk 11** kijken wij naar de slokdarm-maag overgang en we onderzoeken het fenomeen van collision tumoren vanuit een moleculair genetisch perspectief. Hier vonden wij dat wat histologisch gezien een collision tumor genoemd wordt, dit het wellicht niet is na moleculaire analyse met behulp van LOH en *p53* mutatie analyse. [22] Deze bevinding staat haaks op onze verwachting naar aanleiding van de pathologie en laat zien hoe belangrijk het implementeren van de moleculaire diagnostiek kan zijn. In **Hoofdstuk 12** kijken wij naar de dunne darm, waar wij een inflammatoire myofibroblastische tumor met een *ALK/TPM3* translokatie en fusie-eiwit beschrijven,[23] een zeldame tumor die ook wordt gevonden bij jonge patiënten en kinderen.

Samenvattend, uit onderzoek in humane carcinomen en diermodellen, blijkt dat er meerdere genetische afwijkingen in maagkanker voorkomen. Er is echter geen "multistep pathway" van carcinogenese bekend in maagkanker zoals voor andere epitheliale tumoren het geval is, waardoor nauwkeurige klinische en pathologishe correlaten evenmin bekend zijn. Maagtumoren zijn heterogeen wat histopathologische en moleculaire veranderingen betreft en dat bemoeilijkt het ontdekken van opeenvolgende stappen in een moleculaire signaleringsroute. Maag tumoren ontstaan vaak zonder enige consistente mutatie en met een grote verscheidenheid in de pathogenese, variërend van een stapsgewijze progressie van veranderingen (gastritis -> metaplasie ->dysplasie -> invasieve carcinoom) tot tumoren die ontstaan zonder precursor lesie. We kunnen uit de bevindingen in dit proefschrift concluderen dat EOGCs anders zijn dan de conventionele maagtumoren, niet alleen op klinisch-pathologisch niveau maar ook op moleculair niveau. Als dit inderdaad een gevolg is van een minder belangrijke rol van omgevingsfactoren dan zou onderzoek naar deze groep tumoren genetische afwijkingen kunnen ophelderen die belangrijk zijn voor het ontdekken van een "multistep pathway" voor maag kanker. Verder onderzoek naar erfelijke maag tumoren en EOGCs als uniek onderdeel van de maag tumoren kan helpen bij het vinden van een maag kanker signalerings route. Het nadeel van het onderzoeken van erfelijke maagtumoren is dat ze zeldzaam zijn. Het verzamelen van EOGCs aan de andere kant is, hoewel ze niet vaak voorkomen, wel realistisch als ze op een landelijk niveau verzameld worden. Recente ontwikkelingen van technieken die aangepast zijn voor het gebruik van paraffine materiaal zorgen ervoor dat meer tumoren beschikbaar kunnen komen voor onderzoek. Met deze nieuwe technologieën, gecombineerd met het gebruik van EOGC weefsel kan een goede stap vooruit gezet worden in het ontrafelen van het maag carcinogenese vraagstuk.

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Colour Figures

chapter 2

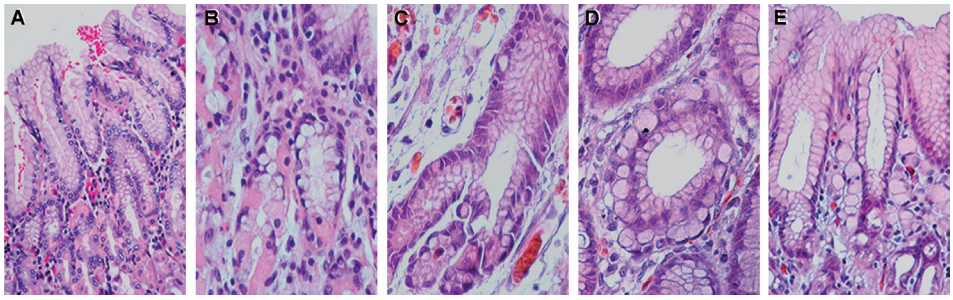


Figure 1 Proposed model for the development of diffuse gastric cancer in E-cadherin mutation carriers: background changes of gastric mucosa encompassing mild chronic gastritis and foveolar hyperplasia (A); in-situ signet-ring cell carcinoma (foveolae and glands with intact basement membrane totally or partially lined by signet ring cells) (B and C); "early"(C) and overt (D) pagetoid spread of signet-ring cells below the preserved epithelium of glands/foveolae; early invasive intramucosal signet-ring cell carcinoma (E).

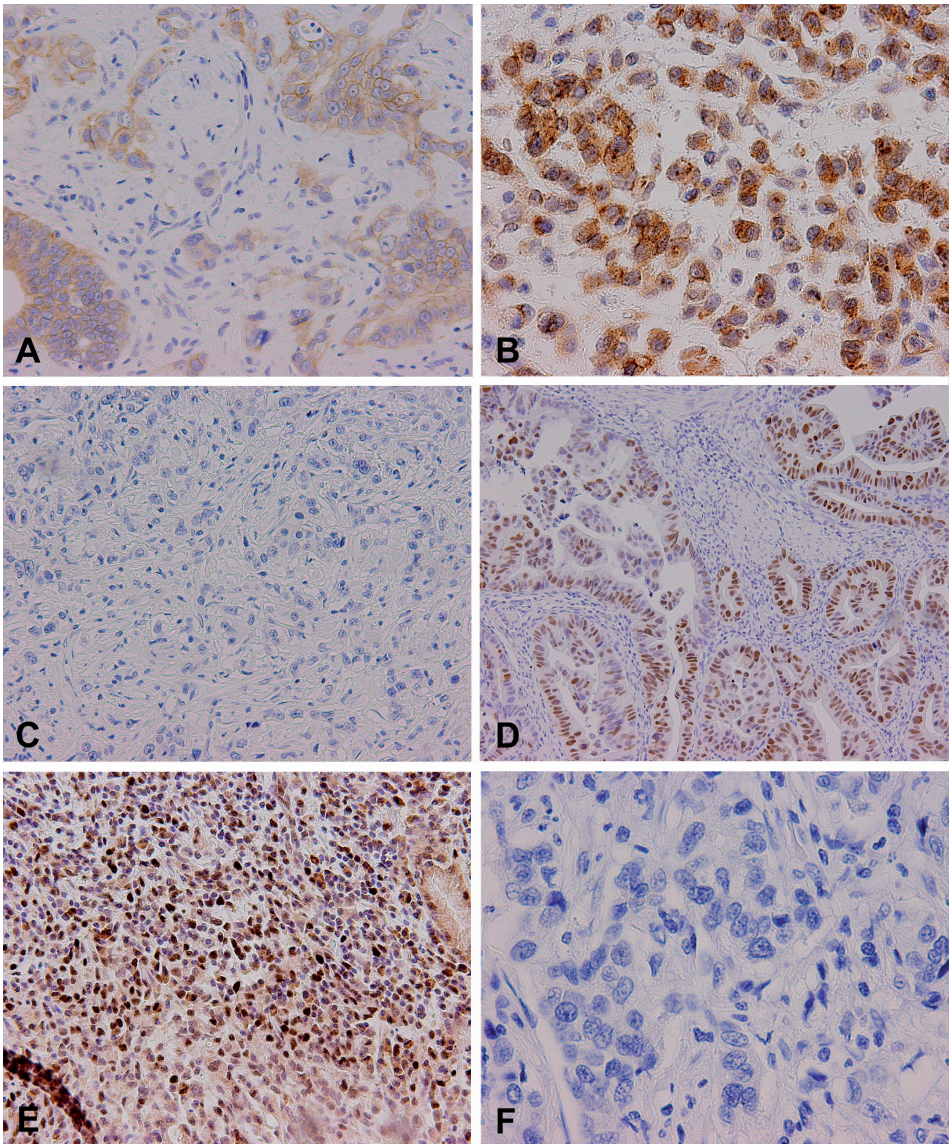


Figure 1 IHC results for E-cadherin (A, B), p53 (C, D) and MSH6 (E, F). Normal protein expression was detected for E-cadherin in case Y13 (A), for p53 in case Y15 (C), and for MSH6 in case Y65 (E). Abnormal protein expression was detected as follows: E-cadherin cytoplasmic expression was seen in case Y28 (B), nuclear p53 overexpression was observed in case Y11 (D), and absence of MSH6 expression was seen in case Y89 (F).

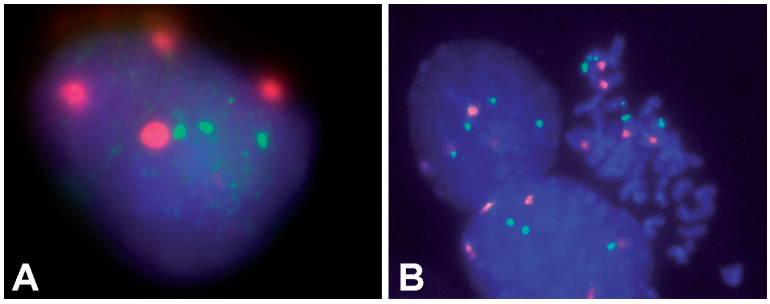


Figure 1 Bicolour FISH analysis of a Y5 gastric tumour cell (A) and HM51 nuclear spreads (B) using a centromere 1-specific probe (red) and a RUNX3-specific probe (green)

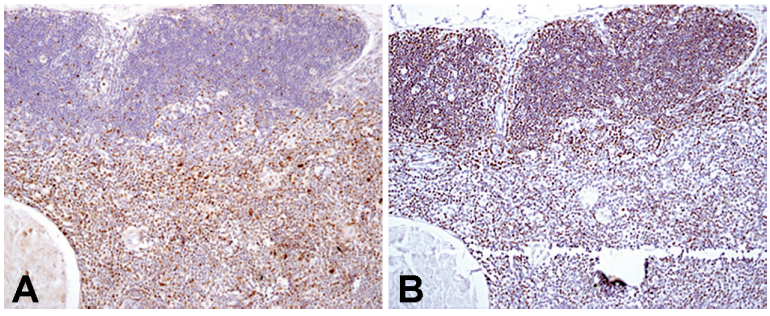


Figure 2 Staining (brown) for RUNX3 (A) and RUNX1 (B) in thymus. RUNX3 expression is seen predominantly in the medulla, whereas RUNX1 expression is mainly observed in the cortex. Nuclei were visualised by counterstaining with haematoxylin-eosin. Visualisation was performed at 10X magnification

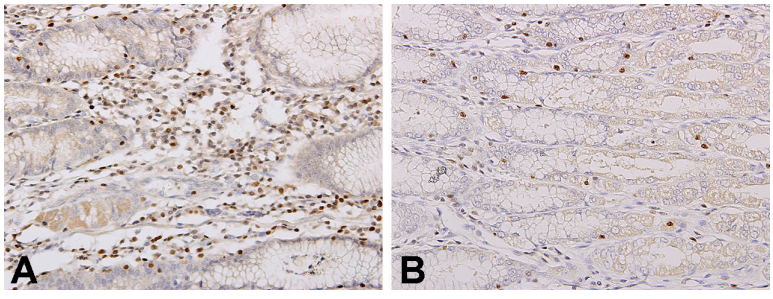


Figure 3 Staining (brown) for RUNX3 in non-neoplastic mucosa of cases Y29 (A) and Y4 (B) showing strong nuclear expression in lymphocytes (A and B) and absence of positivity in non-neoplastic gastric epithelium in the antral and transitional mucosa (A) and in the fundic mucosa (B). Faint cytoplasmatic background staining is observed in both cases. Nuclei were visualised by counterstaining with haematoxylin-eosin. Visualisation was performed at 20X magnification

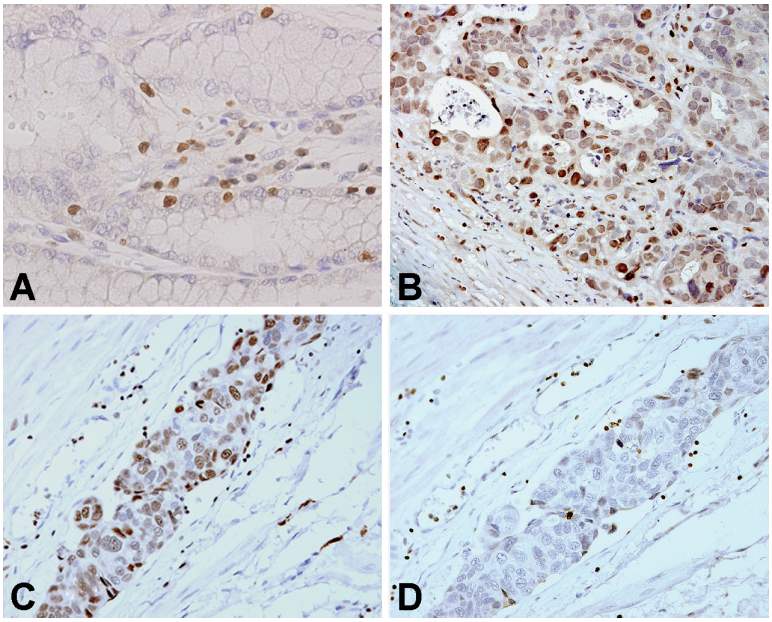


Figure 4 Staining (brown) for RUNX3 in case Y11 (A and B) showing expression in lymphocytes (A and B), absence of positivity in the non-neoplastic epithelial cells of Y11 (A) and positivity in a subpopulation of the tumour cells of Y11 (B). Staining (brown) of the same region of case Y5 for RUNX1 (C) and RUNX3 (D) showing expression in lymphocytes (C and D) and absence of RUNX3 expression in cells that are positive for RUNX1. Nuclei were visualised by counterstaining with haematoxylin-eosin. Visualisation of Y11 (A and B) was performed at 40X magnification, and of Y5 (C and D) at 20X magnification

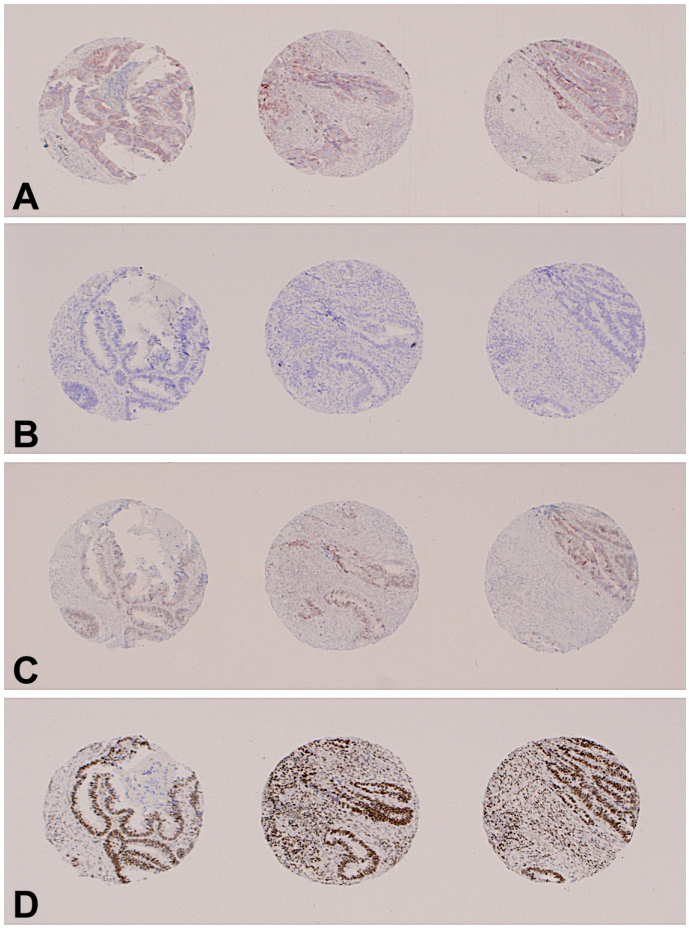


Figure 1 Immunohistochemistry on tissue microarrays for COX-2 (COX-2 High, A), TFF1 (negative, B), HuR (positive cytoplasmic staining, C) and C/EBP-β (positive, D). Magnification 32.5, counterstain haematoxylin.

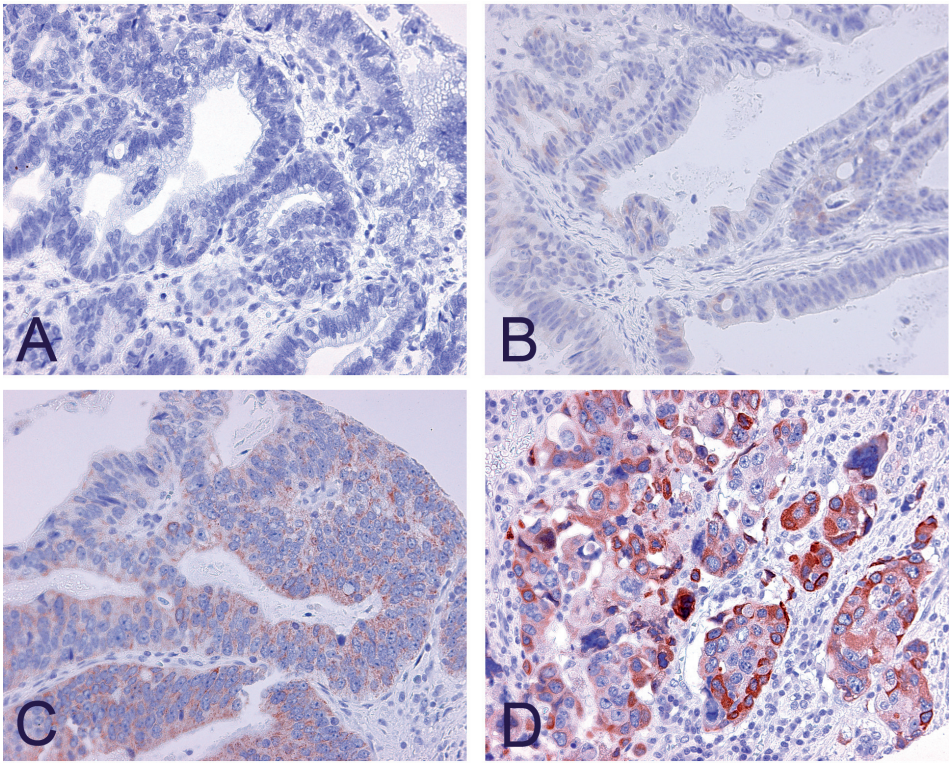


Figure 1 COX-2 Immunohistochemistry. A – category 0, no staining; B – category 1, weak diffuse cytoplasmic staining (may contain stronger intensity in less than 10% of the cancer cells); C- category 2, moderate to strong granular cytoplasmic staining in 10-50% of the cancer cells; D- category 3, >50% of the tumor cells stained with strong intensity. For statistical analysis scores 0 and 1 were categorized as COX-2 low and scores 2 and 3 as COX-2 high.

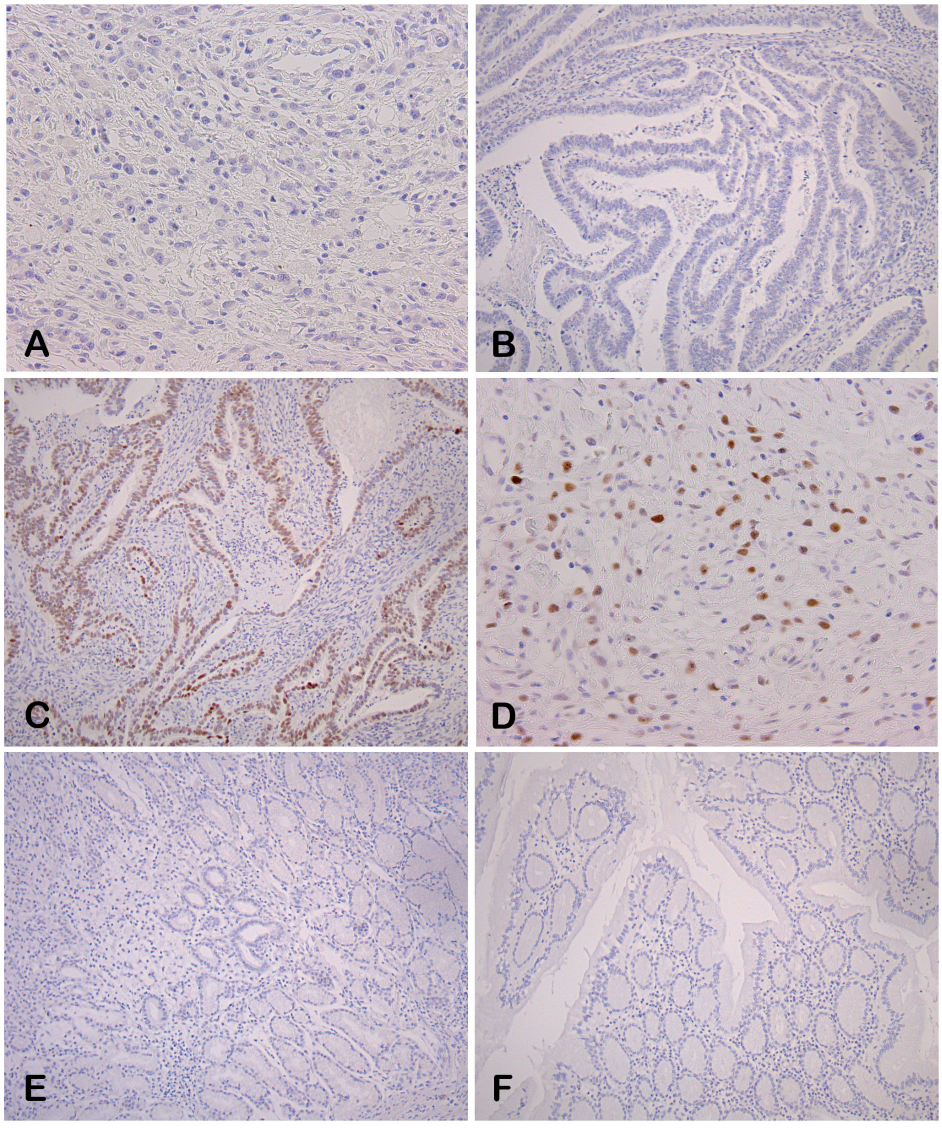


Figure 1 The panel on the left shows immunohistochemical staining (x100) with the N-terminal antibody, negative (A) and positive (C) in tumour tissue and normal gastric mucosa (E). The right panel shows staining with the C-terminal antibody, negative (B) and positive (D) in tumour tissue and in normal gastric mucosa (F). Of note (A) and (D) show staining of the same tumor with different antibodies.

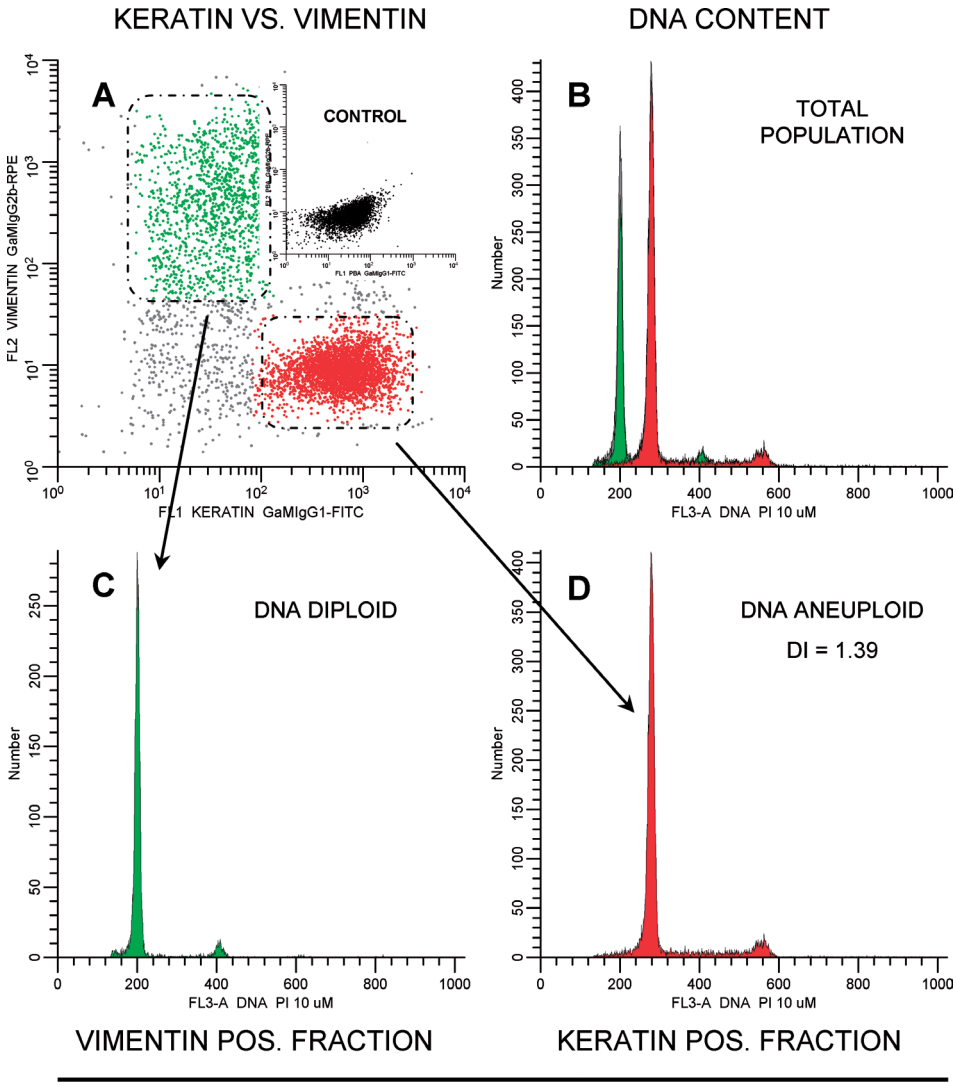


Figure 1 Multiparameter DNA flowcytometry of a FFPE gastric cancer sample. Cells were isolated from a FFPE gastric cancer sample (Y5), stained as described and analyzed on a FACSCalibur flow cytometer. **A**: *keratin versus vimentin dot plot. Inlay: negative control only stained for DNA.* **B**: *DNA histogram of the total cell suspension showing a bimodal DNA histogram.* **C**: *gating on the vimentin-positive cell fraction (green) yields a unimodal DNA histogram representing stromal cells* **D**: *gating on the keratin-positive cell fraction also reveals unimodal DNA histogram, with a DI of 1.39, representing gastric carcinoma cells.*

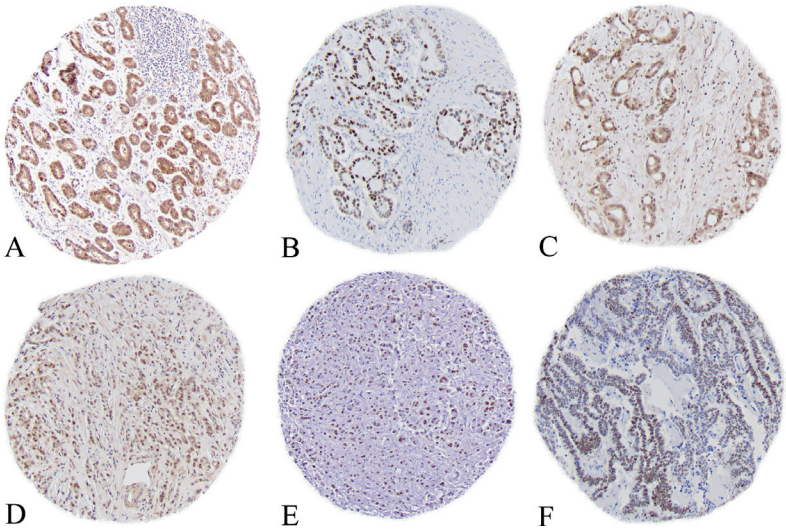


Figure 2 Immunohistochemistry for cdc4 (A), cyclin E (B), c-myc (C), c-jun (D), Notch (E) and p53 (F) (x 100) on tissue microarray cores.

chapter 9

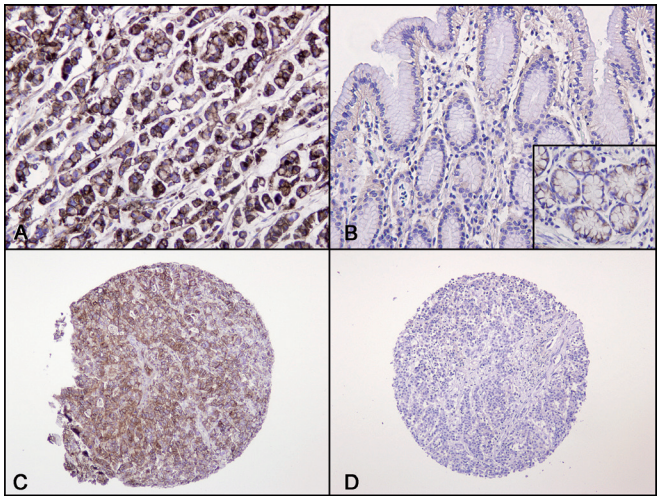


Figure 4 Typical IHC results for CD44v6 on paraffin sections (A and B) and TMAs (C and D): overexpression of CD44v6 can be seen in tumors Y103 (A) and GC25 (C), with membranous and cytoplasmic stain (brown) in tumor cells, and negative staining in surrounding normal tissue; panel B shows absence of expression in the normal epithelium, with the exception of positive stain (brown) in the proliferative region (B, inset); an example of lack of CD44v6 overexpression in a tumor (GC42) is shown in panel D. Nuclei were counterstained with hematoxylin.

chapter 10

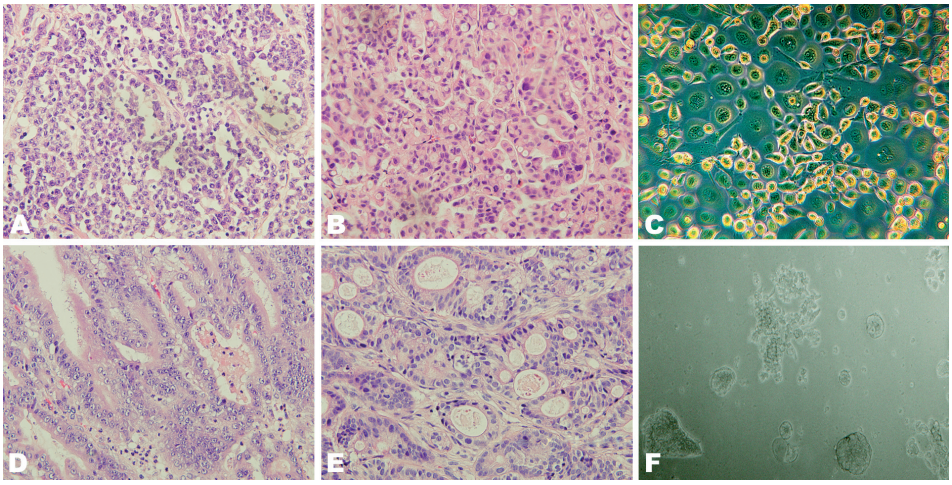


Figure 1 showing the H+E stain (x 40) of P1 (A), M1(B), P8 (D) and M8 (E) together with the cell lines DGC1 (C) and IGC8 (F) in culture, which were established from the corresponding xenografts.

chapter 11

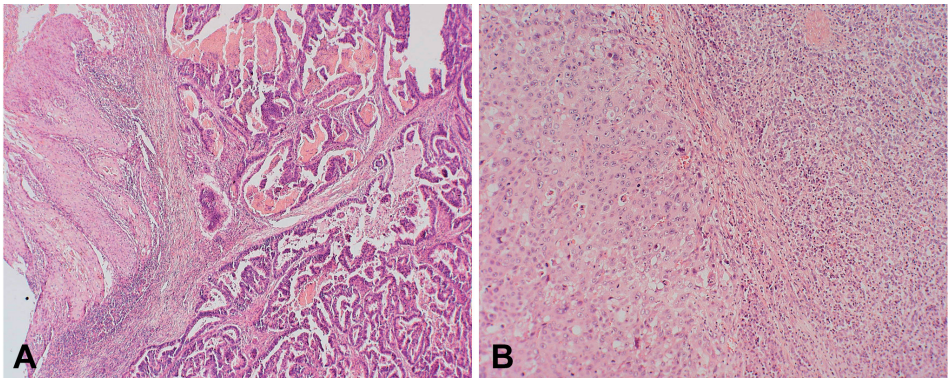


Figure 1 Haematoxylin and eosin staining of cases A (x50) and B (x100) showing the interface of the adenocarcinoma and squamous cell carcinoma components.

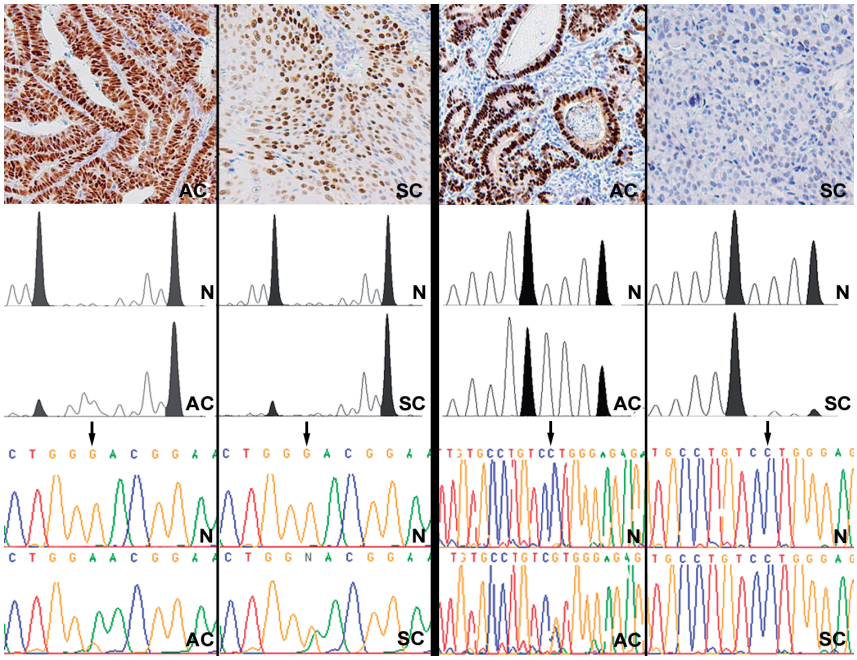


Figure 2 Results for adenocarcinoma (AC) and squamous cell carcinoma (SCC) components of case A (a presumed collision tumor) are represented in the two left hand columns and results for the AC and SC components of case D (a presumed composite tumor) are represented in the two right hand columns. p53 immunohistochemistry (x100) (top) shows positivity in AC and SC for case A, whereas positivity is seen only in the AC component for case D. LOH analysis (middle) shows a loss in the large allele in both components for case A (left) whereas case D (right) a loss of heterozygosity is seen only in the SC component. Arrows point to a TP53 G to A mutation present in both components in case A and point to a C to G TP53 mutation in case D that is present in the AC and absent in the SC component.

chapter 12

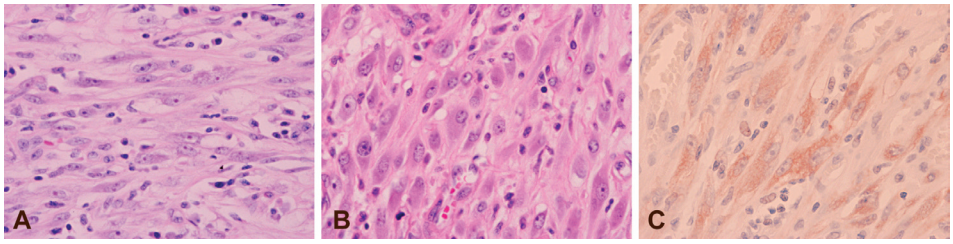


Figure 2 (A) Haemoatoxylin and eosin staining (x250) showing a tumor composed of spindle cells with large round/oval nuclei, prominent nucleoli and plentiful cytoplasm, admixed with an inflammatory infiltrate of numerous lymphocytes, histiocytes, plasma cells and eosinophils (B) Haemoatoxylin and eosin staining (x250) showing ganglion-like cells in the tumor. (C) Immunohistochemistry (x250) for Alk-1 showing diffuse smooth cytoplasmic staining

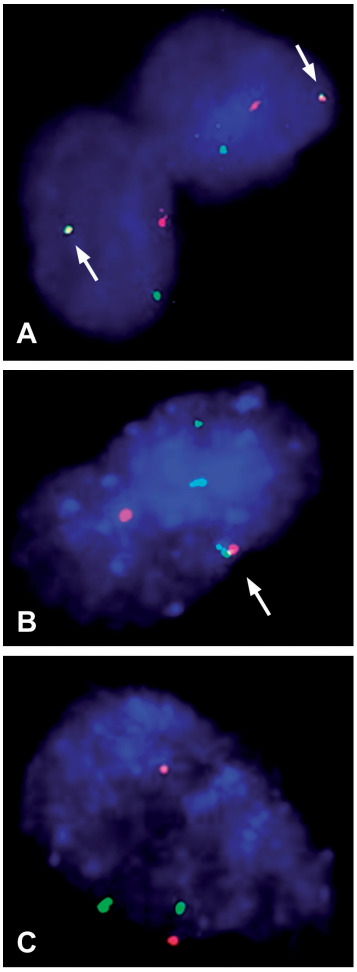
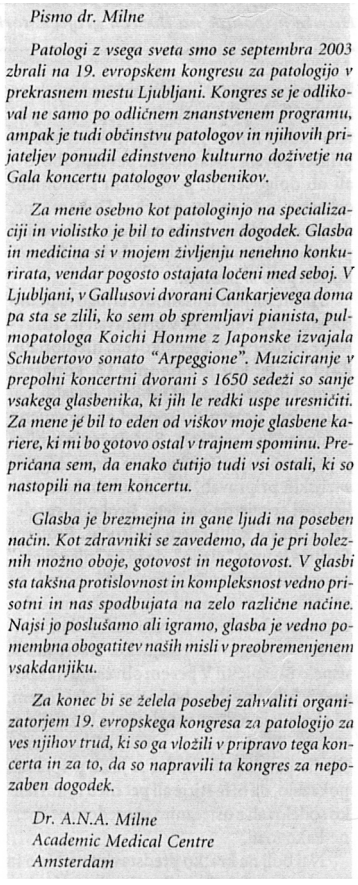


Figure 3 Dual-colour FISH on interphase nuclei from tumor specimen. Using Split-Apart LSI®ALK probe, one overlapping red/green and split apart single red and green signals were detected (arrows), indicating ALK rearrangement (A). Co-hybridization of digoxigenin labelled ALKP1 and biotin labelled RP11-205M9 (that contains TPM3/1q25 gene) probes revealed a single red, a double green and an overlapping red/green signal (arrow), indicating the presence of ALK-TPM3 fusion (B). Co-hybridization of digoxigenin labelled ALKP1 and biotin labelled BAC RP11-121I1 (that contains TPM4/19p13.12 gene) probes showed two normal green and red signals (C).

Reflections

Invited letter, Slovenian Medical Times, 2003, Nov; pg 32



Pismo dr. Milne

Patologi z vsega sveta smo se septembra 2003 zbrali na 19. evropskem kongresu za patologijo v prekrasnem mestu Ljubljani. Kongres se je odlikoval ne samo po odličnem znanstvenem programu, ampak je tudi občinstvu patologov in njihovih prijateljev ponudil edinstveno kulturno doživetje na Gala koncertu patologov glasbenikov.

Za mene osebno kot patologinjo na specializaciji in violistko je bil to edinstven dogodek. Glasba in medicina si v mojem življenju nenehno konkurirata, vendar pogosto ostajata ločeni med seboj. V Ljubljani, v Gallusovi dvorani Cankarjevega doma pa sta se zlili, ko sem ob spremljavi pianista, pulmopatologa Koichi Honme z Japonske izvajala Schubertovo sonato "Arpeggione". Muziciranje v prepolni koncertni dvorani s 1650 sedeži so sanje vsakega glasbenika, ki jih le redki uspe uresničiti. Za mene je bil to eden od viškov moje glasbene kariere, ki mi bo gotovo ostal v trajnem spominu. Prepričana sem, da enako čutijo tudi vsi ostali, ki so nastopili na tem koncertu.

Glasba je brezmejna in gane ljudi na poseben način. Kot zdravniki se zavedemo, da je pri bolezenih možno oboje, gotovost in negotovost. V glasbi sta takšna protislovnost in kompleksnost vedno prisotni in nas spodbujata na zelo različne načine. Najsi jo poslušamo ali igramo, glasba je vedno pomembna obogatitev naših misli v preobremenjenem vsakdanjiku.

Za konec bi se želela posebej zahvaliti organizatorjem 19. evropskega kongresa za patologijo za ves njihov trud, ki so ga vložili v pripravo tega koncerta in za to, da so napravili ta kongres za nepozaben dogodek.

Dr. A.N.A. Milne
Academic Medical Centre
Amsterdam

In the beautiful city of Ljubljana pathologists the world over gathered for the 19th European Congress of Pathology in September 2002. This congress boasted not only an excellent scientific programme, but provided a unique cultural experience through the Gala concert by pathologist musicians, for an audience of pathologists and friends.

From a personal viewpoint, as a viola player and pathologist in training, it was an outstanding event. The music and medicine in my life have constantly competed for my time, and the two areas have often remained quite separate. In Ljubljana, they came together in the Galus Hall, Cankarjev Dom, in a performance of Schubert's Arpeggiona sonata, accompanied (proficiently) by Koichi Honma, a lung pathologist from Japan. The exhilaration of playing to a packed concert hall seating 1,650 is a moment that every musician dreams of, though most never get the opportunity. For me it was one of the highlights of my musical career and will remain with me for quite some time. I am certain that the other participants in this concert have equally memorable feelings.

Music is something that overcomes boundaries and touches people in a special way. As doctors we are aware of the certainties and uncertainties of disease. In music such paradoxes and complexities are constantly apparent, yet it provides us with stimulation in a very different

way. Whether listening or playing, music is an important means for enriching our minds and our busy lives.

On a final note, I would like to thank the committee of the 19th ECP for the hard work that went into organising this concert and for making it such a memorable event. I would also like to thank the Tiniakos Foundation for bestowing me with the prestigious George Tiniakos Award for gastrointestinal research, bringing indeed the two worlds of medicine and music together for me during this conference.

Dr A.N.A. Milne
Academic Medical Centre
Amsterdam

Acknowledgements

So I suppose it all started in Tallaght hospital in Dublin, where I started my pathology training back in 2001 and couldn't believe my luck that something so interesting and stimulating was my new found career. When the opportunity came to move to the Netherlands and the idea came to me to do some research, my plans were enthusiastically supported by Paul Crotty, Michael Jeffers and Barbara Loftus (thank-you all so much, and to Paul of course not only inspiring me in pathology but for also becoming a valued friend). In fact having spent some time working in The Netherlands herself, Barbara was able to supply me with a list of addresses and heads of departments of academic hospitals where I promptly began to send letters…. And so I ended up one day in Jan Weening's office in the Academic Medical Centre, Amsterdam, to whom I am eternally grateful for introducing me to Johan Offerhaus, who catapulted me into in the world of academic pathology. Johan of course was inspiring from the first meeting and imparted his love of learning and infectious enthusiasm from the start- the choice wasn't to difficult to make.

After getting married on sunny day in August 2002, we packed our bags, Dutch tutorial books in hand, and moved to Holland the next day where we had bought a little flat in Amsterdam. And so it began….

Thank-you Marian for teaching me to think critically, for encouraging me to challenge ideas and for teaching me a lot of fundamental techniques. Thank-you Eric, Alex, Miriam and Thera for not only for all the help in the lab, but also for teaching me Dutch! And Folkert – what an amazing colleague who shares his love of work everyday and appears to take pleasure in the countless "favours" asked of him! And Ralph – we moved to Holland at the same time and started our PhD research period on the same day – thank-you as a colleague and a friend for all your help and support. And Marnix for his tireless academic brain, always ready for a discussion on any chosen topic, whose friendship I also treasure. Thank-you Marjon, Lodewijk, Arnout, Robert, Josbert and Steven for all your social and academic contributions. And of course thank-you Wendy, with whom I worked closely over the last fours years and will continue to do so for many more I hope. I also want to give a special mention to Wim Corver with whom I worked for a few months in Leiden University Medical Centre – bedankt voor de tijd en geduld! I want to thank Steven Finn and Ester O'Regan who have remained in close contact with me over the last years and continue to inspire me with their academic (and other) endeavours. We have spent many a conference together with lots of heated debates (scientific and other) and hopefully will share many more!

I am endebted to all the AIOs, technicians and pathologists in the AMC, whom I have not mentioned by name but to whom I am extremely grateful for making my years in the AMC such a pleasure and success. It was a very warm environment to work in, where you were made feel like you belonged and certainly gave me a lot of positive feelings about Dutch culture.

Johan, I want to thank-you so much for the faith you have always shown in me, the challenges you have presented me with and the opportunities you have given me. For the kindness you have shown me on a personal level and for your inspiration that is present without fail. It has been a real boost to the beginning of my career, an example to me for the rest of my life and I realize just how lucky I am to work so closely with you!

And of course the clear advantages that I saw from working in such a group are what prompted my move to the University Medical Centre, Utrecht, along with the other 10 members of the group who realized they were on a winning streak. I want to sincerely thank all the trainees, administrative staff, technicians, researchers and pathologists in the UMCU making me feel so welcome in Utrecht, where I hope to spend many more happy and productive years.

I also want to say a special thank-you to my parents, who despite the odd "your taking on to much" conversation from the age of about 15, have always been supportive of my plans and choices – even if they don't understand them at the time- they have always been very proud of me, as I am of them – go raibh maith agat mamaí agus dadai as gach rud ar thug sibh domh, tá grá mór mór agam daoibh…

And finally and most important of all comes Nick, with whom after 8 years I am still madly in love. A more supportive husband I could not imagine. Someone who is full of inspiring thoughts and ideas. And he is there to pick up the pieces and comfort the Anya not seen by the outside world. He's a dad to our wonderful daughter Luana (01-12-2004) on whom I dote – she surprises and delights me every day. Let's see what bundle of joy this summer will bring…(no. 2)…

Curriculum Vitae

Anya Nic Aoidh Blaney was born prematurely on the 9th August 1974 in Co. Down, Northern Ireland and raised bilingually with both Irish (Gaelic) and English. During secondary school she achieved 11 "A" grades at GSCE and 4 "A" grades at Advanced-level as well as an Outward Bound Scholarship awarded by the Belfast City Council. She started medical school at Trinity College, Dublin in 1993 with an Entrance Exhibition Award and received the First Class Honours Trinity College Dublin Book Prize in 1994 and 1995. Having attained an undergraduate science scholarship from the Health Research Board in 1996, she side-stepped to graduate with honours from Biochemistry in 1997. She then completed her medical training in 2000, graduating with honours in all subjects. In 2001, Anya began her training in Cellular Pathology in The Adelaide and Meath Incorporating the National Children's Hospital in Dublin only to start her full time research PhD position at the Academic Medical Centre in Amsterdam in September 2002. The following year she went on to win not only the poster presentation prize at the Dutch "Pathologendagen" but also the prestigious George Tiniakos Award for gastro-intestinal research at the 19th European Congress of Pathology, Slovenia 2003. Since then Anya has presented frequently at conferences and has been an invited speaker at the 20th European Congress of Pathology Paris, 2005 and the XVIII International Workshop Gastrointestinal Pathology and Helicobacter 2005, Copenhagen.

Having studied viola with Prof. R. Masin at the DIT Conservatory of Music and Drama during her medical studies, Anya has also won various summer school scholarships and competitions and travelled throughout Europe and America as principal viola of among others the National Youth Orchestra of Ireland, European Medical Students Orchestra, Ulster Youth Orchestra and more recently with "Het Orkest" based in Amsterdam.

Anya is currently completing her specialist training in pathology at the University Medical Centre, Utrecht and lives in Utrecht with her husband Nick and daughter Luana, and is expecting her second child this summer.