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**Histopathological, molecular and genetic profile of Hereditary Diffuse Gastric Cancer: Current knowledge and challenges for the future**

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**Abstract**

Familial clustering is seen in 10% of gastric cancer cases and approximately 1-3% of gastric cancer arises in the setting of hereditary diffuse gastric cancer (HDGC). In families with HDGC, gastric cancer presents at young age. HDGC is predominantly caused by germline mutations in *CDH1* and in a minority by mutations in other genes, including *CTNNA1*. Early stage HDGC is characterized by a few, up to dozens of intramucosal *foci* of signet ring cell carcinoma and its precursor lesions. These include *in situ* signet ring cell carcinoma and pagetoid spread of signet ring cells. Advanced HDGC presents as poorly cohesive/diffuse type carcinoma, normally with very few typical signet ring cells, and has a poor prognosis. Currently, it is unknown which factors drive the progression towards aggressive disease, but it is clear that most intramucosal lesions will not have such progression.

Immunohistochemical profile of early and advanced HDGC is often characterised by abnormal E-cadherin immunoexpression, including absent or reduced membranous expression, as well as “dotted” or cytoplasmic expression. However, membranous expression of E-cadherin does not exclude HDGC. Intramucosal HDGC (pT1a) presents with an “indolent” phenotype, characterized by typical signet ring cells without immunoexpression of Ki-67 and p53, while advanced carcinomas (pT>1) display an “aggressive” phenotype with pleomorphic cells, that are immunoreactive for Ki-67 and p53. These features show that the IHC profile is different between intramucosal and more advanced HDGC, providing evidence of phenotypic heterogeneity, and may help to define predictive biomarkers of progression from indolent to aggressive, widely invasive carcinomas.

## 1. Introduction

Gastric cancer (GC) is the fifth leading cause of cancer globally and ranks third in terms of cancer-related mortality [1]. GCs display various morphological phenotypes reflected in a large number of suggested histopathological classification schemes. The most commonly used are the classification of the World Health Organization (WHO) [2] and the classification by Laurén [3]. The Laurén classification is often used to classify GC into three broad categories, namely intestinal type, diffuse type and a remaining group of GC that cannot be placed in one of these two categories [3]. Intestinal type GC is composed of tumour cells with glandular, tubular or papillary growth pattern with various degrees of differentiation. Diffuse type GC consists of solitary or small clusters of poorly cohesive cells that frequently infiltrate in a diffuse pattern with or without a small component of gland formation. Typical signet ring cells (SRCs) often characterize diffuse GC and when the tumour is composed of predominantly (more than 50%) SRCs, the tumour is also referred to as signet ring cell carcinoma (SRCC).

Although most GCs are sporadic, familial aggregation is known to occur in around 10-20% of patients. Incidences described range from 2.8% in Sweden to 36.6% in Japan and are different between low- and high-risk areas [4-7]. Familial gastric cancer can be classified as hereditary diffuse gastric cancer (HDGC), familial intestinal gastric cancer (FIGC) and, when the histopathology of tumours is unknown, as familial gastric cancer (FGC) [8]. Among these groups, only 1-3% are related to known specific genetic causes with the most important GC susceptibility gene for HDGC being *CDH1*.

In 2012, a new hereditary gastric cancer syndrome was identified, and was coined GAPPS (Gastric Adenocarcinoma and Proximal Polyposis of the Stomach), which is an autosomal

dominant condition characterised by fundic gland polyposis with increased risk of developing intestinal type GC and so far unknown genetic cause [9, 10].

Moreover, GC risk is elevated in several other hereditary cancer syndromes, namely Lynch syndrome caused by germline mutations in one of the DNA mismatch repair genes [11-13], Li-Fraumeni syndrome caused by *TP53* germline mutations [14-16], familial adenomatous polyposis caused by *APC* germline mutations [17, 18], Peutz-Jeghers syndrome caused by *STK11* germline mutations [19-21], juvenile polyposis syndrome caused by *SMAD4* or *BMPR1A* germline mutations [22, 23], and hereditary breast or ovarian cancer syndrome caused by *BRCA1* or *BRCA2* germline mutations [10, 24, 25].

In this chapter, we discuss the current knowledge of HDGC, particularly *CDH1* mutation related HDGC, and provide new insights into the phenotypic characteristics of early and advanced HDGCs using immunohistochemical biomarkers of cell adhesion, proliferation, anoikis, epithelial-mesenchymal-transition and cancer cell stemness.

**2. Genetics of HDGC**

Already in 1964, Jones reported familial clustering of GC among a large Māori kindred in New Zealand [26]. However, it took until 1998 to identify germline mutations in *CDH1* in three Maori families as the cause of HDGC by linkage analysis [27]. The E-cadherin gene, *CDH1,* is located on chromosome 16q22.1. The 120 kDa glycoprotein encoded by *CDH1* displays a large extracellular domain, a transmembrane segment and a short cytoplasmic domain [28]. E-cadherin is a transmembrane calcium-dependent protein and is mainly expressed at the basolateral membrane of epithelial cells, where it has important roles in cell-cell adhesion at the *adherens* junctions to maintain epithelial integrity [29, 30]. Heterozygous germline *CDH1* mutations have been described in 18-40% of HDGC families [31-35]. The frequency of *CDH1*

mutations seems to be highly variable, which may be related to the variable incidence of GC across different geographic regions. Overall, in more than 60% of HDGC families, the role of *CDH1* germline deficiency is unclear.

There are a few other genes which are involved in HDGC predisposition, including *CTNNA1*. Like *CDH1*, *CTNNA1* is involved in intercellular cell adhesion. *CTNNA1* encodes the protein α-E-catenin, which functions in a complex with β-catenin where it binds the cytoplasmic domain of E-cadherin to the cytoskeleton [36, 37]. α-E-catenin inhibition has been shown to destabilize *adherens* junctions, weakening the interaction between cells [38]. Currently, three families have been described with *CTNNA1* germline mutations [35, 39]. Loss of α-E-catenin expression with preservation of E-cadherin has been observed in GC identified in *CTNNA1 mutation carriers*. These families show a clinical picture similar to that of *CDH1*-mutation positive families, however there is insufficient data available to make a statement on disease penetrance.

There are HDGC families with mutations in genes associated with other cancer-predisposition syndromes, such as *BRCA2*. Germline *BRCA2* mutations predispose to hereditary breast and ovarian cancer. In some families with *BRCA2* mutations, an increased incidence of GC has been encountered [40-43], with one family fulfilling the HDGC criteria [35].

Germline mutations in *MAP3K6 gene* have been described in families with FGC, although the role of these mutations is yet to be proven [44]. In *CDH1*-negative HDGC families, multiplexed targeted sequencing of cancer associated genes led recently to the identification of new germline mutations in several genes, such as *CTNNA1*, *BRCA2*, *STK11*, *SDHB*, *PRSS1*, *ATM*, *MSR1*, and *PALB2* [35]. It is likely that other HDGC associated genes will be discovered in the near future through next-generation-sequencing (NGS) empowered

methodologies. However, to assess pathogenicity, disease penetrance and management for newly identified gene mutations, multiple mutation positive families have to be studied and outcomes have to be collected at a global level.

### 2.1 Germline *CDH1* mutation and clinical guidelines

HDGC caused by germline *CDH1* mutations is an autosomal dominant cancer-susceptibility syndrome. Germline *CDH1* alterations can affect the entire coding sequence including small frameshifts, splice-site, nonsense, missense mutations as well as large rearrangements [45, 46]. Most truncating mutations in *CDH1* are pathogenic and several missense *CDH1* mutations have been shown to have a deleterious effect on E-cadherin function [47].

Mutation carriers have an increased risk of developing diffuse type GC (DGC) as well as lobular breast cancer (LBC). In a recent study, penetrance data for *CDH1* mutation carriers has been updated based on affected individuals, who presented clinically with DGC or LBC, from 75 families with germline, pathogenic truncating *CDH1* mutations [35]. The cumulative risk of DGC for *CDH1* mutation carriers by the age of 80 years is reported to be 70% for men (95% confidence interval [95% CI], 59-80%) and 56% for women (95% CI, 44-69%), though there is no clear explanation why this risk is different for men and women. Furthermore, the cumulative risk of LBC for women with a *CDH1* mutation by the age of 80 years is estimated to be 42% (95% CI, 23-68%) [35].There is currently no evidence that the risk of other cancer types in individuals with a *CDH1* mutation is significantly increased.

In 1999, the International Gastric Cancer Linkage Consortium (IGCLC) defined families with the HDGC syndrome (OMIM #137215) as those fulfilling one of the two following criteria [8]:

(1) Two or more documented cases of diffuse gastric cancer (DGC) in first- or second-degree relatives, with at least one being diagnosed before the age of 50 years; or

(2) Three or more cases of DGC in first- or second-degree relatives, independent of age of diagnosis.

Families with aggregation of GC and an index case with DGC, but not fulfilling the IGCLC criteria for HDGC, should be classified as familial diffuse gastric cancer (FDGC) [8]. IGCLC criteria were updated in 2010 [45] and again more recently in 2015 [48]. The recently published guideline broadened the clinical criteria to select patients for *CDH1* mutation analysis. The above mentioned criteria were merged into a new criterion: “Two or more GC cases regardless of age, at least one confirmed DGC, in first- and second-degree relatives.” In addition, new criteria were added for genetic testing and family management, including families with: bilateral or multiple cases of LBC without DGC, and families with DGC and cleft lip/cleft palate. The precise criteria are described in **Table 1**.

*CDH1* germline mutation testing should be performed in probands affected by either DGC or LBC. Screening of at-risk individuals is indicated from the age of consent, after counselling in a multidisciplinary team. Since *CDH1* mutation carriers have a considerable increased risk to develop invasive GC, which is associated with high mortality, prophylactic total gastrectomy is advised for individuals with a proven pathogenic germline *CDH1* mutation, as it is the only option to eliminate the risk of DGC development [45, 48]. In the prophylactic gastrectomy specimens of these individuals, multiple SRCCs can usually be found. In individuals with proven pathogenic *CDH1* mutations who decline to undergo prophylactic gastrectomy, endoscopic surveillance with multiple biopsies according to the Cambridge protocol is advised [48]. Endoscopic screening in a research setting is also recommended for patients with a *CDH1* variant of unknown significance, or with HDGC without a proven *CDH1* mutation. In

one case series, intramucosal SRCCs were detected endoscopically in 2 of 7 *CDH1* mutation-negative individuals (1/5 families) [49].

## 3. Somatic changes in HDGC

### 3.1 Inactivation of the 2nd *CDH1* allele

Individuals with a germline *CDH1* mutation, have a single functional *CDH1* allele. When this wild-type allele becomes inactivated by a somatic second-hit molecular mechanism, this leads to biallelic inactivation of the *CDH1* gene and the development of DGC [50-52]. Initial reports indicated that the second-hit that inactivates *CDH1* in HDGC is most commonly promoter hypermethylation [50, 52]. In 2009, Oliveira et al. performed a systematic study to establish the frequency of different types of somatic *CDH1* second-hits occurring in *CDH1*-related GC [53]. This study confirmed that promoter hypermethylation was the most frequent second *CDH1* hit, identified in 32.1% of the lesions analyzed, whereas loss of heterozygosity was found in 25%, both alterations in 17.9% and no alterations in 25%, when both primary GC and lymph node metastases were analyzed [53]. In fact, 50% of primary GC displayed *CDH1* epigenetic modifications as a second-hit, whereas in GC metastases the most common second-hit was loss of heterozygosity. Different neoplastic lesions from the same patient frequently displayed different types of second-hits and different types of second-hits were also found within the same tumour sample [50, 52, 53]. These results demonstrated substantial heterogeneity in the mechanisms that can act as *CDH1* second-hits in a single patient.

### 3.2 Other somatic changes in HDGC

There has been no systematic study of somatic genetic and epigenetic alterations in genes other than *CDH1* in HDGC from *CDH1* germline mutation carriers. Thus, at this moment in time, there remains a lack of understanding of the cascade of genetic or epigenetic events

taking place after *CDH1* inactivation by a second-hit. Such knowledge is necessary to shed light onto the players involved in the evolution from early to invasive HDGC lesions.

Exome sequencing of a single HDGC has been performed. However, in this case, the underlying family predisposing gene was *CTNNA1* and not *CDH1* [39], and somatic mutations at *LMTK3*, *MCTP2*, *MED12*, *PIK3CA*, and *ARID1A* genes have been demonstrated, as well as mutations in other genes recently shown to be part of the molecular signatures of sporadic GC [54-59]. Similar studies in a series of HDGC caused either by *CDH1* or *CTNNA1* germline mutations, and in different progression stages, would undoubtedly help to disclose the somatic mutation landscape of this disease.

## 4. Histopathology of HDGC

### 4.1 Prophylactic gastrectomy

The gross appearances of stomachs from asymptomatic *CDH1* mutation carriers that undergo prophylactic gastrectomy and index patients that present with widely invasive GC differ enormously. The prophylactic gastric specimens generally show macroscopically no specific abnormalities at all [60-62]. Sometimes, subtle “pale” areas are visible endoscopically which may represent small *foci* of SRCs [48, 63]. Macroscopic examination and sampling of prophylactic gastrectomies should follow specific protocols. Pathological analysis of the entire gastrectomy specimen includes a thorough microscopic assessment using Haematoxylin and Eosin (H&E) and periodic acid-Schiff-diastase (PAS-D) stain.

Microscopically, there are almost always multiple, ranging from a few, up to dozens, of invasive intramucosal cancer *foci* (pT1a) identified in prophylactic gastrectomy specimens if these are completely processed into paraffin blocks. These tiny (<0.1 to 10 mm) *foci* are restricted to the superficial lamina propria and composed of SRCs that are relatively small at

the neck-zone level and usually enlarge towards the surface of the gastric mucosa [64]. Considering different studies which reported systematic complete mapping of total gastrectomies, there seems to be no restriction or convincingly preferred location of intramucosal SRCC in the stomach [60-62, 64-68]. The *foci* were identified from cardia to pre-pyloric region and even in gastric metaplasia beyond the pylorus [48]. As all regions can be affected, pathological examination of the resected specimen should include confirmation of the presence of a complete cuff of proximal squamous oesophageal mucosa and distal duodenal mucosa.

Two typical features of intraepithelial SRCC, which are considered as precursors and only described in *CDH1* mutation carriers, include:

- *In situ* SRCC (Tis), which corresponds to the substitution of normal epithelial cells of a gland or foveolae by disorganised SRCs that stay above the basal membrane. These tumour cells have hyperchromatic and depolarised nuclei.
- Pagetoid spread of SRCs (Tis), which corresponds to a row of SRCs between the normal epithelial cells and the still intact basal membrane.

*In situ* SRCC and pagetoid spread of SRCs have so far only been described in germline *CDH1*-mutation related DGC and have not been described in the non-involved stomach of patients with sporadic SRCC. Confirmation of precursors of SRCC by an independent, experienced histopathologist is recommended since various benign 'signet cell-like changes' may mimic these lesions [48]. In most specimens, there are often only a low number or no intraepithelial SRCCs at all identified in contrast to numerous T1a *foci*.

The surrounding gastric mucosa of these prophylactic gastric specimens is often without significant abnormalities. Background changes that are described include mild chronic

gastritis, foveolar hyperplasia with tufting of the surface epithelium and globoid change with clear changes of the superficial epithelium [32, 60]. Intestinal metaplasia, atrophy, dysplasia and infection with *Helicobacter pylori* are very rarely observed.

### 4.2 Gastrectomy with curative intent for advanced HDGC

Advanced HDGCs often present as *linitis plastica* with increased gastric wall thickness, corresponding to diffuse infiltration of all layers of the stomach wall by cancer cells indistinguishable from *linitis plastica* in sporadic cases of DGC, but cases with a localised tumour do occur as well. The predominant histological pattern is a poorly cohesive carcinoma with only a few or no classic SRC morphology, sometimes with a mucinous or (micro-) tubular component particularly when present in lymph node metastasis. These GCs cannot be discriminated based on histology basis alone from advanced sporadic GC. However, if there are *in situ* lesions, pagetoid spread of SRCs or multiple intramucosal SRCC lesions at distance from the tumour bulk, these are important clues in favour of *CDH1*-related GC. *In situ* SRCC and pagetoid spread lesions have not been described so far in sporadic SRC/diffuse type GC [60].

## 5. Immunohistochemical profile of HDGC and its relationship with *CDH1* mutations

Consistent with the bi-allelic *CDH1* inactivation and consequent E-cadherin loss of function, E-cadherin protein expression by immunohistochemistry (IHC) is almost always abnormal in HDGC, in contrast to the normal complete membranous expression in adjacent normal (non-tumoural) epithelium [44, 51-53, 60, 64, 65, 68-70]. Aberrant E-cadherin staining patterns include absence of immunoreactivity as well as reduced membranous, “dotted” and cytoplasmic staining. The “dotted” staining pattern is probably due to the persistence of E-cadherin non-functional domains in the Golgi apparatus [51]. Abnormal immunoreactivity of E-

cadherin has been described in precursor lesions (*in situ* SRCC and pagetoid spread of SRCs) as well as in early or advanced carcinomas, suggesting that the inactivation of E-cadherin is probably a key initiating event in HDGC tumourigenesis [60]. Moreover, normal immunoreactivity of the gastric mucosa between lesions suggests a clonal origin of the individual cancer *foci*.

One report [51] described abnormal patterns of expression of both α- and β-catenin in early HDGCs, suggesting that the absence of a normal E-cadherin protein may lead to the disruption of the cell-cell adhesion complex. Furthermore, β-actin, p120 catenin and Lin7 were shown to be reduced or absent in HDGC in another study [69].

In 2010, da Cunha *et al* [71] investigated the expression of v6-containing CD44 isoforms (CD44v6), in the process of malignant transformation of gastric mucosa comparing precursor lesions and advanced sporadic and hereditary GCs. In the three HDGC cases from *CDH1* germline mutation carriers, a simultaneous loss of E-cadherin expression and overexpression of CD44v6 was observed, and CD44v6 was proposed to be a putative biomarker of early invasive intramucosal HDGC [71].

Based on prophylactic gastrectomy specimens, the microscopic *foci* of intramucosal SRCs are scattered throughout the stomach of *CDH1* germline mutation carriers, and represent early and asymptomatic lesions that have the potential to progress to aggressive and widely invasive carcinomas. However, few studies describe the immunohistochemical profile of these lesions [68, 69].

In general, early HDGCs were described as low-proliferative lesions, with few mitotic cells and low numbers of cells with Ki-67 expression, while advanced HDGCs showed many more Ki-67 positive cells [68, 69]. Ki-67 expression was observed in the small and less differentiated

SRCs located at the base of larger intramucosal lesions [69]; however, in another study, those small and less differentiated SRCs were described as having a low proliferative index, similar to the superficial and more differentiated cells [68].

Since the small and dedifferentiated tumour cells that constitute the bulk of advanced HDGC and the deep layer of early HDGC display a morphology “reminiscent of mesenchymal cells”, Humar *et al* [69] hypothesised that epithelial-mesenchymal transition (EMT) mediates the progression from early to advanced HDGC. The activated kinase c-Src, a well characterised EMT inducer [72] and its downstream targets, such as fibronectin, p-Fak and p-Stat3 were not expressed in small intramucosal *foci* of *SRCCs*, while the immunoreactivity was observed in dedifferentiated neoplastic cells in larger intramucosal lesions, and in advanced HDGCs with increased depth of invasion. These findings, however, could not be confirmed by Barber *et al* [68]. These authors did not observe differences in immunoreactivity between smaller and larger intramucosal SRCCs. Barber *et al* [68] investigated immunoreactivity of cytokeratins (CK) 8/18 and vimentin by dual-label immunofluorescence, and their results failed to demonstrate the evidence of EMT. CK expression in both differentiated and dedifferentiated SRCCs had also been described in previous studies [64, 69]. Moreover, N-cadherin, an EMT marker with increased expression in the presence of EMT [73] was not observed in intramucosal *foci* of SRC [52]. In conclusion, the role of EMT in the development of aggressive and widely invasive HDGC from early and indolent microscopic *foci* is uncertain and further analysis of these lesions is necessary to understand the molecular events required for the progression from indolent intramucosal lesions to widely invasive carcinomas.

The cell differentiation pattern of HDGC has been also investigated: Humar *et al* [69] described MUC5A expression in differentiated, large SRCs at the surface of gastric mucosa,

and MUC6 expression in poorly differentiated cells, at the neck zone, whereas Oliveira *et al* [51] observed a widespread expression of gastric differentiation markers (MUC1, MUC5AC and TTF1) within the lesions.

Another molecular pathway that has been explored in HDGC is the relationship between the disruption of apical-basal cell polarity induced by loss of functional E-cadherin and the resistance of cancer cells to anoikis, a particular form of programmed cell death that is triggered by the loss of cell-cell and cell-matrix normal interactions. The group of Raquel Seruca [74, 75] developed an *in vitro* model to test the pathogenecity of *CDH1* germline missense mutations found in HDGC patients, and observed that the loss of functional E-cadherin renders cells more resistant to taxol apoptotic stimuli, and that an interplay exists between loss of E-cadherin and gain of the anti-apoptotic protein Bcl-2 activity, probably through the aberrant activation of Notch-1 [74, 75]. Such *in vitro* findings were supported by the Bcl-2 cytoplasmic immunoreactivity found in one case of a primary tumour harbouring one of the mutations analysed in the *in vitro* model [75].

### 5.1 New insights in morphological, immunohistochemical and genetic profile of HDGC

As HDGC encompasses a spectrum of precursor and invasive lesions, as described above, and the molecular events that occur in early and advanced carcinomas have not yet been clearly elucidated, we have recently investigated the relationship between the morphology of these lesions and the immunoexpression of biomarkers of cell-adhesion, proliferation, anoikis and EMT (unpublished data). Moreover, we have explored the immunoexpression of a putative biomarker of cancer cell stemness, ALDH1A, a cytosolic protein that catalyses the oxidation of endogenous and exogenous aldehydes in the equivalent carboxylic acids and

their functions are fundamental in physiological processes, including proliferation, survival, differentiation and detoxification. ALDH1A has been described as a biomarker of both normal progenitor and stem cells (hematopoietic, mesenchymal, neural, mammary, prostate and gastrointestinal lineages) and cancer stem cells (CSCs), including head and neck, breast, prostate, ovarian, lung, hepatic, pancreatic, bladder and colon cancers [76]. Moreover, high ALDH1A expression has been associated with adverse prognosis in breast, lung, serous ovarian, pancreatic, bladder, prostate and oesophageal cancer [76]. With regard to GC, the findings are still conflicting and inconclusive [77-83] and, to our knowledge, no previous studies have investigated ALDH1A expression in HDGC and its precursor lesions, to assess the validity of this putative CSC biomarker in this specific setting.

We have recently undertaken a study including twenty-one lesions (from 17 surgical specimens belonging to 12 *CDH1*-related HDGC families), that encompassed 12 intramucosal carcinomas (pT1a) and 9 widely invasive carcinomas (pT>1). The cases were reviewed by an expert pathologist in HDGC (FC) and were analysed by IHC for E-cadherin (clone 4A2C7), Ki-67 (clone 30-9), Bcl-2 (clone 124), p53 (clone 318-6-11), pSrc (clone Y416) and ALDH1A (clone EP1933Y). Furthermore, the study included one case of endoscopic submucosal dissection (ESD) and four biopsy specimens, three of them corresponding to preoperative specimens of patients submitted to surgery, and two obtained from distinct HDGC families, both harbouring the same *CDH1* mutation. The cases were retrieved from the Department of Pathology, Radboud university medical centre, Nijmegen (The Netherlands), the Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York (USA), the Department of Histopathology and Molecular Pathology, Leeds Teaching Hospitals NHS Trust, Leeds (UK), the Department of Histopathology, Cambridge University Hospitals NHS Trust, Cambridge (UK) and the Department of Pathology, Centro Hospitalar de São João,

Porto (Portugal). The morphological phenotype of these lesions included a variable spectrum of precursor, early and advanced lesions, namely: (1) Precursor lesions (pTis), including *in situ* SRCC and pagetoid spread of SRCs; (2) Intramucosal HDGC (pT1a), showing typical SRC morphology ("indolent phenotype") and (3) Advanced HDGC (pT>1), composed by a mixture of SRCs and poorly cohesive, pleomorphic and bizarre cells ("aggressive phenotype"). Interestingly, all these lesions were observed in one case in different locations of the same surgical specimen, as shown in **Figure 1**.

All pTis and pT1a lesions showed "indolent" morphological features, and were negative for p53 and Ki-67 immunoexpression. In contrast, pT>1 carcinomas were characterised by high Ki-67 proliferation index (89%, $p<0.01$) and p53 overexpression (56%, $p<0.01$) in the pleomorphic component of the tumours (**Figure 2**). E-cadherin immunoexpression was abnormal in all precursor lesions, early and advanced SRCCs and showed a heterogeneous staining pattern, from absent or decreased membranous immunoreactivity to "dotted" pattern and cytoplasmic immunoreactivity (**Figure 3**). Expression of ALDH1A and pSrc was higher in intramucosal carcinomas (100% and 58%, respectively) compared to advanced carcinomas (44%, $p<0.01$ and 33%, $p=0.03$ respectively) (**Figure 4**). Bcl-2 was expressed only in one case. The analysis of a putative relationship between biomarkers expression revealed a significant correlation between Ki-67 and p53 immunoreactivity ($p<0.01$), while ALDH1A overexpression inversely correlated with Ki-67 and p53 overexpression ($p<0.01$). We noted a tendency for pSrc overexpression to be associated with absence of Ki-67 and p53 immunoreactivity, but differences were not statistically significant.

The presence of ALDH1A immunoreactivity in normal gastric mucosa, and in all intramucosal SRCCs, together with the loss of such immunoreactivity in advanced HDGCs, exclude the

possible role of ALDH1A as a biomarker of cancer cell stemness in HDGC. The studies of ALDH1A expression in epithelial cancers show that the percentage of ALDH1A positive tumour cells is strongly correlated with the level of ALDH1A expression in the normal counterpart, suggesting that, as a CSC biomarker, ALDH1A may be useful only for tumours with a low level background expression of the protein in the normal counterpart [77]. GC may be added to the list of tumours in which ALDH1A is not useful as a CSC biomarker but, otherwise, may represent a marker of cell differentiation.

A noteworthy study by Fricke *et al* [84] investigated the relationship between E-cadherin and p53 gene mutation and p53, Ki67 and Bcl-2 immuno-expression in a series of 24 sporadic diffuse gastric carcinomas, 16 of which were positive for E-cadherin mutation. P53 overexpression was significantly more frequent in tumours without *CDH1* mutations than in GCs with *CDH1* mutations, while no correlation was found between Ki-67 immunoreactivity and the *CDH1* mutation status. Furthermore, *TP53* mutation was detected in 12.5% of tumours without *CDH1* mutations and in 6.3% in tumours with *CDH1* mutations, though the difference was not statistically significant. These findings may suggest that, in the sporadic GC setting, the presence of *CDH1* mutation can alter the accumulation of p53 protein.

To our knowledge, our study is the first to explore p53 immunoreactivity in the context of HDGC. In sporadic GC, p53 nuclear overexpression by IHC, was found both in intestinal and diffuse GCs and was correlated with tumour progression, poor prognosis and unfavourable response to therapy [84-89]. Moreover, NGS studies have identified *TP53* mutations as one of the most frequently alterations in GCs, and *TP53* has been pointed as a candidate driver gene, especially in intestinal-type GC [54, 56-58, 90]. The evidence of p53 nuclear accumulation in our study suggests that *TP53* may be a key gene involved in GC progression,

also in the hereditary setting.

In the ESD and biopsy specimens from *CDH1* germline mutation carriers, both “indolent” and “aggressive” features of the neoplastic cells were observed (**Figure 5**). Based on available evidence, the finding of GC with an “aggressive” phenotype in a screening biopsy performed in a *CDH1* germline mutation carrier should be taken as a predictive sign of widely invasive carcinoma, and prompt for staging and surgical treatment.

In the patients submitted to surgery, belonging to the 12 *CDH1*-related HDGC families, 11 different germline mutations in the *CDH1* gene were identified: 3 splice-site mutations; 3 frameshift, 1 missense, 1 missense/frameshift, and 3 nonsense mutations. Intramucosal carcinomas associated or not with widely invasive carcinomas were always found in carriers of different mutations, independently of the mutation site or its type. This observation likely reflects a disease mechanism and morphological phenotype that is characteristic of *CDH1* inactivation in the stomach, independent of the germline mutation, intimately associated with the first stages of HDGC development [60].

We also analysed two patients (19 and 20 years old) with metastatic and inoperable disease, from whom only biopsy specimens were available. These two cases, from 2 distinct HDGC families of different countries (Portugal and USA) were caused by the same missense/frameshift mutation c.1901C>T (p.Ala634Val), the GCs displayed an “aggressive” morphological phenotype and had significantly higher expression of Ki-67 and p53, compared to the cases from which surgical specimens were available ($p=0.04$ and $p=0.03$, respectively). Hence the c.1901C>T mutation deserves further analysis for its potential association with an aggressive clinical behaviour.

The appearance of early HDGC lesions, that may or may not evolve to widely invasive carcinomas, is thought to be triggered in the stomach by the somatic inactivation of the remaining *CDH1* wild-type allele [50, 52, 53]. Although it would be conceivable that complete *CDH1* gene inactivation leads to complete loss of E-cadherin protein expression, this is not always the case. In fact, E-cadherin protein expression, detected by IHC can either be lost or maintained, regardless of *CDH1* germline mutation and HDGC tumour stage. Similar observations in HDGC invasive carcinomas have been documented in several studies [52, 53]. Thus, IHC analysis of E-cadherin in HDGC lesions (early and advanced) is not a good marker to detect *CDH1* gene complete inactivation or to predict whether an early lesion will evolve or not to invasive cancer.

Our data provide evidence that intramucosal (pT1a) and widely invasive HDGC carcinomas (pT>1) differ in their IHC expression of Ki-67 and p53, and that the expression of these markers in such lesions is independent from the type and site of the underlying *CDH1* germline mutation. In particular, it was observed that all early intramucosal lesions, in any germline mutation context, display characteristic SRC morphology and lack both Ki-67 and p53 expression, while most invasive carcinomas display a pleomorphic phenotype characterized by Ki-67 and p53 expression. These results show for the first time the proliferative nature of invasive HDGC and its association with abnormal p53 expression, as part of the progression molecular profile of HDGC tumours.

The finding of proliferation associated with p53 positivity in invasive and pleomorphic HDGC cells is of even more importance if one reconciles these data with that from available murine models of diffuse gastric cancer [91-94]. From studies in which three mouse models were developed, the double conditional knockout cell line in which both *CDH1* and *TP53* were

specifically inactivated was by far the most efficient in producing diffuse gastric cancers [93]. These murine tumours were mainly composed of poorly differentiated cells and SRCs similar to those in human advanced HDGC, which developed carcinoma within 12 months with 100% penetrance. This mouse model mimics closely the human disease, and as demonstrated in the present study very likely mimics the natural history of *CDH1*-related HDGC in humans.

**6. Conclusions and practical points**

- Germline *CDH1* mutations are the most important cause for HDGC and give an increased risk of both DGC as well as LBC. Germline *CTNNA1* mutations were described in three families with diagnoses of DGC. There is limited data of other susceptible genes for HDGC, including *MAP3K6,* and its role in GC remains to be determined.
- The updated *CDH1* testing criteria 2015 include families with (1) two or more patients with GC, one confirmed DGC; (2) DGC before the age of 40; (3) families with diagnoses of both DGC and LBC, one before the age of 50.
- Given the high mortality associated with invasive DGC, prophylactic total gastrectomy is advised for individuals with pathogenic *CDH1* mutations.
- Standardised endoscopic surveillance in experienced centres, preferably in a research setting, is recommended for those opting not to have gastrectomy at the current time, those with *CDH1* variants of uncertain significance and those that fulfil HDGC criteria but without germline *CDH1* mutations.
- Characteristic lesions in HDGC are tiny microscopic intramucosal *foci* of typical SRCs, *in situ* SRCC, and pagetoid spread of SRCs.

- Intramucosal SRCCs are invasive lesions that may remain indolent for an uncertain long time; no metastatic disease has been described in prophylactic gastrectomies of *CDH1* mutation carriers with the diagnosis of exclusively intramucosal DGC.

**7. New hypotheses and further research directions**

- It remains unanswered how long early lesions of HDGC can remain indolent, and how to predict their progression to widely invasive carcinomas.
- Intramucosal HDGCs present with an “indolent” phenotype (SRCs; Ki67–; p53–), while advanced carcinomas display an “aggressive” phenotype (pleomorphic cells; Ki67+; p53+). This is the first evidence of phenotypic heterogeneity in HDGC lesions, which may help to define prognostic biomarkers of progression from indolent to widely invasive carcinomas.
- One of the first alterations in HDGC is inactivation of the second *CDH1* allele. Other driver alterations in tumour suppressor genes and oncogenes that may play significant roles in the pathogenic mechanisms of DGC remain to be clarified.
- The molecular background and potential causing germline mutations in patients with HDGC and without germline *CDH1* mutation remains an area of ongoing research. It is likely that more HDGC-associated genes will soon be discovered using NGS methodologies. The management will be difficult until multiple mutation-positive families have been studied.
- Population based germline interrogation of potential gene mutations and single nucleotide polymorphism in familial clusters may also generate noteworthy findings.

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