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E-Cadherin (*CDH1*) and *p53* rather than *SMAD4* and *Caspase-10* germline mutations contribute to genetic predisposition in Portuguese gastric cancer patients

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Abstract

Approximately 30% of all hereditary diffuse gastric cancer (HDGC) families carry *CDH1* germline mutations. The other two thirds remain genetically unexplained and are probably caused by alterations in other genes. Using polymerase chain reaction (PCR)/single-strand conformation polymorphism (SSCP)/sequencing, we screened 32 Portuguese families with a history of gastric cancer and 23 patients with early onset gastric cancer for *CDH1* germline mutations. In probands negative for *CDH1* mutations, we screened genes involved in hereditary cancer syndromes in which gastric cancer may be one of the component tumours, namely *p53* (Li-Fraumeni Syndrome) and *hMLH1* and *hMSH2* (HNPCC). We also screened in these patients for mutations in *Caspase-10*, a gene inactivated in sporadic gastric cancer, and *SMAD4*, a gene whose inactivation in mice is associated with signet-ring cell carcinoma of the stomach. One of the families fulfilling the HDGC criteria harboured a *CDH1* germline mutation, and one of the families with incomplete criteria harboured a *p53* germline mutation. No mutations were identified in *hMLH1* and *hMSH2*, and only sequence variants were found in *SMAD4* and *Caspase-10*. The present work reports for the first time *CDH1* germline mutations in Portuguese gastric cancer families, and highlights the need for *p53* mutation screening in families lacking *CDH1* germline mutations, in a country with one of the highest incidences of gastric cancer in the world. No evidence was found for a role of germline mutations in *SMAD4* and *Caspase-10* in families lacking *CDH1* mutations.

Keywords: E-cadherin germline mutations; Familial gastric cancer; Hereditary diffuse gastric cancer; *p53* germline mutations; Li-Fraumeni syndrome; HNPCC; *SMAD4* mutations; *Caspase-10* mutations

1. Introduction

The incidence of gastric cancer has been decreasing in older patients, but in younger patients and cases with familial clustering the level remains stable [1].

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Aggregation of gastric cancer within families is observed in approximately 10% of the cases [2,3], but only 1–3% of gastric carcinomas arise as a result of inherited gastric cancer predisposition syndromes [4].

In 1999, clinical criteria for the hereditary diffuse gastric cancer (HDGC) syndrome were defined by the International Gastric Cancer Linkage Consortium (IGCLC) [5]. However, gastric cancer may also be seen as part of the tumour spectrum in other inherited cancer predisposition syndromes, such as: hereditary non-polyposis colorectal cancer syndrome (HNPCC), Li-Fraumeni syndrome (LFS), Familial Adenomatous Polyposis (FAP), Cowden syndrome and Peutz-Jeghers syndrome (PJS) [6–10].

The presence of germline *CDH1* mutations in affected family members was shown to be, the genetic defect responsible for a proportion of families with HDGC as first described by Guilford and collaborators in 1998 [11]. Approximately one third of families with an aggregation of gastric cancer, fulfilling the IGCLC criteria for HDGC studied so far, show germline *CDH1* mutations in affected individuals (reviewed in [12]). Most of these families carry truncating mutations, whereas a small percentage carry missense mutations [12].

Approximately two thirds of HDGC families remain genetically unexplained. In kindred negative for *CDH1* germline mutations, other genes are probably involved. In some inherited predisposition syndromes characterised by a higher incidence of gastric cancer, germline mutations of different tumour-related genes have been demonstrated to segregate with the disease. HNPCC occurs due to inactivating alterations of mismatch repair genes (MMR) leading to instability at short tandem repeat sequences – microsatellites – microsatellite instability (MSI), a typical molecular manifestation of MMR deficiency in the tumour tissue of HNPCC patients [13]. In approximately 70% of Li-Fraumeni kindred, which occasionally present with gastric cancer cases, germline mutations in *p53* are found [14]. *SMAD4* has been found to be inactivated in a percentage of PJS, and knockout studies revealed the presence of *foci* of signet-ring carcinoma cells in the stomach of *SMAD4* heterozygous mice [15]. Each of the aforementioned genes, as well as genes found to be inactivated in sporadic gastric cancer (like *Caspase-10* [16]), remain good candidates for familial gastric cancer.

We have selected a series of Portuguese families with a positive history of gastric cancer ($n = 32$), and a series of Portuguese early onset gastric cancer patients ($n = 23$) and screened all probands for *CDH1* germline mutations. In probands negative for *CDH1*, we have screened the entire coding sequence and splice-sites of several candidate genes, namely *SMAD4*, *Caspase-10* and the mutational hotspots corresponding to exons 5–8 of *p53*. Whenever tumour tissue was available, we performed MSI analysis, and in MSI-positive cases *hMLH1* and *hMSH2* coding sequences were studied to exclude HNPCC.

2. Materials and methods

2.1. Patients

The study protocol was reviewed and approved by the appropriate Ethics Committees and blood samples and family histories were obtained with informed consent. Thirty two families of Portuguese origin with a positive history of gastric cancer and 23 early onset gastric cancer patients ($\leqslant 45$ years old) were studied (Table 1). Nine families fulfilled the IGCLC criteria for HDGC. Ten families had an index case with diffuse gastric cancer (FDGC). Three families had an index case with intestinal gastric cancer (FIGC). Ten families had aggregation of gastric cancer, but without histology available (FGC). The nine HDGC families followed the criteria of the IGCLC: (1) two or more documented cases of diffuse gastric cancer in first-second-degree relatives, with at least one diagnosed before the age of 50 years; or (2) three or more cases of documented diffuse gastric cancer in first-second-degree relatives, independent of their age. Sixteen early onset gastric cancer patients had diffuse type gastric carcinoma, two had intestinal type carcinoma and five had developed gastric cancer at an early age, but the histological classification was unknown. Genomic DNA was isolated, using standard methods, either from peripheral blood or from frozen normal mucosa. In a few cases in which fresh material was not available, DNA was isolated from paraffin-embedded normal mucosa (germline mutation screening) or tumour (MSI analysis) by phenol/chloroform extraction, using standard methods, after macrodissection.

2.2. Polymerase chain reaction–single-strand conformation polymorphism analysis (PCR-SSCP)

All coding regions from *CDH1*, intron–exon boundaries and the promoter region of the *CDH1* gene were amplified by polymerase chain reaction (PCR) from germline DNA. Primer sequences and PCR conditions were based on those previously reported in [17]. The same approach was used to amplify *SMAD4*, *p53* and *Caspase-10*. Primers to amplify *SMAD4* exons 1 and 10 were based on those previously reported in [18], and the remaining exons were amplified with newly-designed primers (primer sequences are given upon request). The *p53* mutational hotspot (exons 5–8), was amplified using the primers from the Operon kit (Operon Technologies, Inc. Atlantic City. CA, USA) following the manufacturer's instructions. *Caspase-10* genomic sequences were

Table 1
Germline mutations and sequence variants found in *CDH1* and in other candidate genes in gastric cancer probands

Clinical criteria of the families	$n = 32$	Germline mutations	Sequence variants	Effect	Frequency in controls (%)	Refs.
HDGC	9	1/9 (11.1%) *CDH1*	1/9*CDH1* 1896C>T	Silent	5	[12]
			1/9 *p53* 639A>G	Silent	11	[30]
			1/9 *Caspase-10* IVS5-20C>T	Unknown	3.8	Present work
FDGC	10	0/10	2/10 *CDH1* IVS1+6T>C	Unknown	27	[12]
			1/10 *CDH1* IVS4+10G>C	Unknown	Nd	[12]
			1/10 *CDH1* 1896C>T	Silent	5	[12]
			1/10 *CDH1* 2253C>T	Silent	Nd	[12]
			1/10 *SMAD4* IVS7-31G>A	Unknown	1.1	Present work
FIGC	3	0/3	1/3 *CDH1* IVS1+6T>C	Unknown	27	[12]
			1/3 *Caspase-10* IVS5-20C>T	Unknown	3.8	Present work
FGC	10	1/10 (10%) *p53*	3/10 *CDH1* IVS1+6T>C	Unknown	27	[12]
			1/10 *CDH1* IVS4+10G>C	Unknown	Nd	[12]
			1/10 *Caspase-10* 1217A>T	Ile406Leu	3.4	Present work
			1/10 *SMAD4* 1086T>C	Silent	1.1	[22]
Early onset patients	$n = 23$					
EODGC	16	0/16	2/16 *CDH1* IVS1+6T>C	Unknown	27	[12]
			5/16 *CDH1* IVS4+10G>C	Unknown	Nd	[12]
			2/16 *CDH1* 1896C>T	Silent	5	[12]
			1/16 *CDH1* 2253C>T	Silent	Nd	[12]
			1/16 *Caspase-10* 1217A>T	Ile406Leu	3.4	Present work
			1/16 *SMAD4* IVS7-31G>A	Unknown	1.1	Present work
EOIGC	2	0/2				
EOGC	5	0/5	2/5 *CDH1* IVS4+10G>C	Unknown	Nd	[12]

HDGC, hereditary diffuse gastric cancer; FDGC, familial diffuse gastric cancer; FIGC, familial intestinal gastric cancer; FGC, familial gastric cancer; EODGC, early onset diffuse gastric cancer; EOIGC, early onset intestinal gastric cancer; EOGC, early onset gastric cancer, Nd, not done.

amplified using primer sequences and conditions previously described in [16]. Genomic DNA (25–100 ng) was amplified by PCR using the following cycling conditions: 30 s at 94 °C, 30 s at the appropriate annealing temperature, and 45 s at 72 °C for 35 cycles. Reaction products were subsequently diluted 1:1 with denaturing buffer (formamide with 0.025% xylene cyanol and 0.025% bromophenol blue) and heated up to 99 °C for 10 min before being loaded onto 0.6× and 0.8× mutation detection enhancement (MDE – Flowgen, Rockland, ME, USA) gels. Gels were run at constant temperature for 12–8 h and stained with silver nitrate.

2.3. Sequencing analysis

Samples showing abnormal bands detected by single-strand conformation polymorphism (SSCP) analysis, were re-submitted to PCR and products were purified and sequenced on an ABI Prism 377 automated sequencer (Perkin–Elmer) using the ABI prism dye terminator cycle sequencing kit (Perkin–Elmer, Foster City, CA, USA) and the original primers for both strands. All sequence alterations detected were confirmed in a second independent PCR.

2.4. Microsatellite instability analysis

Available neoplastic lesions from family probands and early onset gastric cancer patients were studied for MSI using a panel of 5 dinucleotide repeat markers and/or BAT 26 [19]. The PCR products from tumour *versus* constitutional DNA were labelled by [$\alpha-^{32}$P] deoxycytidine triphosphate (dCTP) during the amplification reaction, separated by electrophoresis in 6% denaturing polyacrylamide gels, at a constant current over approximately 3 h, and visualised by autoradiography.

2.5. DGGE

Germline mutational analysis of *hMLH1* and *hMSH2* genes in family FGC#1 and FGC#24 which had MSI gastric carcinoma in the proband, was performed by denaturing gradient gel electrophoresis (DGGE) as described in [20].

2.6. Polymorphism analysis

Novel sequence variants detected were tested in at least 156 chromosomes from Caucasian blood donors.

3. Results

From the Portuguese families with an aggregation of gastric cancer, one out of 32 (3.1%) harboured a germline mutation in *CDH1*. This family (FGC#32) was one of the nine (11.1%) kindred that fulfilled the criteria for HDGC (Tables 1 and 2). The proband in this family was heterozygous for a missense mutation at position 1901 (C>T) in codon 634, leading to an amino acid substitution from Ala to Val (Fig. 1(a)). This germline mutation was identified in DNA isolated from paraffin-embedded normal tissue from the proband which developed an invasive signet-ring carcinoma with few *foci* of *in situ* signet-ring cancer at the age of 23 years. This patient had an older brother who died four years before with diffuse gastric carcinoma at the age of 26 years. However, no good quality biological material was available from this patient (only Holland Bouin fluid-fixed biopsies) to search for the presence of the missense mutation (Fig. 1(a)). These two siblings have six other brothers and sisters who remain asymptomatic, as well as their parents aged 63 and 65 years old, who refused any genetic testing (Table 2, Fig. 1(a)). Twelve of the 31 remaining families and 11 of 23 early-onset cancer cases

Table 2
Details of the two families with an aggregation of gastric cancer carrying germline mutations

ID	Clinical criteria	Members with GC	Generations	Mean age (years old)	Age range (years old)	Cancers at other sites	Germline alteration	Exon	Effect	Cancer syndrome
FGC#11	FGC	3	2	38	26–52	Colon, pancreatic	*p53* 471C>G	5	Missense Arg158-Gly	Li-Fraumeni
FGC#32	HDGC	2	1	24.5	23–26	–	*CDH1* 1901C>T	12	Missense Ala634Val	HDGC

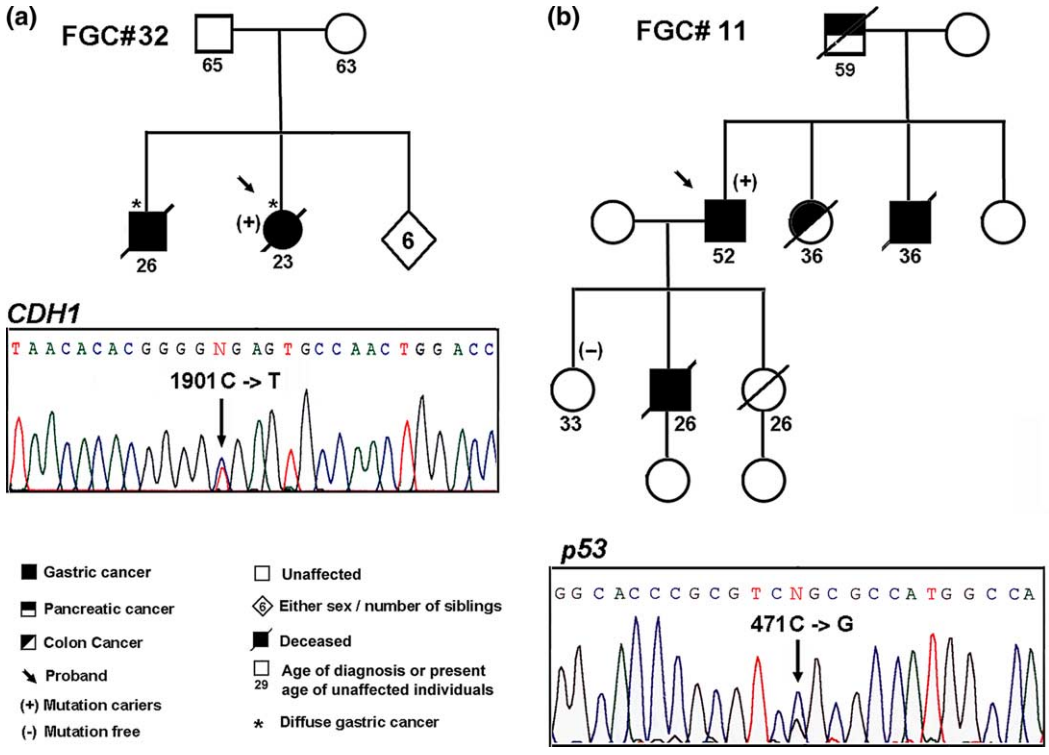

Fig. 1. Family pedigrees and mutation analysis from mutation-positive gastric cancer families. (a) Pedigree from a Portuguese hereditary diffuse gastric cancer (HDGC) family and sequencing analysis of the Ala634Val *CDH1* germline mutation in exon 12. (b) Pedigree from a Portuguese Li-Fraumeni kindred and sequencing analysis of the Arg158Gly *p53* germline mutation in exon 5. The age of onset of the tumours is shown underneath the symbols; (+), Carriers of germline mutation; (–) Subjects that did not carry the mutated alleles in the germline DNA; Arrow, Proband.

showed *CDH1* polymorphisms. The *CDH1* polymorphisms found are described in detail in Table 1.

Since most of our families were negative for *CDH1*, we decided to screen for genes associated with other cancer predisposing syndromes in which gastric cancer also occurs. We screened *p53* gene hotspots for germline mutations (exons 5–8) where 95% of the *p53* mutations cluster [21]. One of the 10 families with familial aggregation of gastric cancer (FGC) (FGC#11) harboured a *p53* germline missense mutation (Tables 1 and 2). This mutation, occurring in *p53* exon 5, was a heterozygous C–G transversion at position 471, leading to an Arg–Gly amino acid change at codon 158 (Table 2, Fig. 1(b)). The proband was diagnosed with gastric carcinoma at the age of 52 years, and had a family history of cancer. Of the three proband's offspring, one died at the age of 26 years with gastric cancer, the other died of an undetermined cause, and the remaining offspring, who is alive and healthy at the age of 33 years screened negative for the *p53* germline mutation. Two of the proband's three siblings died of cancer, one with gastric cancer at the age of 36 years, and the other of colon cancer at the age of 36 years. The proband's father died at the age of 59 years with pancreatic cancer. No material was available from the other affected members of the family.

In all probands negative for *CDH1* and early onset patients, we have also screened the entire coding sequence and splice-sites for *SMAD4* and *Caspase-10*.

For *SMAD4*, two sequence variants were found, not affecting the coding sequence. The first was an alteration in intron 7 (IVS7-31G> A) of unknown significance and not described previously, which was present in 1/10 FDGC and in 1/16 early onset diffuse gastric cancer patients. This sequence alteration was screened in a series of 94 blood donors and one (1.1%) carried the same sequence alteration (Table 1). The second was a silent polymorphism in exon 8, a transition (1086T>C) at codon 362 (Phe362Phe) previously described in the literature, and was present in a FGC family [22]. As for the previous sequence variant, the 1086T>C alteration was present in 1/94 (1.1%) of the blood donors analysed (Table 1).

The *Caspase-10* mutation screening allowed the identification of two new polymorphisms. The first, in intron 5 (IVS5-20C>T) of unknown significance, was present in two families (1/9 HDGC and 1/3 FIGC) and in 3.8% of the 78 blood donors analysed (Table 1). The second polymorphism detected, was a sequence alteration in exon 9 (1217A>T) which changes the Ile406 for a Leucine, and was found in 1/10 FGC and in 1/16 early onset diffuse gastric cancer patients (Table 1). This sequence alteration was also screened in a series of 88 blood donors and three (3.4%) controls carried the same sequence alteration, confirming its polymorphic nature (Table 1).

In cases, in which tumour tissue was available, we performed MSI analysis to exclude HNPCC. We were able to analyse six of the 32 family tumours for MSI and 16 of 23 early onset gastric cancer patient tumours. From the six family tumours, two showed MSI, whereas none of the 16 tumours from early onset gastric cancer patients presented with the MSI phenotype. The two family probands carrying MSI tumours (FGC#1 and FGC#24) were screened for mutations in *hMLH1* and *hMSH2* by DGGE, but no germline mutations of these two genes were found.

4. Discussion

We have collected a series of 55 patients of Portuguese origin which developed gastric cancer, either with a positive family history of gastric cancer or an early age of onset. The sole *CDH1* germline mutation found was present in a family with HDGC. The *CDH1* mutation found in family FGC#32 is a missense mutation (Ala634Val) and was identified in the 23 year old proband. This mutation was shown to harbour dramatic functional consequences *in vitro* [23]. Moreover, Vecsey-Semjen and colleagues [24] found this same Ala634Val mutation in a colon carcinoma cell line and showed that this missense mutation results in the activation of a cryptic splice-site, leading to a premature stop codon. Both experiments support the pathogenic role of this germline missense mutation. Interestingly, none of the parents of the proband of family FGC#32 was affected with gastric cancer, at the time of family history collection, aged 65 and 63 years old. This suggests that either the proband carries a *de novo* mutation in *CDH1*, or that missense mutations in *CDH1*, although proved as pathogenic, may have a lower penetrance in gastric cancer families. The latter hypothesis seems more likely, since the proband's brother had gastric cancer of similar histology, although a *CDH1* study could not be carried out in this patient. This is the first *CDH1* germline mutation described in a Portuguese HDGC family, and represents a low percentage (11.1%) of HDGC families carrying *CDH1* mutations in comparison to the calculated percentage (33.0%) for all the HDGC families studied to date [12]. Portugal is a high-risk country for gastric carcinoma with an incidence of 37 new cases per 100 000 inhabitants per year [25]. This high incidence of gastric cancer in Portugal in comparison to other countries, and the low frequency of germline mutations in high-penetrance genes, raises the possibility that gastric cancer clustering observed in Portuguese families may be related to low-penetrance predisposing genes in association with environmental factors [26,27]. It should be emphasised that the low frequency of *CDH1* germline mutations detect by us is not due to differing sensitivities in our screening methodology (PCR/SSCP/sequencing), since in most (if not all) published studies the methods used were essentially the same [12].

In one of the 10 FGC kindred (FGC#11), we found a *p53* germline mutation, previously described in a Li-Fraumeni kindred [28]. FGC#11 was clinically characterised as a FGC family, since it had three members affected with gastric cancer. This family also had a case of pancreatic cancer and another case of colon cancer at an early age, tumour types which have already been described in HDGC families [12]. Li-Fraumeni syndrome is usually associated with a variety of different tumour types occurring over a wide age range, including in childhood. Although gastric, pancreatic and colon cancers have been previously found in families with this syndrome, paediatric tumours, such as osteosarcomas and brain tumours, are typical tumours associated with the Li-Fraumeni syndrome [8], which makes this family an atypical Li-Fraumeni kindred with excess of gastric cancer. Our results show that *p53* mutations may be involved in a percentage of families with an aggregation of gastric cancer who lack *CDH1* mutations.

We found that two of the families (FGC#1 and FGC#24) carried tumours with MSI, raising the possibility of a HNPCC syndrome. One of these families (FGC#1) that fulfils the HDGC criteria, presented MSI tumours in four affected family members, but no germline mutations of *hMSH2* and *hMLH1* were found [29]. However, in one of the patients the neoplastic lesion showed a lack of *hMLH1* expression which was found to be caused by *hMLH1* promoter methylation, suggesting the possibility of a sporadic tumour occurring within this HDGC family [29]. In the other family (FGC#24) with an aggregation of gastric cancer of unknown histology, MSI was detected in the proband's tumour, but no germline mutations of *hMLH1* or *hMSH2* were found.

In the remaining kindred without an underlying genetic defect, other genes are probably involved. The possibility of *SMAD4* involvement in genetic predisposition to diffuse gastric cancer was raised by the observation of *foci* of adenocarcinoma with signet-ring cells in the stomach of heterozygous knock-out *SMAD4* mice [15]. *Caspase-10* is another candidate gene for familial gastric cancer, possibly through loss of apoptotic function, as suggested by reports of loss of heterozygosity (LOH) and mutations in coding regions of this gene in 15% and 3%, respectively, of sporadic gastric cancer cases [16]. From all of the probands screened in this study, we identified sequence variants in *SMAD4* and *Caspase-10*. However, these sequence variants were either silent or intronic, or a missense alteration that was present with the same frequency as in normal controls (*Caspase-10* 1217A>T), pointing to its polymorphic nature. This observation suggests that these genes are not important candidate genes in explaining the familial gastric cancer cases.

In summary, we report the first *CDH1* germline mutation in Portuguese gastric cancer families. Furthermore, the present report highlights the need for *p53* mutation screening in families with an excess of gastric cancer and demonstrates that these *p53* families should have a distinct clinical follow-up. Finally, the two thirds of HDGC families lacking *CDH1* mutations seemed not to be explained by germline mutations in *SMAD4* and *Caspase-10*.

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