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E-cadherin genetic screening and clinico-pathologic characteristics of early onset gastric cancer

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ABSTRACT

Aim: *CDH1* germline alterations occur in about 40% of hereditary diffuse gastric cancer (HDGC) families. *CDH1* germline mutations are also documented in few early onset diffuse gastric cancer patients (EODGC) without family history, but the real frequency in this setting in unknown. In these patients, the advanced stage at the time of diagnosis remains a clinical burden due to the poor long term survival.

Methods: The entire coding region and exon flanking sequences of the *CDH1* gene was analysed by direct sequencing in 21 EODGC patients aged ⩽50 years. The potential deleterious nature for a new *CDH1* missense variant was assessed by cell–cell aggregation and invasion assays. Somatic *CDH1* mutation, loss of heterozigosity (LOH) and promoter hypermethylation was explored in the tumour from one *CDH1* germline mutation carrier.

Results: Two novel *CDH1* germline variants were identified in 21 EODGC cases, c.670C>T and –63C>A. Functional analysis of the c.670C>T missense variant classified this mutation as non-pathogenic. The analysis of *CDH1* somatic second hits failed to demonstrate E-cadherin structural and epigenetic alterations in the tumour sample.

Conclusion: Data from the present work and a systematic review of the literature revealed that *CDH1* germline mutations occurred in 7.2% of EOGC patients invariably with diffuse of mixed histology. From these, proved *CDH1* mutation pathogenicity has been assigned only to 2.3% of the cases who were recurrently diagnosed before 35 years old. Germline *CDH1* mutation remain the only germline genetic defect described in this type of patients and *CDH1* mutation screening should be recommended for patients with these characteristics.

1. Introduction

Gastric cancer (GC) represents the second most frequent cause of cancer related death and the 4th most common malignancy worldwide.[1–4]

Several genetic, epigenetic and environmental factors interact simultaneously in the early steps of gastric carcinogenesis. Amongst these, human genetic polymorphisms of some inflammatory-related genes are thought to be responsible for an increased risk of GC.[5,6] Other factors such as

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tobacco consumption, dietary habits and *Helicobacter pylori* infection[7–9] have been demonstrated to be involved in the multifactor process of gastric carcinogenesis.

Over the last years, the incidence of GC, especially the intestinal histotype, has been decreasing in older patients, remaining otherwise stable in young patients and in cases with diffuse histotype.[10] This would suggest that the aetiology of GC in young people may be different compared to older subjects. In the older population, the association of different environmental factors and epigenetic changes frequently relates to GC development. In young patients, the role of genetics is presumably greater, environmental carcinogens playing a less significant role.[11]

EOGC is defined as any GC presenting at the age of 45 or earlier and represents approximately 10% of all patients with stomach cancer with reported frequencies varying between 2.7% and 15% according to different populations studied.[12,15–17] GC patients younger than 40–45 years old are believed to develop GCs involving molecular pathways different from those of sporadic carcinomas that occur later in life.[12–14] GC that occurs in patients younger than 30 years is very rare (1.1–1.6%) and most of EOGCs are diagnosed in patients older than 35.[18,19] GC's diagnosed before 20 years of age are exceptional and current literature is limited to a small number of cases.[16,20,21]

EOGC may occur in sporadic or hereditary forms. Germline inactivating mutations and deletions of the E-cadherin gene (*CDH1*) are a well-documented genetic factor associated with diffuse histotype of EOGC (EODGC) or with the HDGC syndrome.[22–24] HDGC is an autosomal dominant cancer disease and represents 1% of all GCs and notably, germline alterations of the *CDH1* gene are detected in about 40% of families that fulfil the clinical criteria for the HDGC syndrome.[24–26]

Germline mutations of *CDH1* gene have also been documented in diffuse EOGC with and without inherited predisposition for the HDGC syndrome.[22,23,27] Considering the apparently sporadic cases, about 6% of diffuse EOGCs are described to carry a constitutional potentially deleterious sequence variant of E-cadherin gene.[28]

In this study, we screened for the presence of *CDH1* germline alterations in 21 EOGC patients with diffuse histotype and without family history of GC. Additionally, we revised the clinico-pathologic and molecular features presented by EOGC described in the literature, namely in those patients carrying *CDH1* germline alterations.

2. Methods

2.1. Patients and sample preparation

From 1993 to 2005, 26 patients with diagnosed sporadic diffuse GC and age at onset $\leqslant$50 years were admitted at the Department of Human Pathology and Oncology, Section of Surgical Oncology, of the University of Siena, Italy; 5 patients were excluded since they not have stored biological material. Twenty-one GC patients younger than 50 years old ($n = 11$ younger than 45 and $n = 10$ older than 45) were therefore included in the study. Family histories were obtained with informed consent and pedigree analysis revealed that all GC cases were apparently sporadic forms of GC. Selected patients presented diffuse histotype of GC according to Laurén classification.[29]

Genomic constitutional DNA was extracted from peripheral whole blood using a standard protocol. To test the frequency of identified alterations, a population of 224 blood donors was used as negative control. The study protocol was reviewed and approved by the local ethical committee.

In one case, DNA was extracted from macrodissected tumour areas with at least 80% of neoplastic cells from paraffin-embedded tissue using Invisorb Spin Tissue Mini Kit (Inviteck, Berlin, Germany). This material was used to characterise molecular alterations in tumour DNA in comparison to constitutional DNA.

2.2. Germline and somatic CDH1 mutation screening, somatic analysis of CDH1 promoter hypermethylation and LOH

CDH1 germline mutations were searched for in 21 EODGC patients. Briefly, each exon and intron–exon boundary of the *CDH1* gene was amplified by PCR and directly sequenced. Primers and conditions were as previously reported[29] (primer sequences available upon request).

Somatic *CDH1* alterations, which could work as *CDH1* inactivating second hits, were screened for in macrodissected tumour material from one EODGC patient that displayed a germline *CDH1* alteration. Somatic mutations were analysed in tumour DNA following the same strategy used for the above mentioned gemline mutation screening. Promoter hypermethylation was evaluated using 200 ng/μl of bisulphite treated (EpiTect Bisulfite Kit, Qiagen, Valencia, California, USA) tumour DNA. An essential region of 221 base pairs of the *CDH1* promoter was amplified using primers flanking but not encompassing CpG sites (primer sequences available upon request). PCR products were purified and directly sequenced. Detailed methods were described earlier.[31] To rule out the possibility of LOH in the tumour, known intragenic single nucleotide polymorphisms (SNPs) in the *CDH1* promoter region [–161 C/A (*rs16260*)], exon 13 [2076 T/C (*rs1801552*)] and 3'-UTR SNP (*rs1801026*), as well as the germline mutation found in one patient, were amplified by PCR and sequenced from normal and tumour material. When for a specific marker, a sample harboured a reduction in the peak area of one allele equal or greater than 90%, in comparison to the other allele, LOH was considered for that specific marker.

2.3. Characterisation of the impact in splicing for the mutation c.670C>T (p.Arg224Cys)

Netgene2 software (http://www.cbs.dtu.dk/services/netgene2) was used to predict the impact of *CDH1* missense mutations in creating cryptic splice sites. In light of such predictive results, RNA was isolated from frozen white peripheral blood lymphocytes (PBLs) from the patient of interest as well as from *CDH1* mutation negative controls using Trizol Reagent (Life Technologies, Inc., Paisley, Scotland) following the manufacture's instructions. Total RNA was used to synthesise first-strand cDNA (Invitrogen SuperScript® III Reverse Transcriptase, Milan, Italy) according to the manufacturer's protocol. *CDH1* transcripts were amplified using a primer set

designed to amplify *CDH1* exons 4–6 that flank the c.670C>T sequence variant (cDNA_Ex4_F: 5'-GCT CAC ATT TCC CAA CTC CTC T-3′; cDNA_Ex6_R: 5′-ATT CGG GCT TGT TGT CAT TCT-3′). To investigate the generation of cryptic splice sites induced by the presence of the missense mutation c.670C>T, RT–PCR products from the carrier and from a control were run in an 2% agarose gel. Band size and migration patterns were compared in both samples.[32]

2.4. Characterisation of the effect of the p.Arg224Cys germline missense mutation on E-cadherin function

The theoretical impact of p.Arg224Cys mutation was firstly evaluated *in silico* by using the *Sorting Intolerant From Tolerant* (SIFT) software.[33] *In vitro* functional relevance was assessed on the basis of its impact on cell–cell adhesion and cell invasion as described earlier.[23] For this purpose, a construct was generated using a pLenti virus based expression vector (pLenti6/V5 Directional TOPO® Cloning Kit; Invitrogen) with an inserted full cDNA sequence of *CDH1* that previously underwent site-directed mutagenesis to include the mutation c.670C>T (that give rise to the mutation p.Arg224Cys in the E-cadherin protein). Chinese Hamster Ovary (CHO) cells were then stably transduced with the following expression constructs: empty vector (mock cells) and wild-type *CDH1* cDNA vector (previously generated in the lab) as well as with the *CDH1* cDNA construct including the mutation p.Arg224Cys. Protein expression levels and localisation were evaluated by Western Blot and immunocytochemistry using the human E-cadherin monoclonal antibody HECD-1 (R&D Systems, 1/200 dilution). For the slow aggregation assay, after trypsinization of each stable cell line, 2×10^4 cells were transferred to an agar gel (0.66%, w/v) in a 96-well plate and then incubated at 37 °C under 5% CO_2 in humidified air. Aggregate formation was evaluated qualitatively after 24 and 48 h using an inverted microscope. The formation of compact cellular aggregates was considered to be a feature of normal cell–cell adhesion. In contrast the presence of small and lose cell clusters was considered as suggestive of impaired cell adhesion.

The same cell lines were tested in the Matrigel invasion assay. Matrigel invasion chambers were purchased from BD (Bedford, USA) and the assay was performed following manufacturer's instructions. In brief, 1×10^5 cells were seeded on top of the Matrigel layer inside the plastic chambers and incubated for 22 h at 37 °C. Invasion indices were expressed as ratios between the number of invasive cells (cells able to degrade the Matrigel layer and cross the semi-permeable membrane at the bottom of the chamber) and the number of cells initially seeded. Each functional assay was repeated at least three times in the same conditions.

3. Results

3.1. CDH1 genetic screening revealed two novel germline potentially deleterious sequence variants

Table 1 encompasses the main clinico-pathologic features of the 21 gastric carcinoma patients considered in this study.

We performed *CDH1* germline mutation screening in our series of 21 EOGC patients and 2/21 (9.5%) were found to display previously unidentified germline *CDH1* sequence variants. None of the remaining 19 patients displayed other potentially deleterious sequence variants of *CDH1*.

The first patient, a 46 years old female with diffuse GC without further history of gastric cancer in the family, presented a heterozygous missense c.670C>T alteration that affects codon 224 of *CDH1* exon 5 and leads to a substitution of an Arginine to a Cysteine (p.Arg224Cys) (Fig. 1A). This sequence variant was identified in a single blood donor subject from our series of 244 cancer free individuals used as controls, indicating a population frequency of 0.4%.

In a second patient with 42 years old male with diffuse GC without further history of gastric cancer in the family, a new *CDH1* sequence variant was identified at the at the 5'UTR region, position –63 from the ATG (Fig. 1B). This sequence alteration was never found in the control population used (0/244).

3.2. The c.670C>T germline sequence variant does not induce cryptic splicing of CDH1

Netgene2 software was used to predict, *in silico*, the potential for the c.670C>T *CDH1* missense mutation in inducing cryptic splicing in the vicinity of the canonical donor splice-site of *CDH1* exon 5. By applying this tool, we verified that a new potential cryptic splice-site was expected to be created 42 base pairs upstream of the canonical splice-site (data not shown). We then tested this possibility by analysing whether this would occur *in vivo* using cDNA produced from PBLs retrieved from the patient harbouring the c.670C>T mutation. In contrast to the *in silico* prediction, the PCR fragment generated using primers flanking the mutation in the mutation carrier and in the control sample did not show differences when run in a 2% agarose gel. This result was indicative that a potential cryptic splicing was not likely to be occurring as a consequence of the c.670C>T missense mutation.

3.3. The c.670C>T (p.Arg224Cys) missense mutation does not impair E-cadherin induced cell adhesion and invasion in vitro

The theoretical impact of p.Arg224Cys mutation was firstly evaluated *in silico* by using the Sorting Intolerant From Tolerant (SIFT) software[33] that predicted a deleterious nature for this novel missense variant.

Two of the most relevant biological effects mediated by E-cadherin are the promotion of homotypic cell–cell adhesion and the ability of suppressing cell invasion. To assess these parameters *in vitro*, we measured slow aggregation in soft agar and cell invasion through a Matrigel matrix of CHO E-cadherin negative cells engineered to stably express the c.670C>T (p.Arg224Cys) mutant, the wild-type *CDH1* cDNA, as well as a negative control (empty vector). We verified that cells expressing the p.Arg224Cys sequence variant maintained the ability to form compact aggregates at a similar level to that of the wild-type *CDH1* expressing cells, while the negative control cells (transduced with the empty vector or mock cells) were not able to form compact cell aggregates (Fig. 2). Moreover, when seeded on Matrigel chambers, the p.Arg224Cys expressing cells behaved in a similar manner to cells transduced with the wild-type *CDH1*, retaining the

Table 1 – Clinico-pathologic characteristics and *CDH1* alterations identified in 21 patients with sporadic diffuse gastric cancer.

ID code	Age onset	Gender	Tumour location	Làuren classification	Depth invasion (pT)	Lymph node involvement (pN)	Stage grouping	*CDH1* variants
MF164	49	Female	Non-cardia	Diffuse	pT 2	pN1	II	–
ZM227	46	Male	Non-cardia	Mixed	pT 2	pN2	III	–
MP245	42	Male	Non-cardia	Diffuse	pT 1	pN0	I	–63C>A
FA286	30	Male	Cardia	Diffuse	pT 3	pN2	III	–
DA290	30	Female	Non-cardia	Diffuse	pT 1	pN0	I	–
PB311	50	Female	Non-cardia	Diffuse	pT 3	pN2	III	–
PA320	38	Male	Non-cardia	Diffuse	pT 2	pN2	III	–
DA328	45	Male	Non-cardia	Diffuse	pT 2	pN3	III	–
PP382	38	Male	Non-cardia	Diffuse	pT 2	pN2	III	–
CS425	48	Male	Cardia	Mixed	pT 3	pN3	IV	–
TF432	50	Female	Linitis plastica	Diffuse	pT 4	pN3	IV	–
SG450	49	Male	Linitis plastica	Diffuse	pT 3	pN1	III	–
DE462	43	Female	Linitis plastica	Diffuse	pT 4	pN3	IV	–
CA471	46	Female	Non-cardia	Diffuse	pT 3	pN3	IV	c.670C>T
ZG1	33	Male	Non-cardia	Diffuse	pT 3	pN3	IV	–
PD8	46	Female	Non-cardia	Diffuse	pT 1	pN1	I	–
CS96	40	Male	Non-cardia	Diffuse	pT 1	pN0	I	–
CC442	46	Male	Non-cardia	Diffuse	pT 2	pN2	III	–
BG414	43	Male	Non-cardia	Diffuse	pT 3	pN1	III	–
MF24	45	Female	Linitis plastica	Diffuse	Not resected	–	–	–
NC410	31	Female	Non-cardia	Diffuse	pT 4	pN2	IV	–

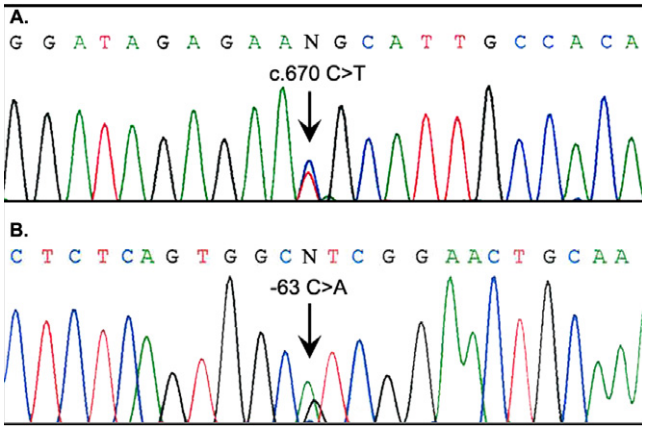

Fig. 1 – Sequencing chromatogram representing novel *CDH1* germline mutations identified in EODGC patients. (A) missense mutation c.670C>T that results in the aminoacid substitution p.Arg224Cys, and (B) promoter substitution –63C>A from the ATG.

capacity to prevent invasion in clear contrast to what was observed for mock cells. These data suggest that the p.Arg224Cys missense mutation is most likely a non-pathogenic *CDH1* sequence variant.

3.4. *The tumour from the c.670C>T CDH1 germline sequence variant carrier did not display additional CDH1 somatic mutations, promoter hypermethylation nor LOH as second hits*

In order to determine whether the tumour from the c.670C>T *CDH1* sequence variant carrier harboured a second somatic hit, inactivating the remaining germline unaltered allele, we analysed DNA extracted from an area enriched in neoplastic cells. We initially screened for somatic mutations and verified that no somatic mutations were detectable in the tumour DNA. Moreover, neither *CDH1* promoter hypermethylation nor LOH were also detected. We further verified that heterozygosity at SNPs either in the *CDH1* promoter region (–161C/A [*rs16260*]) or in the mRNA sequence (2076T/C [*rs1801552*] and 3′-UTR [*rs1801026*]) as well as at the site of the germline sequence variant was retained in the tumour.

Due to lack of tumour material, we were not able to characterise the second hit inactivating the remaining germline unaltered allele in the patient carrying the –63C>A sequence variant.

4. Discussion and review of the literature

4.1. *CDH1 germline sequence variants and EOGC*

CDH1 encodes the E-cadherin tumour suppressor protein that acts as a trans-membrane calcium dependent glycoprotein and plays an essential role in the intercellular adhesion between epithelial cells.[34]

Decreased E-cadherin expression is often found in several epithelial neoplasias during tumour progression and is correlated with a higher infiltrative and metastatic ability of the tumour[35] due to loss of cell adhesion and increased cell motility.[36]

Typically, inactivating E-cadherin alterations are present in a fraction of both sporadic and hereditary diffuse GC.[22,24,31,37,38] Moreover, several authors identified *CDH1* germline alterations in apparently sporadic EOGC, always presenting diffuse or mixed histotype.[23,39–42] The largest series of diffuse EOGC screened for *CDH1* germline mutations was reported by Bacani and colleagues who identified eight germline sequence variants in 81 (11.1%) patients younger

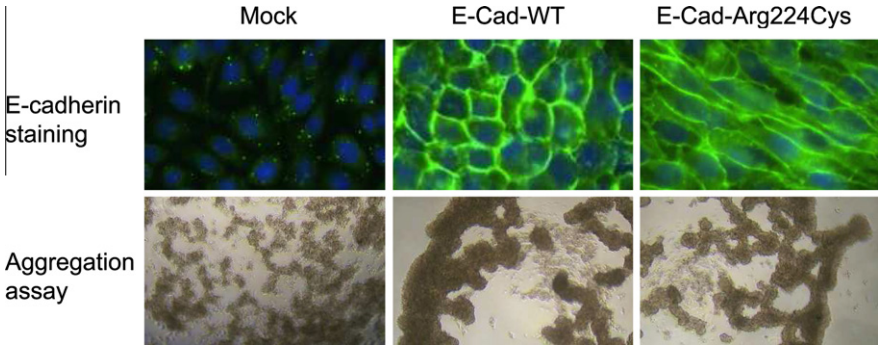

Fig. 2 – Functional analysis of *CDH1* missense mutation p.Arg224Cys. The upper panel displays representative images of the E-cadherin localisation in CHO transduced cells (Mock; E-cad-WT and p.Arg224Cys) by immuno-fluorescence analysis. The lower panel displays representative images obtained after the aggregation assay performed in soft agar with the same cell lines.

than 50 year old, in the Central-East Ontario region, Canada, a region of low incidence of GC.[40] Suriano and Oliveira also described a large series of 54 diffuse or mixed type EOGC patients for which five (9.3%) germline sequence variants were reported.[30]

We selected a series of diffuse EOGC patients younger than 50 years old with apparently negative GC family history, from a region in Italy with high incidence of GC (4.2/10000 inhabitants; ISTAT 2002), and analysed the presence of *CDH1* germline sequence variants. Considering that Bacani[40] identified *CDH1* germline mutations also in sporadic DGC patients with age of onset of 50 years, we have established the 50 years at diagnosis as the cut-off limit. Herein, we report the presence of CDH1 germline sequence variants in 2/21 cases (9.5%). This frequency is similar to that obtained when combining all reports on the same issue available in the literature, in which 7.2% of the EOGC cases were reported to harbour germline *CDH1* sequence variants, most of them with unknown functional significance (Table 2).

Apart from analysing the frequency of germline *CDH1* alterations in EOGC, we also addressed the potential *in silico* and *in vitro* functional impact of one missense sequence alteration (p.Arg224Cys) herein identified. Despite obtaining a deleterious nature for this alteration using *in silico* prediction, the E-cadherin protein function was preserved when *in vitro* functional analysis was performed, as ability to mediate cell–cell adhesion and suppress cell invasion was retained. This single nucleotide alteration (c.670C>T – p.Arg224Cys) was searched for in a control population and its frequency was proved to be very low (0.4%). Moreover, the other identified alteration also found in the present report (–63C>A) was never found in the population and its functional effect was not possible to address. The C-nucleotide at this position is conserved in *Homo sapiens*, *Pan troglodytes* and *Gorilla gorilla* but is not conserved in other species (data not shown) and therefore an alteration at this position may not have a great deleterious effect. Therefore, it is likely that both alterations are new rare polymorphic variants rather than germline sequence variants associated with increased susceptibility of GC. Another possibility, that was not addressed in this report, is the putative role of these two sequence variants in the activation of other E-cadherin related pathways such as the EGFR pathway. Although not likely for the -63C>A, as it localizes at the promoter region and does not impact the protein sequence, the c.670C>T mutation may have a role in the modulation of E-cadherin downstream effector pathways.

To date, 264 apparently sporadic diffuse or mixed EOGC patients aged 51 years old or less have been screened for the presence of *CDH1* germline sequence variants (for references see Table 2). From these 264 EOGC cases, 19 (7.2%) carried *CDH1* constitutional germline sequence variants but only 2.3% (6/264) of them did in fact represent variants with a proven potentially deleterious effect (Table 2). This predicted pathogenicity that is depicted in Table 2 was based on the type of mutation (frameshift) or on the results obtained from *in vitro* functional analysis.

Five of the six germline pathogenic *CDH1* mutations were detected in EOGC patients from low or moderate incidence GC areas (Table 2), whereas only one arose in a patient from Portugal, a high incidence GC area. Although CDH1 germline mutations have been searched for in EOGC patients from other areas where high incidence rates of GC are verified, namely in countries like China, Korea, Japan, and even Italy, no deleterious *CDH1* germline variants have been identified (Table 2).

Gathering all data on germline sequence variants reported in diffuse and mixed EOGC, both from the literature and herein presented, functional assessment is the only currently available mean to shed light into the role of *CDH1* missense mutations. Although *in silico* tools for prediction of cryptic splicing, protein function and deleterious effects, as well as for conservation analysis may be used to weigh up missense, intronic and promoter mutation impact, such an evaluation frequently fails when used on its own.

The presence of genetic and epigenetic second hit events affecting the remaining *CDH1* wild-type allele, in tumour DNA from EOGC patients carrying *CDH1* germline variants, may also help in interpreting the deleterious effect of a given sequence variant. In line with our previous results, we were unable to detect *CDH1* somatic mutation, promoter methylation or LOH in the tumour from the p.Arg224Cys mutation carrier, re-enforcing the thesis that this alteration is most probably not deleterious.

Table 2 – Summary of reported diffuse and mixed histotype EOGC cases screened for *CDH1* germline sequence variant.

Ref.	Total EOGC screened *n* (age setting)	*CDH1* Sequence variants (%)	Ethnicity	Histotype	Age onset	Population frequency	*CDH1* sequence variant	Mutation Type	Gene position	Predicted protein change	Predicted Pathogenicity
39	1	1	**New Zeland**	**Diffuse**	**31**	**Nd**	**c.1487del7**	**Frameshift**	**Exon 10**	**PTC_556**	**Yes**
30	**54[b] (age⩽51)**	**5 (7.6%)**	Africa/USA	Diffuse	43	1%	c.1849G>A	Missense	Exon 12	p.Ala617Thr	No
			Africa/USA	Diffuse	43	1%	c.1849G>A	Missense	Exon 12	p.Ala617Thr	No
			Portugal	**Diffuse**	**30**	**0%**	**c.1901C>T[a]**	**Missense/ splice-site**	**Exon 12**	**p.Ala634Val**	**Yes**
			England	Diffuse	51	0%	c.532-18C>T	Intronic	Intron 4	Nd	No
			Portugal	Mixed	42	0%	c.532-18C>T	Intronic	Intron 4	Nd	No
23	**10 (age⩽35)**	**2 (20%)**	**USA**	**Diffuse**	**27**	**Nd**	**c.1285C>T**	**Missense**	**Exon 9**	**p.Pro429Ser**	**Yes**
			USA	**Diffuse**	**27**	**Nd**	**c.1063delT**	**Frameshift**	**Exon 8**	**PTC_355**	**Yes**
41	**15 (age⩽45)**	**1 (6.7%)**	**Europe**	**Diffuse**	**29**	**Nd**	**c.1619_1620insG**	**Frameshift**	**Exon 11**	**PTC_546**	**Yes**
40	81 (age <50)	8 (9.9%)	USA	Diffuse	Na	Nd	-117G>A	5′-UTR substitution	Promotor	Nd	Nd
			USA	Diffuse	Na	3.90%	-71C>G	5′-UTR substitution	Promotor	Nd	Nd
			Europe/USA	**Diffuse**	**30**	**Nd**	**c.41delT**	**Frameshift**	**Exon 1**	**PTC_55**	**Yes**
			USA	Diffuse	Na	0%	c.48-5G>C	Intronic	Intron 1	Nd	Nd
			USA	Diffuse	Na	Nd	c.48-15C>G	Intronic	Intron 1	Nd	Nd
			USA	Diffuse	Na	Nd	c.387+26C>T	Intronic	Intron 3	Nd	Nd
			USA	Diffuse	Na	Nd	c.2295+53G>A	Intronic	Intron 14	Nd	Nd
			USA	Diffuse	Na	Nd	c.2439+31G>A	Intronic	Intron 15	Nd	Nd
Present report	21 (age⩽50)	2 (8.9%)	Italy	Diffuse	50	0%	–63C>A	5′-UTR substitution	Promotor	Nd	Nd
			Italy	Diffuse	45	0.4%	c.670C>T	Missense	Exon 5	p.Arg224Cys	No
63	9 (age<35)	–	Japan	Diffuse	<35	–	–	–	–	–	–
43	9 (age<50)	–	USA	Diffuse	<50	–	–	–	–	–	–
64	24 (age<50)	–	China	Unknown	<50	–	–	–	–	–	–
13	40 (age<45)	–	Europe and USA	21 Diffuse 9 mixed	<45	–	–	–	–	–	–
Total	**264**	**19 (7.2%)**	–	–	–	–	–	–	–	–	**6/264 (2.3%)**

Note. PTC, premature termination codon; Na, not available; Nd, not determined.

[a] This mutation also generates a cryptic splice-site at the site of the missense change leading to the generation of premature termination at codon 653.

[b] The whole series encompassed 66 EOGC cases, from which only 54 were of diffuse or mixed histology; bold, predicted pathogenicity based on mutation type (frameshift) or *in vitro* functional analysis.

The most interesting finding from the previous analysis is the fact that the 2.3% EOGC cases displaying deleterious *CDH1* germline alterations were always younger than 35 years old and their tumours always presented diffuse histology (Table 2). These results are important as they impact the clinical management, counselling and molecular diagnosis to be offered to EOGC patients in the near future. This observation has been also previously advanced in smaller series of cases[42,43] and based on these strong evidences, *CDH1* mutation screening is now recommended for young patients with diffuse GC and the cut-off for the age of diagnosis of EOGC was accepted to be decreased from 50 to 35 years.[26]

4.2. *Clinical and pathological features of EOGC and older GC patients*

EOGC patients and patients with GC at older ages clearly display very different clinical and pathological features. Those younger than 30 years are most commonly females,[17,44,45] while older ones are most frequently male patients.[9] Based on these findings, it has been hypothesised that the development of GC in young women may be significantly influenced by natural, biological and hormonal factors,[46] nevertheless this has never been proven.

Positive family history of GC has been reported in about 10% of young patients with diffuse histotype of GC,[47] and therefore these patients are frequently considered strong candidates to be part of HDGC families. In such cases, families should be offered genetic counselling and should be tested for the presence of germline alterations at the *CDH1* gene.[25,48]

EOGC occur more frequently in the proximal stomach at the gastroesophageal junction[49,50] with diffuse histotype and signet ring cell features while these features are less frequent in older patients.[49–53]

Patients with EOGC are frequently reported to display a worse long-term prognosis due to a delay in diagnosis and a more aggressive course of the disease,[44,54,55] but this view is debatable as long-term results have also been reported to be similar to those of the older population with GC,[56–61] instead stage of disease was the most important prognostic factor.[49]

The advanced stage of the tumour in many young patients represents a clinical burden for the physician who has little if no therapeutic options for patients. The lack of symptoms in the early stages of GC may be the main cause of the delayed diagnosis with the subsequent poor clinical outcome and the cause of the advanced disease in young patients.[62,63]

5. Conclusion

Approximately 2.3% of EOGC cases, without family history of GC, display deleterious *CDH1* germline alterations. These tumours invariably present partial (mixed) or complete diffuse histology and occur in patients younger than 35 years old. Germline *CDH1* mutation screening is now recommended for patients with these characteristics.

From the clinico-pathologic stand point, EOGC preferentially occur in female gender presenting tumours with diffuse histology, multifocal appearance, proximal location and poor outcome. In EOGC patients, the advanced stage at the time of diagnosis remains a clinical burden due to the poor long term survival.

Aetiological factors of EOGC remain unknown as environmental agents represent a minor component and therefore a high impact of genetic causes is predicted. *CDH1* germline mutations in well documented diffuse EOGC cases remain the only germline genetic defect described in this type of patients.

Conflict of interest statement

None declared.

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