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Is there a link between environmental factors and a genetic predisposition to cancer? A lesson from a familial cluster of gastric cancers

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Abstract

To determine the prevalence of gastric precancerous lesions and mucosal genetic alterations in relatives of a cluster of familial gastric cancer (FGC), we studied a kindred spanning two generations. The founder, daughter and niece underwent surgery for gastric cancer (GC); a son and other two daughters of the founder, presented with chronic dyspepsia. In all subjects, gastric mucosa samples were analysed for pathological features, *Helicobacter pylori* infection, microsatellite (MIN) and chromosomal (CIN) instability. The overexpression of mp53 and c-myc, and cytoplasmic β-catenin delocalisation were found in the 2 younger cancer patients. All GC and gastritis patients had normal E-cadherin expression and were MIN-negative. Aneuploidy characterised all GC cases, and mixed euploid and aneuploid cell populations were present in the gastric biopsies from two of three 'at-risk' relatives. These two subjects, one of whom had severe active gastritis, and gastric mp53 and c-myc expression, were CagA-positive *H. pylori*-infected. DNA aneuploidy, p53 and c-myc expression disappeared after *H. pylori* eradication. In this FGC cluster, genetic abnormalities were found in first-degree relatives (3 patients) only in presence of *H. pylori* infection (2 cases *H. pylori*-positive versus 1 case *H. pylori*-negative) supporting the hypothesis that, besides the influence of a genetic profile, FGC may be, at least partly, mediated by intrafamilial clustering of *H. pylori* infection.

Keywords: Gastric cancer; Family history; *Helicobacter pylori*; p53; Genomic instability

1. Introduction

Gastric cancer (GC) is a major clinical challenge because of its frequency, mortality, poor prognosis and limited treatment options [1]. There is a well-recognised familial predisposition to GC, and the risk of developing it is greater in relatives of patients with familial cases of the cancer than in relatives of sporadic cancer [2]. Familial gastric cancer (FGC), in which more than two relatives in two successive generations have been affected, is a rare syndrome accounting for approximately 1% of all GC [4]. Earlier studies claimed as familial cancer the diffuse type only, but more recent reports found similar proportions of intestinal and diffuse gastric cancer types [5,6].

The familial clustering and the development of cancer at a young age suggest that genetic factors play a main role in the pathogenesis of this condition [3]. An understanding of the molecular changes underlying the pathogenesis of GC is a prerequisite for the identification of early markers of cell transformation, which in turn might allow us to estimate the individual risk of cancer.

There are at least two molecular phenotypes of genomic destabilisation in GC: the microsatellite mutator phenotype (MMP) [7] and a phenotype associated with chromosomal and intrachromosomal instability (CIN) [8]. The microsatellite mutator phenotype is caused by

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mismatch repair (MMR) deficiency and is associated with mutational inactivation of one of at least five MMR genes, i.e. *hMLH1*, *hMSH2*, *hMSH6*, *hPMS1* and *hPMS2* [7]. This condition, identified as microsatellite instability (MIN), is reported in GC, particularly in the diffuse types, and in FGC [7,9].

CIN is characterised by chromosomal rearrangements and losses or gains of chromosomes, which in turn can induce oncogene activation and/or tumour-suppressor-gene inactivation, thereby causing the development and progression of GC [8,9].

Mounting evidence suggests that MMP alterations, DNA aneuploidy and expression of the products of cancer-related genes are early markers of cell transformation, and may serve to identify the genetic pathway of gastric carcinoma in patients with and without a familial background of the disease [10,11].

As yet, there are no generally agreed upon recommendations for relatives of FGC patients, and the relationship between their genetic predisposition and environmental factors is obscure. We have studied gastric disease findings, mucosal genetic alterations (MIN and CIN status), and *Helicobacter pylori* infection in affected and unaffected relatives from a well-characterised FGC kindred. Our data suggest an interplay between the genetic profile of the host and *H. pylori* gastric infection.

2. Patients and methods

The FGC kindred studied (Fig. 1) was identified at our Endoscopy Unit and consists of two generations: the GC-affected male in the first generation died at age 78 years from GC; one of his four children (three females and one male) died of gastric cancer at age 37 years. The other three, aged 46, 51 and 54 years, are alive and all suffer from chronic dyspepsia. The niece of GC-affected male succumbed to GC at 37 years of age. Her brother (57 years) also suffers from dyspepsia, but was not included in the study. There was no history of colorectal cancer on the paternal and maternal sides of the family. Other hereditary cancer syndromes were also excluded. All subjects gave their informed consent to this study and to use the archival materials of their relatives.

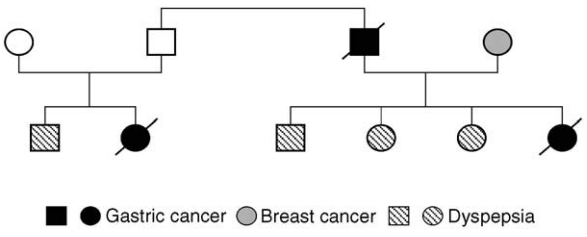

Fig. 1. Gastric cancer kindred: three cases of gastric cancer presented in two generations. All affected individuals had an intestinal type gastric cancer. General symbols: squares, males; circles, females; solid black symbols: gastric cancer; all symbols with a diagonal line, deceased.

Formalin-fixed, paraffin-embedded tumour and gastric mucosa samples of the GC-affected patients were retrieved from the surgical files of the Pathology Department, and at least three antral and three corporal gastric biopsies from the relatives were obtained at upper endoscopy. Gastric cancers were histologically classified according to Lauren's criteria [12]. *H. pylori* was identified with a modified Giemsa staining procedure. Each biopsy sample was examined for *H. pylori*, degree of inflammatory reaction, glandular atrophy, intestinal metaplasia and cellular dysplasia, which were classified according to the updated Sydney system [13]. Serum samples of each alive subject were stored frozen until the detection of anti-CagA antibody by use of a commercial western blot (Helico Blot 2.0 Genelabs diagnostics, Singapore). The serum of deceased GC patients was unavailable. One month after antibiotic therapy performed according to the Maastricht consensus report [14], the *H. pylori* eradication was evaluated by a ^{13}C-urea breath test that was repeated yearly. In case of persistent infections, in spite of therapy, new endoscopy with gastric mucosal samples for histology and culture tests should be offered.

2.1. Immunohistochemistry

For each case, 4-μm thick serial sections were cut from paraffin blocks, mounted on acid-cleaned glass slides, and heated to 55 °C for 60 min. Slides were dewaxed, rehydrated and processed using the avidin–biotin–peroxidase complex procedure (ABC standard, Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine as a substrate according to the method of Hsu and colleagues [15]. The primary antibodies and dilutions used were: anti-E-cadherin, 1:150 (Santa Cruz Biotechnology Inc., CA, USA); anti-β-catenin, 1:700 (Transduction Laboratories, Lexington, KY, USA); anti-p62 c-myc, 1:150 (Oncogene Science, San Diego, CA, USA); anti-p53 (wild-type) and anti-mp53 (mutated type), 1:800 (Santa Cruz Biotechnology, CA, USA). Negative controls in which normal mouse serum replaced the specific primary antibodies were included in each run. The positive controls were a colon adenocarcinoma that is positive for a mutated p53 protein, and a normal lymph node for c-myc and proliferating cell nuclear antigen (PCNA) expression. Sections were considered positively stained only in instances of unequivocal nuclear staining for p53 and PCNA, and of nuclear and cytoplasmic staining for c-myc. A membranous or cytoplasmic signal was considered positive for E-cadherin and β-catenin. A total of 400 cells was counted in random fields from more representative histological areas, and the degree of immunopositivity was evaluated semi-quantitatively.

Table 1
Clinical and histological findings of cancer patients

Patients	Age* (years)	Gender	Histological characteristics		
			Type	Grading	Staging
1	76	M	Intestinal	Well	T1N1M0
2	37	F	Intestinal	Poor	T4N2M1
3	35	F	Intestinal	Poor	T3N2M1

*Age at diagnosis; M, male; F, female.

2.2. DNA ploidy

We used the Feulgen (sulphuric fucsin-acid) technique for nuclear DNA staining of paraffin-embedded sections. Briefly, sections were deparaffinised in xylene and rehydrated through decreasing ethanol concentrations. Cellular DNA was quantified with a microprocessor-controlled image analysis system (Leica-Quantimet 500IW analyser, Sony 3CCD camera mod. DXC-950P and Leica DMLB microscope). The Leica-QWIN V0200A software was used for data analysis. At least 400 cells from each patient were examined in non-consecutive random fields of representative areas of the lesions. The following indexes were determined for each measurement: normal diploid DNA content (2c), 2c-deviation index (2cDI), 5c exceeding events (5cEE) and DNA-malignancy grade (DNA-MG) [16].

2.3. DNA extraction and microsatellite analysis

Five-μm-thick paraffin-embedded sections were collected on microscope slides. Areas representative of tumour and of normal tissue (*muscularis propria* and/or microscopically normal mucosa, with no evidence of intestinal metaplasia or dysplasia) were identified within single unstained sections and microdissected into 1.5-ml polypropylene vials, using an haematoxylin–eosin-stained step section as a guide. DNA was extracted as previously reported in Ref. [17]. Genomic DNA was extracted from peripheral blood lymphocytes according to standard procedures [17]. Microsatellite instability was evaluated at the BAT 25, BAT 26 and SMAD2 mononucleotide repeats using a polymerase chain reaction (PCR)-based assay. These three loci are considered sensitive indicators of MIN [17]. Primers, PCR mixture, cycling conditions, electrophoretic separation and autoradiography are described elsewhere in Ref. [17]. Paired genotypings of cases positive for microsatellite alterations were confirmed in triplicate experiments.

3. Results

The clinical and histological characteristics of the FGC kindred, 3 patients with GC and three GC-unaffected first-degree relatives, are listed in Tables 1 and 2.

All three GC were of the intestinal type. The cancers were poorly differentiated and advanced in the younger first- and second-generation patients, T4N2M1 and T3N2M1, respectively (Table 1). All GC showed an aneuploid pattern while none had MIN, as evaluated with the very sensitive BAT 25, BAT 26 and SMAD 2 mononucleotide repeat sequences (Table 3). Immunohistochemistry showed that E-cadherin was normally expressed, with a membranous pattern, while β-catenin had a normal membranous localisation in case #1 and was de-localised to the cytoplasm in the poorly differentiated and advanced tumours from the 2 younger patients. Both the poorly differentiated and advanced tumours showed mp53 and c-myc expression.

The three first-degree relatives were referred to our Endoscopy Unit for chronic dyspepsia. *H. pylori* infection was found in two of these subjects, associated with a chronic gastritis. All three subjects were analysed for serum *H. pylori* test; positivity for anti-CagA antibody was present in both *H. pylori*-positive patients. One of these (case #2) was affected by severe active gastritis, mostly at the neck region of the glands, associated with moderate atrophy in antrum and body and focal mild dysplasia in the antrum (Table 2 and Fig. 2). This case, manifesting histological alterations associated with a

Table 2
Clinical and histological findings of gastritis patients: a 2 years follow-up

Patients	Age (years)	Gender	Controls	Histological characteristics						
				Atrophy	IM	Dysplasia	Lymphocytes	PMN	*Hp*	Cag-A
1	54	M	06/05/99	–	–	–	+ +	+ +	+ +	+
			08/07/00	–	–	–	+	–	–	
2	51	F	05/03/99	+ +	–	+ +	+ + +	+ + +	+ +	+
			30/03/00	+ +	–	+ +	+ + +	+ + +	+ +	
			29/09/00	+	–	+	+ +	+	+	
			08/09/01	+	–	–	+	–	–	
3	46	F	04/04/99	–	–	–	+	–	–	–
			01/03/01	–	–	–	+	–	–	

IM, intestinal metaplasia; PMN, polymorpho-leucocytes; *Hp*, *Helicobacter pylori*.

cancer risk, showed an increase in cell proliferation, as witnessed by PCNA expression, and was positive for c-myc expression and for the expression of wild and mutated types of p53 (Table 3), the latter being observed in less than 10% of cells. A mixed euploid and aneuploid cell population was found in the mucosal biopsies from the two subjects with *H. pylori* infection (Fig. 3). All three first-degree relatives did not have MIN and showed normal expression of E-cadherin and β-catenin (Table 3).

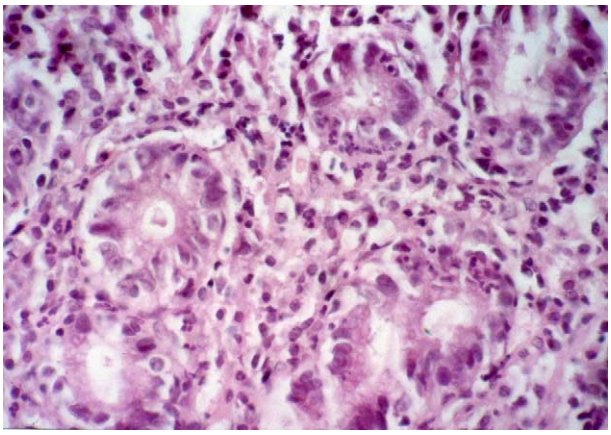

Fig. 2. *Helicobacter pylori*-positive chronic gastritis (case #2) showing severe activity and sparse nuclear atypia (haematoxylin–eosin (HE), ×250).

Cases #1 and #2 were treated with conventional *H. pylori*-eradication therapy that was only effective in Case #1. Case #2 was successfully eradicated after three antibiotic cycles according to the Maastricht guidelines and culture test. Interestingly, 12 months after *H. pylori* eradication, gastric atrophy improved (disappearing in the body and decreasing in the antrum), dysplasia completely regressed and aneuploidy, p53, mp53 and c-myc expression disappeared (Table 2).

4. Discussion

Familial clustering of GC has long been recognised. The most famous FGC pedigree is that of the Bona-

Table 3
Microsatellite instability, DNA ploidy and oncoproteins expression in familial cluster

	Cancer patients			First-degree relatives		
	1	2	3	1	2	3
Microsatellite instability						
BAT 25	–	–	–	–	–	–
BAT 26	–	–	–	–	–	–
SMAD2	–	–	–	–	–	–
DNA aneuploidy	+	+	+	+	+	–
Oncoprotein expression						
PCNA	+ + +	+ + +	+ + +	+ +	+ + +	+ +
E-Cadherin	+	+	+	+	+	+
β-Catenin	+	Cytoplasmic	Cytoplasmic	+	+	+
p53	+	+	+	–	+	–
mp53	–	+	+	–	+	–
c-myc	–	+ + +	+ + +	–	+	–

PCNA, proliferating cell nuclear antigen.

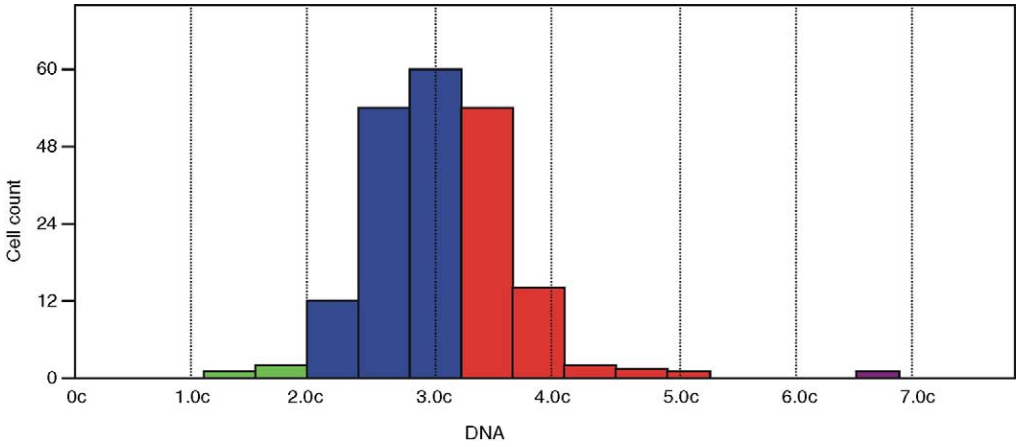

Fig. 3. Representative DNA ploidy patterns in *Helicobacter pylori*-positive chronic gastritis (case #2), image analysis Quantimet 500C.

parte family: the Emperor, two sisters, one brother, the father and, probably, a paternal aunt died from stomach cancer [18]. Predictive genetic testing is an essential step for the clinical management of affected families. MIN and E-cadherin are the most frequent genetic alterations associated with a family history of GC [17,19,20]. At present, the International Gastric Cancer Consortium recommends testing for E-cadherin alterations in patients with diffuse GC and suggests prophylactic gastrectomy in carriers of E-cadherin germline mutations belonging to FGC [4]. In our FGC kindred, neither GC cases nor unaffected first-degree relatives had MIN or deregulated E-cadherin expression in gastric tissues (Table 3). Unfortunately, fresh blood samples from the GC-affected members of the kindred were not available for germline mutation analysis. However, it is unlikely that alterations in the *E-cadherin* gene are involved in our FGC kindred. In fact, germline mutations of the *E-cadherin* gene, responsible for hereditary diffuse gastric cancer [4], have not been reported in intestinal type FGC kindreds, while somatic *E-cadherin* mutations have been frequently detected only in sporadic diffuse-type GC [21–23].

The lack of a clear genetic basis for FGC lends weight to the notion that familial aggregations might reflect not only a hereditary predisposition, but also exposure to risk factors in the familial or community environment [24,25]. *H. pylori* infection is one of the most important environmental risk factors associated with sporadic GC [26]. The disease risk can be further modulated by *H. pylori* genotypes. In Western populations, subjects with CagA-positive strains of *H. pylori* have an enhanced risk of developing atrophic gastritis, peptic diseases and GC compared with individuals with CagA-negative strains [27]. Exposure of gastric epithelial cells to *H. pylori* results in the generation of reactive oxygen species and an increased level of inducible nitric oxide synthase; both in turn may cause genetic alterations which may lead to cancer [28,29]. Alterations indicative of a CIN phenotype during *H. pylori* infection are not rare. Altered expression of p53, APC, K-*ras*, c-myc and an aneuploidy status have been described in chronic atrophic gastritis, intestinal metaplasia and dysplasia, and in populations at high risk of GC [10,11,28–30].

Huang and colleagues in a meta-analysis of 19 cohort and case–control studies found an approximately 2-fold risk of GC among infected individuals [31], while Brenner and colleagues found a 16-fold risk of non-cardiac GC in patients with *H. pylori* infection and a familial aggregation of gastric cancer compared with uninfected subjects [32]. Therefore, familial aggregation of GC could be mediated by *H. pylori* infection, which suggests an interplay between *H. pylori* and the genetic profile of the host. In a Caucasian population, a DNA polymorphism in the interleukin-1 (IL-1) gene cluster resulted in atrophy of the gastric corpus and an increased risk for GC in response to *H. pylori* infection [33]. The absence of the DQA10102 allele is associated with a greater incidence of glandular atrophy and GC in a Japanese population with *H. pylori* infection [34].

In this study, we describe, for the first time, a familial cluster of FGC in which unaffected first-degree relatives (3 cases, 2 *H. pylori*-positive and 1 *H. pylori*-negative) with chronic gastritis have CIN alterations only in presence of CagA-positive *H. pylori*. One of these patients (case #2) was affected by severe active gastritis associated with moderate atrophy and mild dysplasia (Table 2 and Fig. 2). Initially, this patient did not respond to three cycles of *H. pylori* eradication therapy. Interestingly, in this patient, CIN alterations disappeared 12 months after *H. pylori* eradication, which indicates a close relationship between the genetic predisposition of the host and *H. pylori* infection. Alterations of CIN have been reported by several authors in gastric mucosa of patients with *H. pylori*-positive chronic gastritis [10,11,28–30]. At present in FGC kindred, no data concerning CIN changes in relation to *H. pylori* status are available, probably because hereditary GC is a rare disease.

In conclusion, the results reported herein, even if obtained in one FGC cluster (3 GC cases and three GC-unaffected relatives), support the hypothesis that besides the influence of genetic profile, hereditary GC may be, at least partly, mediated by intrafamilial clustering of *H. pylori* infection. Therefore, screening for and, eventually, treatment of *H. pylori* infection should be offered to reduce the risk of GC in first-degree relatives of patients with FGC and, taking into account the rate of *H. pylori* infection recurrence, these procedures should be repeated yearly. Finally, our finding provides scientific backing for the recommendation of the Maastricht 2-2000 Consensus Report that patients with a family history of GC should undergo *H. pylori*-eradication therapy [14].

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