Quantification of Epigenetic and Genetic 2nd Hits in *CDH1* During Hereditary Diffuse Gastric Cancer Syndrome Progression

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Background & Aims: **Hereditary diffuse gastric cancer (HDGC) families carry *CDH1* heterozygous germline mutations; their tumors acquire complete *CDH1* inactivation through "2nd-hit" mechanisms. Most frequently, this occurs via promoter hypermethylation (epigenetic modification), and less frequently via *CDH1* mutations and loss of heterozygosity (LOH). We quantified the different 2nd hits in *CDH1* occurring in neoplastic lesions from HDGC patients.** ***Methods:*** **Samples were collected from 16 primary tumors and 12 metastases from 17 patients among 15 HDGC families; *CDH1* mutations, LOH, and promoter hypermethylation were analyzed. E-cadherin protein expression and localization were determined by immunohistochemistry.** ***Results:*** **Somatic *CDH1* epigenetic and genetic alterations were detected in lesions from 80% of HDGC families and in 75% of all lesions analyzed (21/28). Of the 28 neoplastic lesions analyzed, promoter hypermethylation was found in 32.1%, LOH in 25%, both alterations in 17.9%, and no alterations in 25%. Half of the *CDH1* 2nd hits in primary tumors were epigenetic modifications, whereas a significantly greater percentage of 2nd hits in metastases were LOH (58.3%; $P = .0274$). Different neoplastic lesions from the same patient frequently displayed distinct 2nd-hit mechanisms. Different 2nd-hit mechanisms were also detected in the same tumor sample.** ***Conclusion:*** **The 2nd hit in *CDH1* frequently occurs via epigenetic changes in HDGC primary tumors and LOH in metastases. Because of the concomitance and heterogeneity of these alterations in neoplastic lesions and the plasticity of hypermethylated promoters during tumor initiation and progression, drugs targeting only epigenetic alterations might not be effective, particularly in patients with metastatic HDGC.**

Gastric cancer is the 4th most common malignancy worldwide, although in recent decades a decline has been observed in its incidence and mortality.[1,2] Diffuse gastric cancer accounts for about 30% of all gastric carcinomas and somatic mutations in E-cadherin (*CDH1* gene), an adhesion molecule and a tumor suppressor protein, were found in >50% of these cases.[3,4] Inactivating *CDH1* mutations were also described in the germline of families with hereditary diffuse gastric cancer (HDGC; OMIM No. 137215), an autosomal-dominant disease characterized by clustering of early onset documented diffuse gastric cancer.[5,6] So far, 82 of these families were found to carry 65 different *CDH1* inactivating germline mutations; 83.1% are predicted to generate truncated E-cadherin transcripts (nonsense, splice-site, and frameshift mutations), and 16.9% are missense mutations.[7,8]

The diminished or absent E-cadherin immunoreactivity observed in diffuse gastric cancer cells (hereditary and sporadic) harboring *CDH1* mutations, is consistent with biallelic *CDH1* inactivation by a 2nd-hit mechanism leading to E-cadherin loss, and determining diffuse cancer development.[9–13] In sporadic diffuse gastric carcinomas harboring somatic *CDH1* mutations, promoter methylation was the most frequently found 2nd-hit inactivation mechanism.[13]

To date, tumors from 13 of 82 (15.9%) HDGC families harboring *CDH1* germline mutations were analyzed for *CDH1* 2nd-hit inactivation mechanisms.[10–12,14] Apart from the low number of cases analyzed, a single neoplastic lesion was investigated per patient, which constitutes a clear drawback in the analysis, because HDGC is a disease with multiple and apparently unrelated tumor foci, scattered in the stomach of mutation carriers.[15,16] Data from 27 tumors arising in 27 patients from the 13 HDGC families studied so far still indicate *CDH1* promoter hypermethylation as the most common 2nd-hit mechanism of inactivation.[10,11,14] In contrast with studies on the 2nd-hit inactivation mechanism of genes causing other neoplastic syndromes,[17–20] somatic genetic alterations (mutations and loss of heterozygosity [LOH]) of

Abbreviations used in this paper: HDAC, histone deacetylase; HDGC, hereditary diffuse gastric cancer; IHC, immunohistochemistry; LOH, loss of heterozygosity; PCR, polymerase chain reaction.

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CDH1 were uncommonly found.[10–12,14] As a consequence, *CDH1* promoter hypermethylation has been suggested as the basis for development of early detection tools as well as for chemoprophylaxis in unaffected *CDH1* mutation carriers.[21]

Among many anticancer drugs collectively named "targeted or molecular therapies," epigenetic drugs constitute an attractive possibility for disease control, because they inhibit histone deacetylases (HDAC) and DNA methyltransferases, leading to gene re-expression. *CDH1* gene became demethylated and selectively re-expressed in human cancer cell lines upon treatment with the histone deacetylation inhibitor trichostatin A,[22] raising the hypothesis that such a treatment would benefit HDGC patients. This therapeutic approach would have a clinical and therapeutic limitation in HDGC patients presenting with tumors harboring somatic *CDH1* mutations or LOH as the 2nd-hit inactivation mechanism.

We decided to characterize the 2nd-hit in 28 neoplastic lesions (16 primary and 12 metastases) from 17 *CDH1* germline mutation carriers belonging to 15 different HDGC families, to understand the clinical potential of epigenetic drugs in the therapeutic management of HDGC patients. Moreover, it is also our aim to clarify whether different neoplastic lesions from the same individual display different 2nd-hit inactivation mechanisms. Our study encloses the largest series of HDGC tumors from families harboring *CDH1* germline mutations analyzed so far, and our results demonstrate that not all HDGC patients are likely to benefit, in the near future, from epigenetic pharmacologic therapies.

Materials and Methods

Patients and Samples

Twenty-eight neoplastic foci from 17 individuals from 15 HDGC families carrying distinct *CDH1* mutations and fulfilling the International Gastric Cancer Linkage Consortium criteria[23] were selected for analysis: (1) ≥2 documented cases of diffuse gastric cancer in 1st-/2nd-degree relatives, with ≥1 diagnosed before the age of 50; or (2) ≥3 cases of documented diffuse gastric cancer in 1st-/2nd-degree relatives, independent of age. The study protocol was reviewed and approved by the appropriate ethics committees. Family histories were obtained with informed consent. Family history, *CDH1* germline mutation status, and representative paraffin-embedded material from tumor and normal gastric mucosa were obtained from the British Columbia Cancer Agency in Vancouver, Canada (n = 11 individuals), from the Department of Clinical Genetics of Santa Casa de Portalegre, Brazil (n = 1 individuals); from the National Cancer Center Research Institute, Tokyo, Japan (n = 1 individual); from Hospital de S. Joao, Porto, Portugal (n = 2 individuals); from Coimbra University Hospital, Coimbra, Portugal (n = 1 individual); and from the First Affiliated Hospital of Zhengzhou University, Zhengzhou, China (n = 1 individual). Twenty-eight neoplastic foci were analyzed, 16 were representative of primary tumors (14 gastric and 2 colorectal), and 12 were representative of metastatic foci (2 localized in the peritoneum, 5 in lymph nodes, 1 in the colon mesentery, 1 in the small bowel mesentery, and 3 in the ovary; Table 1).

Hematoxylin and eosin-stained, 4 μm sections from each case were used to label tumor and normal mucosa. Sections of 5–10 μm were used to macrodissect tumor and normal mucosa. High molecular weight DNA was isolated from paraffin-embedded tissue using the Invisorb Spin Tissue Mini Kit (Invitek, Berlin, Germany) following the manufacturer's instructions. DNA extracted from macrodissected areas with ≥75% of neoplastic cells, identified by an experienced pathologist (FC), was used to characterize molecular alterations in tumor DNA in comparison with DNA extracted from matched normal mucosa.

CDH1 *Mutation Screening and LOH Analysis*

After DNA extraction from tumor and normal mucosa, 30 ng of template DNA was used in 2 multiplex polymerase chain reaction (PCR) reactions. Reaction 1 was performed with a primer set to amplify the exon where the *CDH1* germline mutation lays (to exclude contamination with other samples) and 4 primer sets to amplify exons 7–10 where somatic mutations commonly cluster. Reaction 2 was performed with a primer set to amplify the exon where the *CDH1* germline mutation lays and 2 primer sets to amplify *CDH1* common polymorphisms (the promoter −160C/A transvertion [rs16260] and a silent substitution 2076C/T at exon 13 [rs1801552]) that were used as intragenic markers for LOH analysis. In cases where these polymorphisms were homozygous, and, whenever possible, another silent substitution at exon 14 (2253C/T [rs33964119]) was used. From these 2 multiplex PCR products, of not >35 cycles each, we used 20 ng to specifically and separately re-amplify each of the aforementioned amplicons. After this 2nd round of amplification, PCR products were run and excised from agarose gels, purified, and sequenced. To analyze LOH, 3 proximal and distal microsatellite markers (D16S3025, D16S496, D16S3067) flanking the *CDH1* locus were amplified, in a multiplex fluorescent PCR and run on a ABI3100 Genetic Analyzer, from tumor and matched normal mucosa samples. All experiments were repeated at least twice using paraffin DNA as template. Only informative markers were considered for LOH analysis: A sample harboring a reduction in the pick area of 1 allele of ≥90%, in comparison to the other allele, was considered to display LOH.

Table 1. Clinical and Molecular Data From HDGC Families and Patients Analyzed for 2nd-Hit Mechanisms Affecting the *CDH1* Gene

Family ID	Patients	Age	Tissue type	Neoplastic lesion site	Germline mutation	Germline mutation type
1[35]	1	39	Tumor Foci 1	Stomach	283C>T	Nonsense
2[36]	1	32	Tumor Foci 1	Stomach	382delC	Frameshift
	1		Metastasis 1	Peritoneum		Frameshift
	1		Metastasis 2	Peritoneum		Frameshift
3[37]	1	67	Metastasis 1	Small bowel mesentery	1003C>T	Nonsense
	2	57	Tumor Foci 1	Stomach		Nonsense
4[6]	1	38	Tumor Foci 1	Colorectum	1018A>G	Missense
	1		Tumor Foci 2	Colorectum		Missense
	1		Metastasis1	Pericolic lymph node		Missense
	2	47	Tumor Foci 1	Stomach	1018A>G	Missense
	2		Tumor Foci 2	Stomach		Missense
	2		Metastasis 1	Perigastric lymph node		Missense
	2		Metastasis 2	Perigastric lymph node		Missense
5[37]	1	28	Tumor Foci 1	Stomach	1063delT	Frameshift
6[38]	1	28	Tumor Foci 1	Stomach	1137G>A	Splicing
	1		Tumor Foci 2	Stomach		Splicing
	1		Metastasis 1	Ovary		Splicing
	1		Metastasis 2	Ovary		Splicing
	1		Metastasis 3	Ovary		Splicing
7[36]	1	51	Tumor Foci 1	Stomach	1225T>C	Missense
8[36]	1	34	Metastasis 1	Colon mesentery	1476del(AG)	Frameshift
9[36]	1	42	Tumor Foci 1	Stomach	1779insC	Frameshift
10[39]	1	23	Tumor Foci 1	Stomach	1901C>T	Missense/Splicing
11[36]	1	24	Tumor Foci 1	Stomach	2061delTG	Frameshift
12*	1	28	Metastasis 1	Lymph node	2195G>A	Missense/Splicing
13[35]	1	39	Metastasis 1	Lymph node	2398delC	Frameshift
14[40]	1	56	Tumor Foci 1	Stomach	2494G>A	Missense
15[41]	1	38	Tumor Foci 1	Stomach	2269G>A	Missense

*Unpublished family results.

CDH1 *Promoter Methylation Analysis*

We analyzed 12 CpG sites within the 90 base pairs upstream of the *CDH1* translation start site (ATG). Owing to DNA amount limitations, we restricted the study of *CDH1* promoter methylation status to this fraction of CpG sites from CpG island 3, because methylation at this location is commonly associated with E-cadherin expression loss. Moreover, this CpG island has been described as a hallmark of promoter methylation in human cancers.[24,25] *CDH1* promoter methylation analysis was performed in all tumor DNA samples. The EpiTect Bisulfite Kit (Qiagen, Valencia, Calif) was used to treat 200 ng of DNA. Unmethylated cytosines were converted to uracil, whereas methylated ones remained unmodified. Bisulfite treated DNA from white blood cells was in vitro methylated with M.*Sss*I DNA MeTase and used as a positive control for methylation determination. The region encompassing 12 CpG sites was PCR amplified using flanking primers (sequences available upon request), specifically designed for bisulfite treated DNA sequences without CpG sites, and sequenced for methylation status determination. Re-amplification of the products was performed whenever recurrent failure in the 1st-round PCR occurred. Independent PCR amplifications were performed at least twice for each sample. Neoplastic lesions displaying methylation/hemimethylation at ≥25% of CpG sites (from the 12 analyzed) were considered as methylated lesions.

Individual Identification by In/del Profiling

In 4 individuals from 3 families (families 2, 4, and 6), for whom >1 lesion was analyzed per patient, a 32 insertion/deletion patterning was performed to confirm the common origin of the samples. All markers were typed in a single multiplex PCR, using a short amplicon strategy (<160 bp) to improve the amplification of degraded samples. Moreover, these markers present high genetic diversi-

Table 2. Second-Hit Molecular Mechanisms in 28 Neoplastic Lesions

Second-hit mechanism	Total lesions (n = 28)	Primary tumors (n = 16)	Metastases (n = 12)	
Methylation alone	9 (32.1%)	8 (50.0%)	1 (8.3%)	P=.0274
LOH alone	7 (25%)	2 (12.5%)	5 (41.7%)	
Methylation + LOH	5 (17.9%)	3 (18.8%)	2 (16.7)	
No alterations	7 (25%)	3 (18.8%)	4 (33.3%)	

Table 3. Description of 2nd-Hit Mechanisms in *CDH1* and *CDH1* Hypermethylation Pattern in Neoplastic Lesions From HDGC Patients

Family ID	Patient's lesion	Age	Germline mutation	Second hit	*CDH1* promoter methylation pattern
1	Pat 1 - T1	39	283C>T	Methylation	
2	Pat 1 - T1	32	382delC	LOH	
	M1			Methylation + dLOH	
	M2			Methylation + dLOH	
3	Pat 1 - M1	67	1003C>T	LOH	
	Pat 2 - T1	57	1003C>T	Not found	
4	Pat 1 - T1	38	1018A>G	Methylation	
	T2			iLOH	
	M1			iLOH	
	Pat 2 - T1	47	1018A>G	Methylation	
	T2			Methylation + dLOH	
	M1			LOH	
	M2			LOH	
5	Pat 1 - T1	28	1063delT	Not found	
6	Pat 1 - T1	28	1137G>A	Methylation	
	T2			Methylation	
	M1			Not found	
	M2			Not found	
	M3			Not found	
7	Pat 1 - T1	51	1225T>C	Methylation + LOH	
8	Pat 1 - M1	34	1476delAG	LOH	
9	Pat 1 - T1	42	1779insC	Methylation + iLOH	
10	Pat 1 - T1	23	1901C>T	Not found	
11	Pat 1 - T1	24	2061delTG	Methylation	
12	Pat 1 - M1	28	2195G>A	Methylation	
13	Pat 1 - M1	39	2398delC	Not found	
14	Pat 1 - T1	56	2494G>A	Methylation	
15	Pat 1 - T1	38	2269G>A	Methylation	

LOH, loss of heterozygosity at informative proximal and distal flanking markers and *CDH1* intragenic markers, when informative or LOH at informative *CDH1* intragenic markers and proximal and distal flanking markers, when informative; dLOH, LOH only at the most distal marker (D16S3067); iLOH, LOH only at intragenic markers with no loss or no information at flanking markers. ○, unmethylated CpG site; ●, Completely methylated CpG site; ⊙, partially methylated CpG site; ◘, not determined.

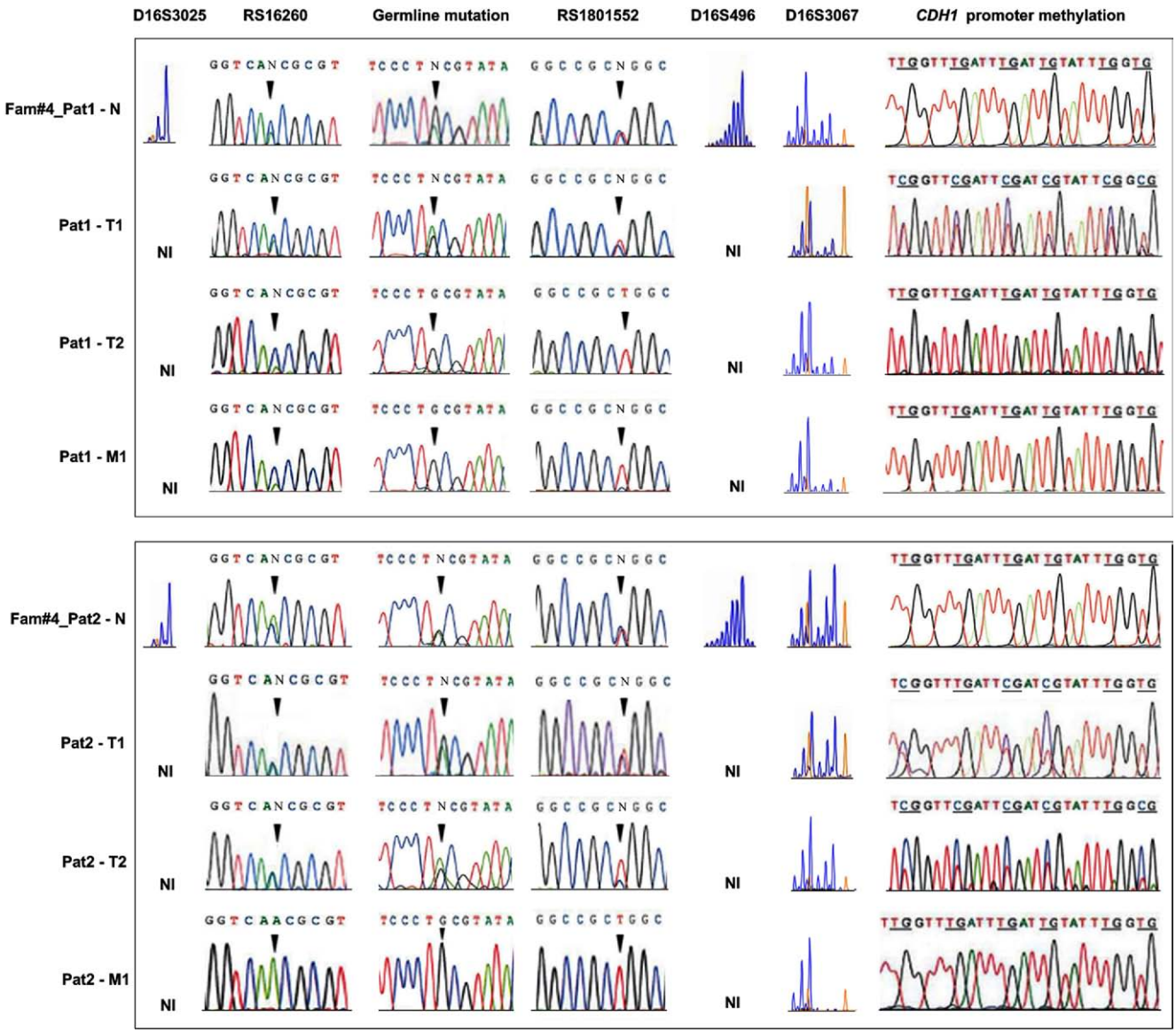

Figure 1. Examples of 2nd-hit inactivating mechanisms in HDGC tumors from 2 family members from family 4. LOH analysis using *CDH1* flanking markers, intragenic markers, and the mutation site, and *CDH1* promoter hypermethylation analysis are depicted for every case. Variation sites, in sequencing schemes, are marked with *arrowheads*; CpG sites at the *CDH1* promoter are *underlined* in the sequencing scheme. Whenever a LOH marker was not informative, *NI*, replaced the LOH pick. N, normal; T, primary tumor; M, metastases.

ties in major population groups and high capacity for individual discrimination (1 in >100 billions), thus providing unique genetic profiles (LG and RP, unpublished data).

E-Cadherin Immunohistochemistry

Consecutive sections from paraffin embedded tissue were used for DNA extraction and immunohistochemistry (IHC), to determine consistency or inconsistency of 2nd-hit mechanisms and E-cadherin IHC status. Human E-cadherin monoclonal antibody 4A2C7 (Zymed, Invitrogen) was used for IHC expression analysis of 3- to 5-μm slides, according to manufacturer instructions. Stained sections were analyzed by an experienced pathologist (FC) and the percentage of E-cadherin positive neoplastic cells as well as the pattern of E-cadherin expression were ascertained.

Statistical Analysis

The statistical analysis was performed using the χ^2 test. Differences were taken to be significant when $P < .05$.

Results

Description of the Series of Patients and Neoplastic Lesions

We selected for this study 17 patients belonging to 15 HDGC families carrying distinct *CDH1* germline mutations and meeting the International Gastric Cancer Linkage

rs #		rs3047269	rs2307579	rs16624	rs2368242	rs2308026	rs1610871	rs1160956	rs2307526	rs2307839	rs2308137	rs2307978	rs35769550	rs2307740	rs5895447	rs16402	rs2067294	rs33972805	rs10688868	rs34811743	rs2067238	rs1610919	rs2308171	rs2308020	rs2067208	D16S3025	*e*16260	*Germline mutation*	rs7007552	D16S496	D16S3067	rs3051300	rs34511541	rs2307689	rs3604036	rs34541393	rs10929077	rs35605984	rs2307700
Chrom		1	1	2	3	4	5	5	5	6	6	7	8	8	8	9	9	11	11	11	12	12	13	15	16	16	16	16	16	16	17	18	19	19	20	21	21	22
	Pat 1 - N	22	22	12	22	12	12	11	12	11	22	12	11	22	22	12	12	12	12	12	11	12	12	12	22	12	12	12	12	ni	12	11	11	22	11	22	11	11	12
Fam#2	Pat 1 - T1	22	22	12	22	12	12	11	12	11	22	12	11	22	22	12	12	12	12	12	11	12	12	12	22	11	22	22	22	ni	22	11	11	22	11	22	11	11	12
	Pat 1 - M1	22	22	12	22	12	12	11	12	11	22	12	11	22	22	12	12	12	12	12	11	12	12	12	22	12	12	12	12	ni	22	11	11	22	11	22	11	11	12
	Pat 1 - M2	22	22	12	22	12	12	11	12	11	22	12	11	22	22	12	12	12	12	12	11	12	12	12	22	12	12	12	12	ni	22	11	11	22	11	22	11	11	12
	Pat 1 - N	22	22	12	12	22	22	12	11	22	12	11	12	22	11	22	22	22	12	12	12	22	22	12	22	ni	12	12	12	ni	ni	12	12	12	12	11	22	22
	Pat 1 - T1	22	22	12	12	22	22	12	11	22	12	11	12	22	11	22	22	22	12	12	12	22	22	12	22	ni	12	12	12	ni	ni	12	12	12	12	11	22	22
Fam#4	Pat 1 - T2	22	22	12	12	22	22	12	11	22	12	11	12	22	11	22	22	22	12	12	12	22	22	12	22	ni	12	22	22	ni	ni	12	12	12	12	11	22	22
	Pat 1 - M1	22	22	12	12	22	22	12	11	22	12	11	12	22	11	22	22	22	12	12	12	22	22	12	22	ni	12	22	12	ni	ni	12	12	12	12	11	22	22
	Pat 2 - N	12	12	12	22	22	22	11	11	22	11	11	12	22	11	22	22	22	11	11	11	22	22	12	22	ni	12	12	12	ni	12	12	22	12	11	12	22	22	12
	Pat 2 - T1	12	12	22	22	22	22	11	11	22	11	11	12	22	11	22	22	22	11	11	11	22	22	12	22	ni	12	12	12	ni	22	12	22	12	11	12	22	22	12
Fam#4	Pat 2 - T2	12	12	22	22	22	22	11	11	22	11	11	12	22	11	22	22	22	11	11	11	22	22	12	22	ni	12	12	12	ni	12	12	22	12	11	12	22	22	12
	Pat 2 - M1	12	12	22	22	22	22	11	11	22	11	11	12	22	11	22	22	22	11	11	11	22	22	12	22	ni	22	22	22	ni	11	12	22	12	11	12	22	22	12
	Pat 2 - M2	12	12	22	22	22	22	11	11	22	11	11	12	22	11	22	22	22	11	11	11	22	22	12	22	ni	12	22	22	ni	11	12	22	12	11	12	22	22	12
	Pat 1 - N	11	12	11	22	12	12	12	12	22	12	22	22	12	12	12	12	22	22	11	12	12	22	12	12	ni	12	12	12	12	ni	12	22	12	12	12	22	22	12
	Pat 1 - T1	11	12	11	22	12	12	12	12	22	12	22	22	12	12	12	12	22	22	11	12	12	22	12	12	ni	12	12	12	12	ni	12	22	12	12	12	22	22	12
Fam#6	Pat 1 - T2	11	12	11	22	12	12	12	12	22	12	22	22	12	12	12	12	22	22	11	12	12	22	12	12	ni	12	12	12	12	ni	12	22	12	12	12	22	22	12
	Pat 1 - M1	11	12	11	22	12	12	12	12	22	12	22	22	12	12	12	12	22	22	11	12	12	22	12	12	ni	12	12	12	12	ni	12	22	12	12	12	22	22	12
	Pat 1 - M2	11	12	11	22	12	12	12	12	22	12	22	22	12	12	12	12	22	22	11	12	12	22	12	12	ni	12	12	12	12	ni	12	22	12	12	12	22	22	12
	Pat 1 - M3	11	12	11	22	12	12	12	12	22	12	22	22	12	12	12	12	22	22	11	12	12	22	12	12	ni	12	12	12	12	ni	12	22	12	12	12	22	22	12

Figure 2. Genetic profiling of 4 individuals from 3 families for which multiple samples were analyzed. Analysis of 32 in/del markers in 20 chromosomes, 3 dinucleotide LOH markers flanking *CDH1* and *CDH1* intragenic markers. Chromosome 16 markers are flanked by *2 vertical black lines*; markers displaying LOH are inside *darker squares*.

Consortium criteria (Table 1). The mean age of the patients was 39.5 ± 12.6 years (range, 23–67). We independently analyzed a total of 28 neoplastic lesions, collected from 17 patients for *CDH1* promoter hypermethylation, LOH, and mutations within the hotspot exons 7–10, where *CDH1* somatic mutations cluster.[26] We did not extend the mutation analyses to all *CDH1* exons owing to a lack of material. To dilute contamination with DNA from normal cells, DNA was extracted from macrodissected areas enriched in neoplastic cells. This approach allowed us to specifically analyze molecular alterations occurring in neoplastic cells that are easily masked when a large population of normal cells contaminates tumor samples.

Frequency of 2nd-Hit Inactivation Mechanisms in HDGC Families and Neoplastic Lesions

We found somatic epi/genetic alterations in ≥1 neoplastic lesions from 80.0% of the families analyzed (12/15 probands).

These somatic epi/genetic alterations were present in 75% of the neoplastic lesions analyzed (21/28 lesions): 81.3% in primary carcinomas (13/16) and 66.7% in metastases (8/12) (Tables 2 and 3). In 3 primary tumors and 4 metastases, no 2nd-hit mechanism was identified. All alterations found in HDGC neoplastic lesions were absent from constitutional DNA, representing the 2nd-hit inactivation mechanism.

Epigenetic and Genetic CDH1 Alterations Occur Either Alone or Simultaneously in HDGC Neoplastic Lesions

From the 28 HDGC lesion analyzed, *CDH1* promoter hypermethylation was found in 9 (32.1%) cases, LOH in 7 (25.0%), and both alterations in 5 cases (17.9%), most probably reflecting tumor population heterogeneity. Examples of a 2nd-hit profile in HDGC neoplastic lesions are depicted in Figure 1. We did not find somatic mutations, in the regions analyzed, in any of the neoplastic lesions.

In HDGC primary tumors (n = 16), we found *CDH1* promoter hypermethylation in 8 (50%) cases, LOH in 2 (12.5%), and both alterations in 3 (18.8%; Table 1; Figure 1). *CDH1* epigenetic alterations alone were present in 50% of the cases, whereas LOH, concomitantly or not with epigenetic alterations, was present in 31.3% of the cases (Table 2; Figure 1). The 8 neoplastic lesions harboring *CDH1* promoter hypermethylation only, retained both alleles and did not show somatic mutations.

In metastatic lesions (n = 12) we found *CDH1* promoter hypermethylation in 1 (8.3%) case, LOH in 5 (41.7%), and both alterations in 2 (16.7%). In metastases, 8.3% of the lesions displayed *CDH1* epigenetic alterations only, whereas 58.3% displayed LOH concomitantly or not with *CDH1* hypermethylation (Table 2).

The comparison of 2nd-hit mechanisms in primary tumors and metastatic lesions showed that primary tumors displayed preferentially epigenetic alterations (50%) as single events, whereas metastatic lesions displayed preferentially genetic (LOH) concomitantly or not with epigenetic alterations (58.3%; P = .0274; Table 2).

The tumor-associated methylation observed in our series of HDGC patients is very unlikely age related because the majority of patients were <50 years old at diagnosis (Tables 1 and 3). Sixty-nine percent of patients <50 years old (9/13) displayed *CDH1* promoter hypermethylation in their tumors whereas 50% (2/4) of patients >50 years old had hypermethylation in their tumors.

Different Lesions From the Same Patient Display Distinct Patterns of 2nd-Hit Mechanisms

In 3 families (families 2, 4, and 6), multiple lesions from the same patient were analyzed. In family 2, we

analyzed 1 primary tumor foci and 2 metastases and found that, although the primary tumor displayed LOH, both metastases displayed methylation concomitant with distal LOH (Table 3). Moreover, the LOH pattern displayed by the primary tumor foci showed loss of the complete *CDH1* coding region while in both metastases only the distal 3′ LOH marker (D16S3067) showed loss (Figure 2).

In family 4, we studied 2 patients: 2 primary tumor foci and 1 metastasis from patient 1 and, 2 primary tumor foci and 2 metastases from patient 2. Primary tumors from patient 1 displayed different 2nd-hit mechanisms; 1 displayed methylation and the other LOH at ≥2 intragenic markers (germline mutation and rs1801552). The metastasis displayed LOH at a single intragenic marker (germline mutation; Table 3 and Figure 1). As for patient 2, primary tumor foci displayed also different 2nd-hit mechanisms; 1 displayed methylation only and the other displayed methylation concomitantly with distal LOH (D16S3067). The 2 metastases from patient 2 displayed LOH only, but with different patterns of loss (Table 3 and Figure 1).

The results in these 2 families likely indicate that the different lesions analyzed in each patient's stomach/metastases arose as a result of independent 2nd-hit mechanisms, acting somatically at the *CDH1* locus.

In family 6, several lesions were analyzed from a single patient and a different scenario was observed: The 2 primary tumor foci analyzed showed methylation only, but in the 3 ovarian metastasis no methylation, LOH, or somatic mutation in any of the 16 *CDH1* exons (exceptionally in this case, enough DNA was available for *CDH1* complete mutation screening) was found as a 2nd-hit mechanism (Table 3).

We validated all 2nd-hit results by confirming the common origin of all neoplastic lesions and matched normal tissue, from each patient, with a large set of insertion/deletion markers in 20 different chromosomes. This analysis allowed us to infer whether each lesion arose independently in each patient's stomach/metastases or whether all lesions were likely to arise as a result of a common trigger mechanism. Moreover, we used the insertion/deletion patterning to exclude allele dropout as a potential reason for loss at single *CDH1* LOH markers. LOH was commonly observed in HDGC tumors at the *CDH1* locus while no allele dropout occurred in the PCR amplification of the 32 insertion/deletion markers.

The E-Cadherin Immunoexpression in HDGC Tumors Is Generally Consistent With the Combination of Germline and/or Somatic Defects

We performed E-cadherin IHC in 26 of 28 lesions using an antibody that recognizes the cytoplasmic domain of the protein and therefore is expected to stain E-cadherin full-length or near full-length proteins only. We assessed the percentage of positive and negative cells for E-cadherin immunoreactivity as well as the cellular localization of the protein (Table 4). For all cases, we had as internal control normal E-cadherin expression either at the foveolar zone or in nondisrupted glands, adjacent to all tumor areas. All lesions analyzed contained neoplastic cells displaying abnormal or absent E-cadherin protein expression and localization, with the exception of neoplastic cells belonging to tumor from family 12, which displayed only homogeneous membrane expression. In most cases we observed consistency of IHC results and the combination of germline and somatic defects (Table 4). All these results as well as their interpretation are provided in Table 4.

Both the percentage of expressing cells and the cellular localization of the protein were independent of type and site of *CDH1* germline mutation as well as from type of *CDH1* somatic inactivation mechanism and type of lesion analyzed (primary tumor or metastasis).

The Type of 2nd-Hit Mechanism Is Not Dependent on the Type and Site of CDH1 *Germline Mutation*

We performed association studies in an effort to understand whether the type of 2nd-hit in HDGC tumors was dependent on the *CDH1* germline mutation type. We analyzed whether individuals carrying either truncating or missense mutations displayed any specific type of 2nd-hit inactivation mechanism and verified that no association exists between these 2 parameters.

In addition, no association was found between the site of *CDH1* germline mutation and the type of *CDH1* somatic inactivation mechanism. Somatic promoter hypermethylation alone and LOH alone or with concurrent promoter hypermethylation occurred in tumors from carriers harboring germline mutations dispersed throughout the *CDH1* gene (Figure 3).

Discussion

Individuals carrying germline mutations in cancer-related genes are at increased risk of developing neoplasia during their lifetime. Overall, carriers of *CDH1* germline mutations have a >70% lifetime risk (penetrance) of developing diffuse gastric cancer; female mutation carriers have an additional risk of 40% of developing breast cancer.[27]

Asymptomatic *CDH1* mutation carriers are offered the opportunity to undergo total prophylactic gastrectomy, therefore preventing the disease to develop in the target organ. However, this is a dramatic decision for apparently healthy carriers of *CDH1* germline mutations; if prophylactic gastrectomy is not performed, the overall outcome of HDGC patients is expected to remain poor. Most HDGC patients carrying a *CDH1* mutation develop preclinical, not detectable by endoscopy, multifocal tumors with an increased ability for metastatic spread.[15] When these patients present to the clinics, commonly the dis-

Table 4. Interpretation of E-Cadherin Immuno-Expression in HDGC Neoplastic Lesions and Its Relationship With Germline and Somatic *CDH1* Defects

Family ID	Patient's lesion	Germline mutation	Mutation type	Second hit	E-cadherin–positive cells (% and pattern)	Comment	E-cadherin–negative cells (%)	Comment
1	Pat 1 - T1	283C>T	Truncating	Meth	0	—	100	Consistent with methylation of WT allele and no protein derived from germline mutant allele
2	Pat 1 - T1	382delC	Truncating	LOH	0	—	100	Consistent with LOH of WT allele and no protein derived from germline mutant allele
	Pat 1 - M1			Meth + dLOH	50 (hetero memb or dotted cyto)	Consistent with dLOH leading to aberrant expression (?) and no protein derived from germline mutant allele	50	Consistent with methylation of WT allele and no protein derived from germline mutant allele.
	Pat 1 - M2			Meth + dLOH	50–75 (dotted cyto)	Consistent with dLOH leading to aberrant expression (?) and no protein derived from germline mutant allele	25–50	Consistent with methylation of WT allele and no protein derived from germline mutant allele
3	Pat 1 - M1	1003C>T	Truncating	LOH	0	—	100	Consistent with LOH of WT allele and no protein derived from germline mutant allele
	Pat 2 - T1			Not found	75 (dotted cyto)		25	
4	Pat 1 - T1	1018A>G	Missense	Meth	0	—	100	Consistent with biallelic methylation
	Pat 1 - T2			iLOH	Not done	Not analyzed	Not done	Not analyzed
	Pat 1 - M1			iLOH	100 (memb)	Consistent with LOH of WT allele and membrane expression of the germline mutant allele*	0	—
	Pat 2 - T1	1018A>G		Meth	0	—	100	Consistent with biallelic methylation
	Pat 2 - T2			Meth + dLOH	50 (homo and hetero memb)	Consistent with expression germline mutant allele or with dLOH leading to aberrant expression (?)	50	Consistent with biallelic methylation
	Pat 2 - M1			LOH	25–50 (hetero memb)	Consistent with LOH of WT allele and membrane expression of the germline mutant allele*	50–75	Inconsistent
	Pat 2 - M2			LOH	0	Inconsistence	100	Inconsistence
5	Pat 1 - T1	1063delT	Truncating	Not found	<25 (hetero memb)		>75	
6	Pat 1 - T1	1137G>A	Truncating	Meth	25–50 (hetero memb; dotted cyto)	Consistent with methylation of WT allele and aberrant protein derived from germline mutant allele	50–75	Consistent with biallelic methylation
	Pat 1 - T2			Meth	25–50 (hetero memb; dotted cyto)	Consistent with methylation of WT allele and aberrant protein derived from germline mutant allele	50–75	Consistent with biallelic methylation
	Pat 1 - M1			Not found	<25 (hetero cyto)		>75	
	Pat 1 - M2			Not found	<25 (dotted cyto)		>75	
	Pat 1 - M3			Not found	<25 (dotted cyto)		>75	
7	Pat 1 - T1	1225T>C[42]	Missense	Meth + LOH	25–50 (hetero memb or dotted cyto)	Consistent with methylation of WT allele and aberrant protein derived from germline mutant allele[42]	50–75	Consistent with biallelic methylation (?)
8	Pat 1 - M1	1476delAG	Truncating	LOH	25–50 (hetero memb)	Inconsistence*	50–75	Consistent with methylation of WT allele and no protein derived from germline mutant allele.
9	Pat 1 - T1	1779insC	Truncating	Meth +iLOH	<25 (hetero memb or dotted cyto)	Inconsistence*	>75	Consistent with LOH or methylation of WT allele and no protein derived from germline mutant allele
10	Pat 1 - T1	1901C>T	Truncating/ missense	Not found	0		100	
11	Pat 1 - T1	2061delTG	Truncating	Meth	0	—	100	Consistent with methylation of WT allele and no protein derived from germline mutant allele

Table 4. (Continued)

Family ID	Patient's lesion	Germline mutation	Mutation type	Second hit	E-cadherin–positive cells (% and pattern)	Comment	E-cadherin–negative cells (%)	Comment
12	Pat 1 - M1	2195G>A[42]	Truncating/ missense	Meth	100 (memb)	Consistent with methylation of WT allele and membrane expression of the germline mutant allele (missense)[42]	0	Consistent with biallelic methylation
13	Pat 1 - M1	2398delC	Truncating	Not found	Not done		Not done	
14	Pat 1- T1	2494G>A	Missense	Meth	<25 (dotted cyto)	Consistent with methylation of WT allele and aberrant expression of the germline mutant allele	>75	Consistent with biallelic methylation
15	Pat 1 - T1	2269G>A	Missense	Meth	25 (memb)	Consistent with methylation of WT allele and membrane expression of the germline mutant allele[41]	75	Consistent with methylation of WT allele and lack of membrane expression of the germline mutant allele[41]

NOTE. [42,unpublished data]The mutant E-cadherin protein carrying missense mutations frequently localizes to the membrane.
Meth, methylation; memb, membrane; cyto, cytoplasm; hetero, heterogeneous; homo, homogeneous; WT, wild type.
*No protein is expected to be produced from the germline mutant allele and if leakage occur, the truncated peptide generated by the germline mutation does not encompass a transmembrane nor a cytoplasmic domain of E-cadherin domain hampering antibody binding; [41]The 2269 mutation generates a protein that may or may not localize to the membrane.

ease is advanced and spread, and they only receive palliative treatments. This illustrates the reason why prophylactic gastrectomy is recommended before clinical disease presentation, that is, before the third decade of life,[28] and why efforts should be made to determine specific molecular mechanisms, occurring in these tumors, that may constitute potential targets for therapy.

Current knowledge shows that HDGC tumors initiate when the remaining *CDH1* wild-type allele becomes inactive in mutation carrier's stomachs, mainly through promoter hypermethylation,[10–12,14] according to Knudson's "2nd-hit" hypothesis.[9] Therefore, HDGC patients displaying *CDH1* promoter hypermethylation, as a 2nd-hit, in their neoplastic lesions, would benefit from the administration of drugs leading to the reversion of *CDH1* promoter methylated state. HDAC inhibitors and DNA-demethylating agents emerge, in this view, as attractive drugs to use in combination with classical chemotherapy agents. The use of such a strategy implies the definitive proof that hypermethylation is the most common mechanism of *CDH1* wild-type allele inactivation in HDGC neoplastic lesions, and the unique mechanism in different neoplastic lesions from the same patient. This knowledge is critical for the therapeutic management of HDGC patients.

The present report encloses the largest series of neoplastic lesions from patients belonging to *CDH1* germline mutation positive HDGC families, ever analyzed for *CDH1* 2nd-hit inactivating mechanisms (Table 1). Moreover, in this work we systematically analyzed all lesions for *CDH1* promoter hypermethylation using CpG methylation-independent PCR and sequencing, LOH with intragenic and flanking markers of the *CDH1* locus, and somatic mutations at *CDH1* hotspot regions, using multiplex PCR having the germline mutation as an internal control. Moreover, we used a large genome covering insertion/deletion patterning to confirm the common origin of different neoplastic lesions and matched normal samples from each individual as well as to exclude allele dropout in the LOH analysis. This approach was never used in previous studies of *CDH1* 2nd-hit analysis, in HDGC patients, precluding a trustworthy comparison of results. Nevertheless, by combining our results with those previously published in this disease,[10–12,14] *CDH1* 2nd-hit inactivation mechanisms are currently identified in ≥1

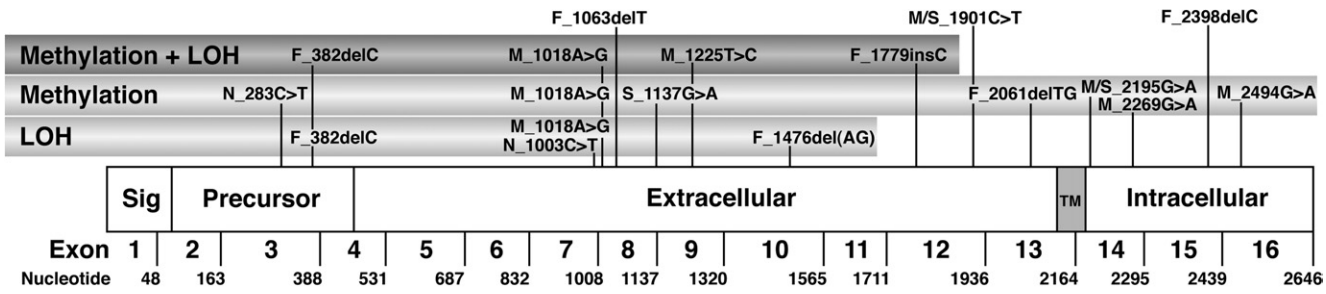

Figure 3. Schematic representation of 2nd-hit inactivating mechanisms superimposed with germline mutations type and site. *CDH1* germline mutations are preceded by letters indicating the type of mutation N (nonsense), F (frameshift), M (missense), and M/S (missense/splicing), and are aligned according to the 2nd-hit inactivating mechanism.

tumor from 34.1% ($n = 28$) of the HDGC families reported to date ($n = 82$).

Seventy-five percent of HDGC neoplastic lesions, herein analyzed, displayed somatic *CDH1* epi/genetic inactivation. This result is higher than that previously obtained combining all HDGC tumors analyzed (29.6%),[10–12,14] and similar to that described for sporadic gastric tumors with diffuse components (90%).[13] Reasons for this discrepancy are likely related to the methodologic approach used, as well as with the lack of information on *CDH1* LOH analysis in previous reports. In other cancer-associated syndromes, the frequency of 2nd-hit mechanisms identified is similar to that herein described as 60% of hereditary nonpolyposis colorectal cancer tumors displaying *hMLH1* hypermethylation and LOH,[17,18] 75% of familial ovarian cancer displaying *BRCA2* LOH,[19] and 83% of familial adenomatous polyposis desmoid tumors displaying *APC* somatic mutations and deletions.[20]

To have a full picture of how *CDH1* are inactivated in this invasive and spreading disease, we have analyzed a series of primary tumors and metastasis as well as several primary tumor foci and metastatic lesions from the same patient, whenever possible. This analysis allowed us to verify that (1) the majority of primary tumors displayed epigenetic alterations, (2) the majority of metastatic lesions displayed LOH, (3) neoplastic lesions, primary or metastatic, frequently display >1 *CDH1* 2nd-hit inactivating mechanism, and (4) different lesions from the same patient display distinct *CDH1* 2nd-hit inactivating mechanisms.

The initial observation of *CDH1* promoter hypermethylation as the most common 2nd-hit mechanism[10,11,14] is consistent with the fact that only primary HDGC tumors have been analyzed. Our own results demonstrate the same when only primary tumors are considered (11/16; 68.8%). Nevertheless, when the analysis is extended to multiple foci from the same patient and/or to metastatic lesions, this scenario is completely changed and *CDH1* hypermethylation becomes 1 among other 2nd-hit mechanisms in each patient, at the same time that LOH emerges as the most prevalent mechanism in metastases.

The biological reasons underlying our results are probably related to the nature of HDGC itself. One to >100 small foci of signet-ring cell gastric carcinoma are found in the stomachs of asymptomatic mutation carriers.[15,16] These lesions evolve and give rise to invasive cancer that populates the whole stomach as well as peripheral and distant lymph nodes.[15] In light of our present results, each of these foci is likely to arise from independent, 2nd-hit–inactivating mechanisms affecting the *CDH1* gene. Moreover, it is also plausible to believe that the vicinity of different initial foci, originated from distinct 2nd-hit mechanisms, cluster together creating a mixed population of neoplastic cells, heterogeneous at the molecular level, that ultimately constitute the bulk of the tumor. Neoplastic cells from these mixed populations are those probably traveling to distant sites and giving rise to metastases carrying distinct or even concomitant 2nd-hit mechanisms. Because our analysis was performed in advanced tumors, most probably the finding of >1 *CDH1* 2nd-hit–inactivating mechanism in the same lesion reflects the initial intratumor heterogeneity. Although rare, more than one 2nd hit has been described in tumors from other hereditary, cancer-related syndromes.[17,18]

The observations herein reported are consistent with those that we previously described in colorectal cancers: Primary tumors harbor either *BRAF* or *KRAS*, and corresponding lymph node metastases accumulate mutations in both genes.[29] These results demonstrate that somatic alterations either occur during progression to metastasis, in some cases, or and in accordance with Bernards and Weinberg,[30] that important components of the genotype of metastasis are already implanted early in tumorigenesis, in small primary tumor cells populations that have a greater ability to dispatch metastatic pioneers to distant sites in the body.

The most important consequence of our observations in HDGC neoplastic lesions is related to the clinical intervention and therapeutic approach to be used in a disease that remains without treatment. The design of any therapeutic approach, at this point, needs to take into account the targeting of different and independently arising populations of neoplastic cells. An approach based on epigenetic drugs only will fail because of the expected insensitivity of cells harboring *CDH1* somatic mutations or LOH. This hypothesis of insensitivity to a specific drug by a subpopulation of tumor cells, although in a different context, is elegantly illustrated by the results obtained by Taniguchi et al,[31] who described that patients with non–small-cell lung cancer constituted by 2 subpopulations of tumors cells displaying different molecular features (mutated and nonmutated for epidermal growth factor receptor [EGFR]) did not respond well to gefitinib. Moreover, the overall survival of these non–small-cell lung cancer patients after gefitinib treatment was significantly reduced compared with patients with EGFR-mutant tumor cells only, demonstrating the lack of benefit after this intervention.

The use of HDAC inhibitors and DNA-demethylating agents would likely be useful in the setting of patients where malignancy remains preclinical, a setting where at least half of the tumor foci may be already hypermethylated. Nevertheless, for those patients who present with tumors displaying both epigenetic and genetic alterations, new therapeutic strategies need to be developed.

Our group has showed that cells transduced with mutant forms of E-cadherin harboring mutations in the extracellular domain or displaying complete loss of *CDH1* expression modify the stability of E-cadherin/EGFR heterodimer, enhancing cancer cell motility through the activation of an EGFR downstream target. This effect was shown to be reverted upon EGFR pharmacologic inhibi-

tion and cells lacking functional E-cadherin stopped moving.[32] Moreover, E-cadherin negative ovarian cancer cells have been recently shown to overexpress α-5-integrin induced by activation of the EGFR pathway.[33] The use of $\alpha 5$-$\beta 1$-integrin–blocking antibody in mice xenografted with an ovarian cancer cell line, overexpressing $\alpha 5$-integrin, induced by E-cadherin down-regulation, significantly reduced the number of metastases and increased survival of the mice. In this same study, patients with high levels of $\alpha 5$-integrin died 9 months earlier than those displaying low $\alpha 5$-integrin expression.[33] Following a similar rational, Kawajiri et al[34] recently showed that the transforming growth factor-β-R inhibitor, A-77, decreased the adhesive and invasive abilities of diffuse gastric cancer cells to the mice peritoneum, as well as the size and number of metastatic nodes, and demonstrated that this effect was mediated by decreased expression of integrins.

All these studies describe drugs that reduce tumor cell migration and metastasis potentially increasing patient survival, that specifically act in cells lacking E-cadherin expression. Such drugs are expected to target cells displaying genetic *CDH1* alterations in the same way that demethylating agents and HDAC inhibitors are expected to re-induce E-cadherin expression in cells displaying *CDH1* promoter hypermethylation. The concurrent use of these 2 types of approaches may be applicable to HDGC patients with tumors displaying epigenetic and genetic alterations. When used early, epigenetic drugs would be expected to restore *CDH1* expression in small foci of primary tumor, eventually arresting them in an epithelial state. Genetic alterations, as LOH, which seem to be acquired later in HDGC progression, could be then targeted with drugs such as EGFR and $\alpha 5$-$\beta 1$-integrin–blocking antibodies or transforming growth factor-β-R small synthetic molecules to prevent cells from migrating and metastasis from establishing.[35–40]

In conclusion, our data support the previously published rate of *CDH1* promoter hypermethylation as a 2nd-hit in HDGC primary tumors, and adds LOH as a key mechanism, mainly in metastatic lesions. Because of the concomitance and heterogeneity of these alterations in neoplastic lesions and the plasticity of hypermethylated promoters during tumor initiation and progression, drugs targeting only epigenetic alterations might not be effective, particularly in patients with metastatic HDGC.

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Conflicts of interest

The authors disclose no conflicts.

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