

# Allele-specific *CDH1* downregulation and hereditary diffuse gastric cancer

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**Hereditary diffuse gastric cancer (HDGC) is an autosomal dominant cancer susceptibility syndrome characterized by early-onset diffuse gastric cancer (DGC) and lobular breast cancer. E-cadherin (*CDH1*) heterozygous germline mutations and deletions are found in 40% of families. Independent of *CDH1* alterations, most HDGC tumours display mislocalized or absent E-cadherin immunoreexpression, therefore undetected defects at the *CDH1* locus may still be involved. We aimed at determining whether *CDH1* mutation-negative probands display germline *CDH1* allele-specific expression (ASE) imbalance, using a single-nucleotide primer extension-based procedure and tried to uncover the underlying molecular defect. *CDH1* ASE analysis was performed using three intragenic SNPs in RNA extracted from the blood of 21 cancer-free individuals and 22 HDGC probands (5 *CDH1* mutation carriers and 17 *CDH1* negative). Germline promoter methylation, deletions and haplotype-related susceptibility at the *CDH1* locus were analysed. Both *CDH1* alleles from cancer-free individuals displayed equivalent expression levels, whereas monoallelic *CDH1* expression or high allelic expression imbalance (AI) was present in 80% of *CDH1* mutant and 70.6% ( $n = 12$ ) of *CDH1*-negative HDGC probands. Germline deletions and promoter hypermethylation were found in 25% of probands displaying high *CDH1* AI. No particular haplotype was found to be associated with *CDH1* high AI. Germline *CDH1* AI is highly frequent among *CDH1* mutation-negative probands but was not seen in cancer-free individuals. This implicates the *CDH1* locus in the majority of mutation-negative HDGC families.**

## INTRODUCTION

Hereditary diffuse gastric cancer (HDGC) (OMIM No. 137215) is an autosomal dominant cancer-associated syndrome characterized by clustering of early-onset diffuse gastric cancer (DGC) (1) and lobular breast cancer (LBC) (2). Approximately 40% of HDGC families harbour heterozygous germline inactivating alterations of E-cadherin (*CDH1*) segregating with the disease (3,4). We have recently reported that HDGC is not only caused by *CDH1* mutations but also by large deletions affecting the *CDH1* locus (4). Although many additional high- and low-penetrance genes have been studied in HDGC, we and others failed to identify other germline genetic causes for cases that remain without molecular diagnosis.

DGC occurring in *CDH1* germline mutation carriers displays abnormal or absent E-cadherin protein expression, due to the inactivation of the remaining wild-type allele through somatic promoter methylation, loss of heterozygosity or a second mutation (5–7). In our experience, tumours from families with clustering of DGC display similar morphological features and abnormal E-cadherin expression pattern, independent of harbouring germline *CDH1* alterations (unpublished data). Therefore, we believe that other *CDH1* germline genetic and epigenetic defects may be the cause of DGC clustering in families that remain genetically unexplained.

Recently, autosomal genes have been demonstrated to be the subject of random monoallelic inactivation (8). Yet, *CDH1* was not one of those genes and was shown to be

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biallelically expressed in normal conditions (8). Approximately 10% of 4000 human autosomes analysed display random monoallelic expression a feature shared with imprinted genes or those encoded by the X-chromosome (8–10). Allelic expression imbalance (AI) for breast susceptibility genes *BRCA2* and *BRCA1* and for the colon cancer susceptibility gene *APC*, has also been associated as cancer-associated risk factors (11,12). More recently, germline AI of *TGFBR1* was shown to confer increased risk of colorectal cancer, and two major haplotypes were predominantly found among cases displaying AI. Nevertheless, none of the previous reports identified the AI-causing mechanism (13,14). The measurement of *CDH1* allele-specific expression (ASE) in the germline of *CDH1*-negative HDGC probands could be used to identify individuals with potential heterozygous germline genetic and epigenetic *CDH1* abnormalities. Tan *et al.* (15) used common single-nucleotide polymorphisms within the *CDH1* transcripts (cSNPs) to demonstrate the AI of *CDH1* and other autosomal genes in a familial pancreatic cancer patient.

We studied whether patients with familial clustering of gastric cancer (GC) mainly of the diffuse type (HDGC) that tested negative for *CDH1* germline alterations display germline *CDH1* AI and attempted to identify the genetic abnormality underlying this phenomenon.

## RESULTS

In this study, we aimed at investigating whether families with GC aggregation, namely HDGC families, that proved negative for *CDH1* germline alterations display *CDH1* AI in RNA derived from peripheral blood lymphocytes (PBLs). Highly polymorphic SNPs at the *CDH1* mRNA (coding SNPs: rs1801552 and rs33964119, and 3'-UTR SNP rs1801026) were selected and used as allele discriminators for *CDH1* AI determination.

### Cancer-free individuals display equivalent germline RNA expression of *CDH1* maternal and paternal alleles

Three SNPs were genotyped from PBLs' RNA from 50 control cancer-free individuals to select a series of heterozygous individuals for ASE analysis. Twenty-one of the control cancer-free individuals were heterozygous at SNP rs1801552 ( $n = 14$ ), rs33964119 ( $n = 1$ ) and/or rs1801026 ( $n = 10$ ), and their cDNAs used to determine the relative expression of *CDH1* maternal and paternal alleles. In all cases, T and C alleles were identically represented (Fig. 1A), and a range of normalcy values was defined with an upper boundary for normal allelic expression ratio. The mean expression ratio in the cancer-free individuals' germline RNA was  $1.32 \pm 0.14$ , and the ratio between alleles did not change significantly when a different SNP was used in the same sample (Fig. 1B). Moreover, these RNA results were similar to those obtained when using matched genomic DNA (gDNA) (Fig. 1C). The fact that the ratio remains equivalent independent of nucleic acid and SNPs used demonstrates that this assay is suitable for ASE quantitative measurement.

### HDGC *CDH1* germline mutation carriers display germline *CDH1* AI

We applied the ASE quantification method, established for cancer-free individuals, in PBLs' RNA from five HDGC probands shown elsewhere to be germline *CDH1* mutation carriers (Table 1). Our aim was to understand whether *CDH1* AI would reflect the presence of a *CDH1* germline mutation.

After confirming that all five probands were constitutively heterozygous for SNP rs1801552 using polymerase chain reaction (PCR) sequencing, ASE analysis was conducted in germline RNA: 80% (4/5) of *CDH1* mutation carriers showed high AI, which was not observed in gDNA using the same primer extension assay (Fig. 2A). One of the five mutation carriers lacked AI and displayed similar ASE mean ratios in RNA and DNA (data not shown). This specific patient carried a missense mutation, whereas the other four were carriers of truncating *CDH1* mutations.

### *CDH1*-negative probands display germline *CDH1* AI

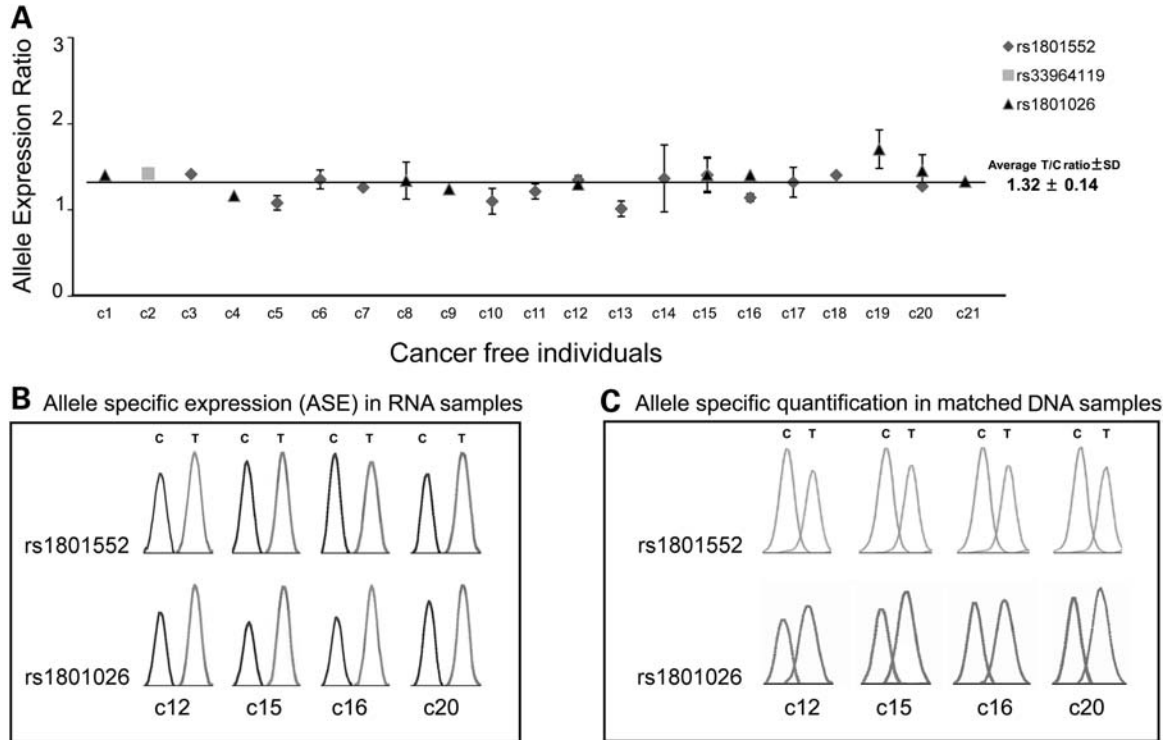
Of 27 *CDH1* mutation-negative probands, 17 showed to be heterozygous for at least one of the SNPs ( $n = 16$  for rs1801552,  $n = 1$  for rs33964119 and  $n = 1$  for rs1801026) and represented families with: (i) two or more GC cases with at least one DGC in first-degree relatives diagnosed before age 50 ( $n = 9$ ); (ii) three or more GC in first-degree relatives with at least one DGC diagnosed at any age ( $n = 1$ ); (iii) DGC before 45 years of age ( $n = 1$ ); (iv) LBC before 45 years of age ( $n = 1$ ); (v) clustering of GC cases of unknown histology ( $n = 5$ ) (Table 1).

Fifteen out of 17 (88.2%) heterozygous *CDH1*-negative probands presented AI with mean ratios above the proposed normal values (Fig. 2B), whereas gDNA data showed equivalent representation of both alleles (Fig. 2C). In 70.6% (12/17) of patients with AI, the mean ratio was higher than 6, whereas the remaining demonstrated a more modest overrepresentation of one allele (Table 1). Complete reduction to monoallelic expression was observed in 41.2% (7/17) of cases (Table 1). The analysis of gDNA in these cases was repeated using a different DNA sample and the heterozygous state at the SNP position confirmed (data not shown). In four mutation-negative probands, we used a different single-base extension (SBE) primer to the same SNP to show that results were primer independent (Fig. 2C).

### *CDH1* germline promoter hypermethylation and germline large deletions occur in probands with germline *CDH1* AI

To disclose putative epigenetic and structural mechanisms that could underlie the presence of high AI in the germline of *CDH1*-negative probands, we studied the *CDH1* promoter methylation status as well as the presence of genomic rearrangements at the gene locus in PBLs' DNA.

We studied germline *CDH1* promoter hypermethylation in 12 probands with high *CDH1* AI. A single proband from Family 11 displayed promoter hypermethylation at least in 11/19 (57.9%) CpG sites (Fig. 3A). Although not completing the HDGC criteria, this family has a history of at least two GC of unknown histology, and several cases of breast and colorectal cancer, which



**Figure 1.** *CDHI* ASE analysis in cancer-free individuals. (A) *CDHI* allelic expression ratio in cancer-free individuals. (B) ASE in RNA samples from four heterozygous individuals. (C) Allele-specific quantification in gDNA samples from four heterozygous individuals.

**Table 1.** Features of probands from GC families selected for ASE analysis

Family ID	Geographic origin	Inclusion criteria <sup>a</sup>	<i>CDHI</i> germline alteration (type)	ASE ratio rs1801552	ASE ratio rs33964119	ASE ratio rs1801026
21 cancer-free individuals	Portugal	NA	NA	1.27 ± 0.06	1.42 ± 0.13	1.38 ± 0.02
Family 1 (16)	Brazil	i	<b>Mutation (1137G&gt;A)</b>	<b>10.00 ± 0.00</b>	NA	NA
Family 2 (17)	Canada	i	<b>Mutation (2276delG)</b>	<b>10.00 ± 0.00</b>	NA	NA
Family 3 (18)	Canada	i	<b>Mutation (1792C&gt;T)</b>	<b>9.07 ± 1.24</b>	NA	NA
Family 4 (19)	Canada	ii	<b>Mutation (187C&gt;T)</b>	<b>8.53 ± 1.97</b>	NA	NA
Family 5 (20)	Portugal	i	<b>Mutation (2269G&gt;A)</b>	2.11 ± 0.89	NA	NA
Family 6	Portugal	i	Negative	<b>8.87 ± 1.51</b>	NA	<b>10 ± 0.00</b>
Family 7	Portugal	v	Negative	<b>10.00 ± 0.00</b>	NA	NA
Family 8	Portugal	i	Negative <sup>b</sup>	<b>10.00 ± 0.00</b>	NA	NA
Family 9	China	i	Negative	<b>6.67 ± 2.02</b>	NA	NA
Family 10	Canada	i	Negative	<b>10.00 ± 0.00</b>	NA	NA
Family 11	Canada	v	<b>Promoter hypermethylation</b>	<b>8.63 ± 1.82</b>	NA	NA
Family 12	Canada	v	Negative	<b>10.00 ± 0.00</b>	NA	NA
Family 13	Ireland	i	Negative	<b>10.00 ± 0.00</b>	NA	NA
Family 14	Italy	iv	Negative	<b>9.93 ± 0.09</b>	NA	NA
Family 15	Ireland	ii	Negative	<b>8.44 ± 2.08</b>	NA	NA
Family 16	Portugal	v	Negative <sup>b</sup>	3.24 ± 0.64	NA	NA
Family 17	Canada	v	Negative	2.87 ± 1.36	NA	NA
Family 18	Canada	iii	Negative	2.48 ± 0.54	NA	NA
Family 19	Spain	i	Negative	1.96 ± 0.08	NA	NA
Family 20	Portugal	i	Negative	1.69 ± 0.30	NA	NA
Family 21 (4) <sup>c</sup>	Canada	i	<b>Deletion</b>	<b>10 ± 0.00</b>	NA	NA
Family 22 (4) <sup>d</sup>	Canada	i	<b>Deletion</b>	NA	<b>10 ± 0.00</b>	NA

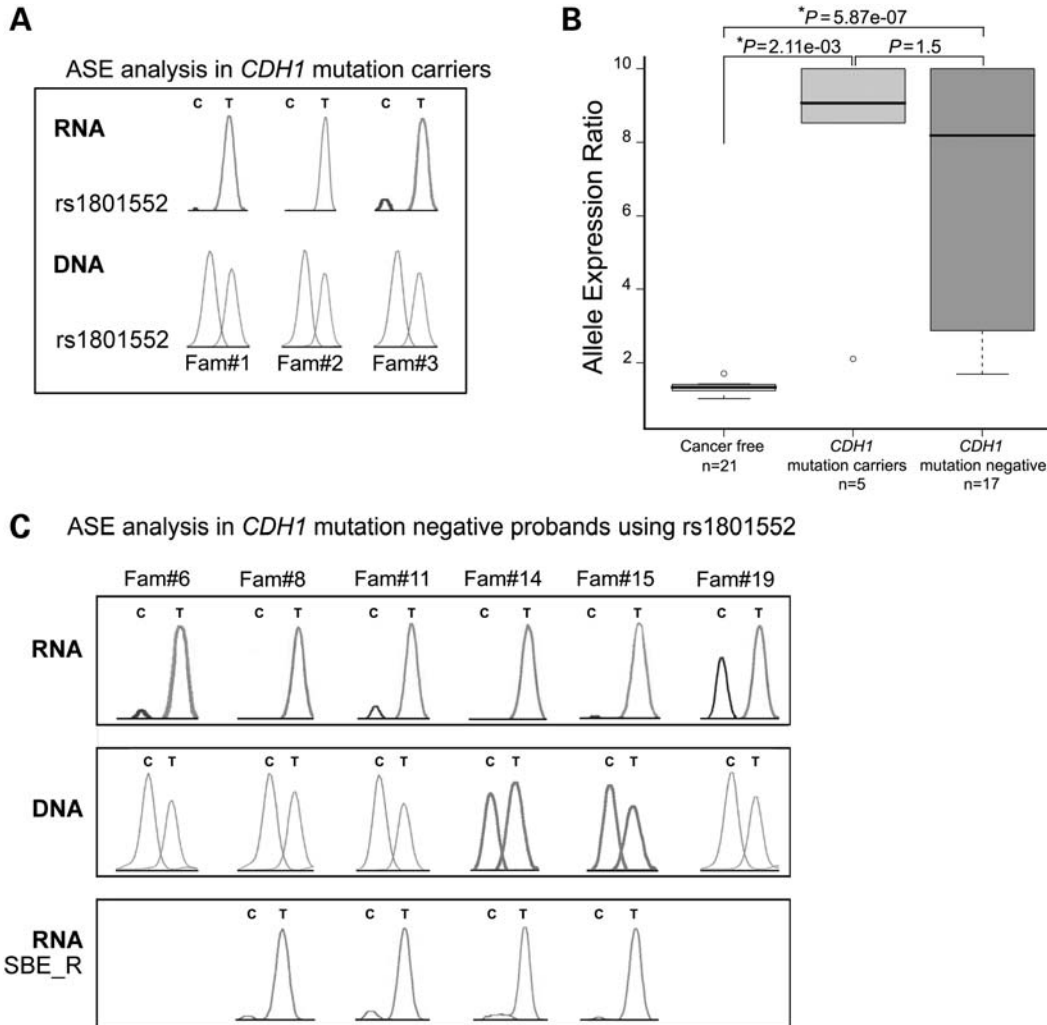
NA, not applicable; high A1 values and *CDHI* alterations are highlighted in bold.

<sup>a</sup>See Results section.

<sup>b</sup>Mutation negative and not tested for large deletions.

<sup>c</sup>Family 1.

<sup>d</sup>Family 4 in reference (4).



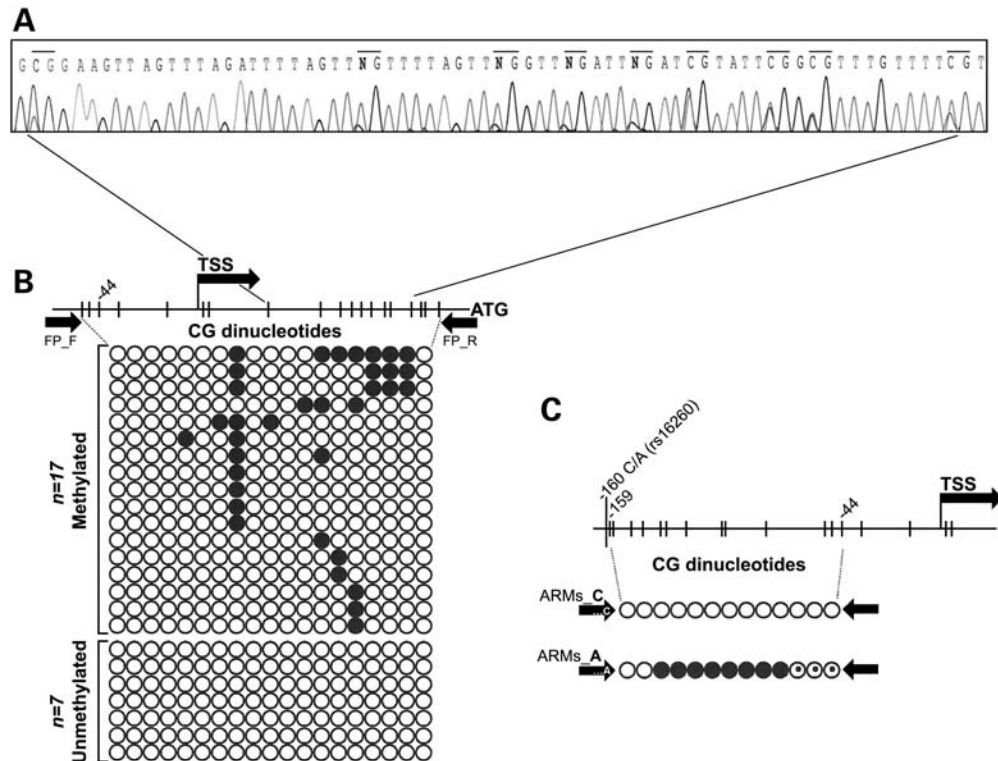
**Figure 2.** *CDH1* allele-specific quantification in *CDH1*-mutated and *CDH1*-negative probands. (A) ASE in *CDH1* mutation carriers' RNA and matched DNA. (B) Conventional boxplot (with whiskers) of allele expression ratio in cancer-free individuals ( $n = 21$ ; IQR = 0.17; Q1 = 1.24; Q3 = 1.41), *CDH1* mutation carriers ( $n = 5$ ; IQR = 3.23; Q1 = 6.77; Q3 = 10) and *CDH1* mutation-negative probands ( $n = 17$ ; IQR = 7.02; Q1 = 2.96; Q3 = 9.98). *P*-values refer to Wilcoxon test (BC correction:  $\times 3$ ).

may also occur in the tumour spectrum of HDGC. Even so, all affected members in this family have deceased, and therefore a transgenerational study could not be performed. We had access to PBLs' DNA from a probands' brother and granddaughter (both non-affected by cancer so far) and observed that neither had *CDH1* promoter hypermethylation.

To understand whether *CDH1* hypermethylation could be allele-specific as well as to determine the extent of methylation in *CDH1* alleles of this case, 24 cloned alleles were sequenced (Fig. 3B). A significant proportion of alleles displayed variable allele-to-allele hypermethylation, supporting that germline allele-specific methylation could be occurring in this patient. To address this issue, we took advantage of patients' heterozygosity at the common *CDH1* promoter SNP rs16260 ( $-161C/A$ ) to perform amplification refractory mutation system (ARMS)-based PCR (ARMS-PCR) and amplify each allele separately from bisulphite-converted DNA. With this approach, we showed that the methylated clones observed in Figure 3B are likely all derived from the

$-161A$  allele, since only this allele was hypermethylated (Fig. 3C). In parallel with the current study, a large series of *CDH1* mutation-negative HDGC probands, which included 15/17 probands from the present *CDH1*-negative series, was screened for large genomic rearrangements at the *CDH1* locus on chromosome 16 by MLPA and array CGH (4). By crossing our *CDH1* AI data with the data arising from the rearrangement mapping, we verified that 2/12 (16.7%) probands with high *CDH1* AI displayed large deletions at the *CDH1* locus. One deletion (193,593 bp) was found in Family 21 (referred as Family 1 in our previous report) and encompassed the full sequence of *CDH3*, extending to position IVS2+57,595 in *CDH1* (4). The other deletion found in Family 22 (referred as Family 4 in our previous report) was a 150 bp removal, encompassing the *CDH1* TSS, with break-points located 125 bp upstream and 25 bp downstream of the TSS (4).

Our results show that 25% (3/12) of probands previously described as mutation negative display high *CDH1* AI,



**Figure 3.** Germline *CDH1* promoter hypermethylation analysis. (A) Partial electropherogram from Family 11 showing methylation at 9/19 CpG sites. (B) Schematic representation of CpG dinucleotides encompassed by the flanking PCR. Each line of circles represents an isolated allele; an open circle, a non-methylated CpG; and a black circle, a methylated CpG. (C) ARMs PCR scheme covering 13 CpG. The rs16260\_A allele is methylated in at least eight CpG sites and partially methylated in three (circles with a black dot).

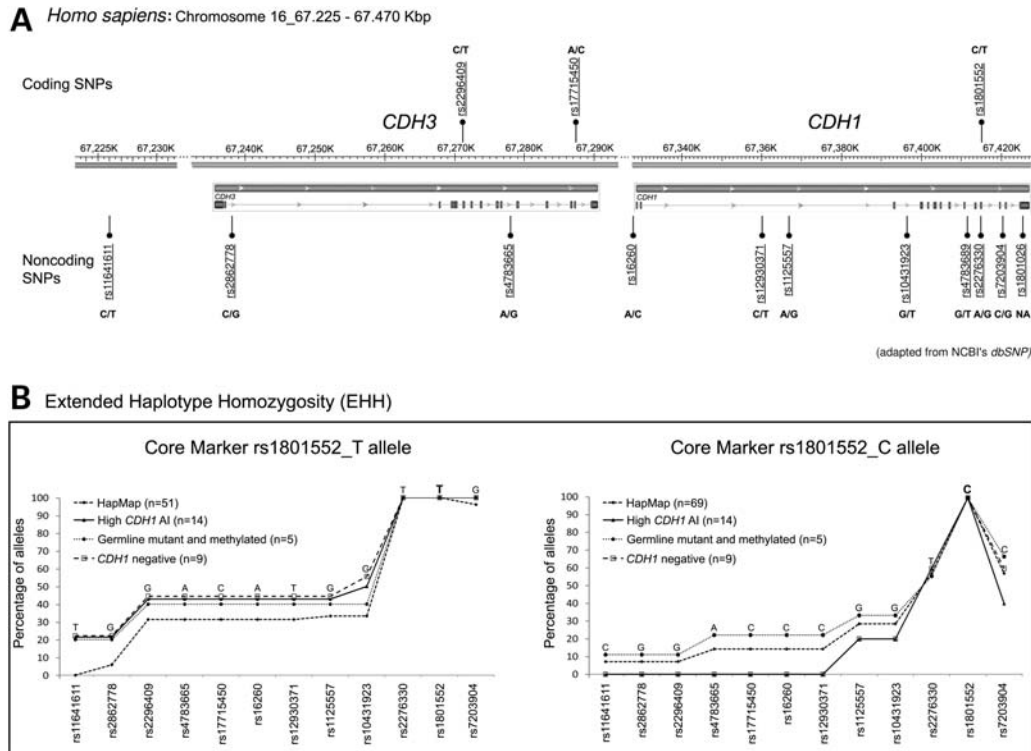
which is likely generated by germline *CDH1* methylation and large genomic rearrangements at the gene locus.

### Germline *CDH1* AI is not associated with a specific extended haplotype

A finding derived from the present ASE analysis was the exclusive underexpression of the rs1801552\_C allele in all samples, independent of the level of AI detected as well as of the *CDH1* germline alteration status, when PBLs' RNA was compared with gDNA (Fig. 2A and C). This result was consistent when both flanking and SBE primers were changed (Fig. 2C, last panel), supporting the hypothesis of a rs1801552\_C allele-associated downregulation, possibly reminiscent of an ancestral disease-associated haplotype and potentially associated with the occurrence of germline *CDH1* alterations always in the C-allele. Supporting this idea, the haplotype reconstruction in Family 11 (displays high *CDH1* AI and allele-specific *CDH1* germline promoter methylation) showed that the hypermethylated rs16260\_A *CDH1* promoter allele was in phase with the underexpressed rs1801552\_C allele.

To test this hypothesis, and assuming that, whatever the mechanism, downregulation of *CDH1* was occurring always in the ancestral rs1801552\_C-allele, we genotyped 12 SNPs, encompassing distinct haplotype blocks along *CDH3* and *CDH1* regions from chromosome 16 (Fig. 4A, Supplementary Material, Fig. S1) in 14 probands with high

*CDH1* AI (four with germline *CDH1* mutation, one with germline *CDH1* promoter methylation and nine without *CDH1* alteration). Haplotypes were reconstructed using PHASE 2.02 software (16,17). HapMap haplotypes from a control population ( $n = 120$ ) with a known phase were used to improve inference of probands' haplotypes. The GOLD (graphical overview of linkage disequilibrium) application (18,19) shows, for our SNP data set, a common region of strong linkage disequilibrium ( $|D'| \geq 0.84$ ) between rs1801552, rs7203904 and rs2276330 (Supplementary Material, Fig. S2). However, the block structure observed in the HapMap control data (Supplementary Material, Figs S1 and S2A) for the region encompassing rs1801552, rs7203904 and rs2276330 was not maintained for the group of 14 probands with high *CDH1* AI alone (Supplementary Material, Fig. S2B). Even when the nine probands with high *CDH1* AI were analysed separately, similar results were obtained, suggesting that the proband series with high *CDH1* AI is somewhat more diverse than the HapMap one (Supplementary Material, Fig. S2B1, 2C and 2A). Nevertheless, when we assessed the breakdown of rs1801552\_T and rs1801552\_C haplotypes (Fig. 4B—left and right panels, respectively) from probands and controls is made clear that the higher diversity of *CDH1* high AI may be explained by rs1801552\_C haplotypes alone. Moreover, the lack of a homogeneous region in the vicinity of rs1801552\_C, common to all probands, refutes the hypothesis of high AI being associated with a specific extended haplotype.



**Figure 4.** Haplotype analysis. (A) Adapted NCBI representation of 193 kb encompassing 12 coding and noncoding SNPs chosen along *CDH3* and *CDH1* loci. (B) Decay over distance of common haplotypes from rs1801552\_T and rs1801552\_C alleles. The most frequent haplotype is represented above the lines for each considered subgroup (HapMap, high *CDH1* AI, germline mutant and methylated and *CDH1* negative).

## DISCUSSION

To date, ~40% of families with well-defined clustering of DGC, described as HDGC, display *CDH1* germline inactivation (4). A lower frequency of *CDH1* alterations has also been described in isolated early-onset DGC patients, in families that do not fulfil the widely accepted clinical criteria and in families with clustering of early-onset LBC, without DGC involvement (3,20). Most reported germline *CDH1* alterations lead to the production of truncated inactive peptides that lack normal function.

Given that a fair proportion of eligible families for germline *CDH1* mutation screening lack such alterations despite displaying E-cadherin protein expression impairment in their tumours (data not shown), we propose that, in these cases, *CDH1* is still compromised due to alterations that escape detection by conventional techniques.

Mutations leading to protein truncation constitute the majority of inactivating mutations in cancer susceptibility genes (3,21–24). Transcripts containing premature termination codons (PTCs), mainly as a result of frameshift and nonsense mutations, are eliminated by the nonsense-mediated mRNA decay (NMD) (25). Owing to the instability of PTC-containing transcripts, many mutations remained undetected by RNA-based techniques and only became discovered upon usage of DNA-based methods. Conversely, as the latter rely mostly on the amplification of individual exons with intronic flanking primers, large genomic deletions and changes in more distant noncoding regions were also missed, until

recent technical improvements have been achieved (MLPA and arrayCGH) allowing their detection. Undiscovered alterations may therefore be revealed by techniques that permit individual assessment of paternal and maternal allelic expression. Without disregarding this interesting possibility, one has to consider that monoallelic expression with random choice between maternal and paternal alleles is no longer exclusive of imprinted and X-inactivated genes, as a group of autosomal gene families has been recently identified as a target of monoallelic downregulation (8).

In the last decade, at least three studies have been published re-enforcing the role of germline AI as a major risk factor for cancer development in sporadic and hereditary cohorts. These studies identified AI in *BRCA1*, *BRCA2*, *APC* and lately in *TGFBR1*, using well-selected cohorts of cancer patients and cancer-free control individuals (11–13). All these studies made use of different methodological approaches to analyse ASE in patients' germline RNA; nevertheless, none described the molecular mechanism underlying the observed effect on RNA monoallelic downregulation.

In the present study, we established a reliable and straightforward assay to evaluate *CDH1* ASE using SNPs within *CDH1* mRNA. We demonstrated that *CDH1* expression is biallelic in PBLs from heterozygous cancer-free individuals and that both *CDH1* alleles express equivalent RNA levels, as mentioned previously (8). Moreover, the contribution of each allele was found to be independent from the SNP tested or SBE primers used (Fig. 1B and 2C). This result enabled us to set an upper artificial boundary for *CDH1*

normal expression ratio in cancer-free individuals. A valuable test of the approach reliability came from results obtained in four HDGC *CDHI* germline-truncating mutation carriers, which showed that *CDHI* AI in mutant probands was statistically higher than that of cancer-free individuals ( $P = 2.11 \times 10^{-3}$ ), as expected after PTC degradation by NMD (25,26). Similar results have been previously obtained for *BRCA1*, *BRCA2* and *APC* (11,12).

Having established the assay, we had the proper tool to disclose whether this finely tuned biallelic expression was getting disrupted in our series of *CDHI*-negative probands. Our main concern was to select a series of patients that, although negative for *CDHI* germline alterations, was, in all features, similar to any series previously used for *CDHI* mutation screening. This selection was expected to increase the probability of finding a mechanism of disease, although it was highly dependent on RNA availability from probands' PBLs. After the selection of 17 *CDHI*-negative probands, from different geographic backgrounds and fulfilling well-defined clinical criteria (see Results), we investigated the possibility of these probands presenting germline *CDHI* AI. An impressive and unexpected proportion of these patients (70.6%) revealed AI ratios  $>6$ , with recurrent downregulation of the rs1801552\_C allele. Coincidentally, a recent study reporting ASE in a familial pancreatic cancer patient showed that the same rs1801552\_C allele was underexpressed (15). Therefore, this allele was assumed as a preferential target for a germline downregulation mechanism so far unidentified.

Recent reports demonstrated that h*MLH1* or h*MSH2* monoallelic germline hypermethylation caused HNPCC and could be transmitted through generations (27,28). These studies established epimutations as the cause of a novel cancer susceptibility pattern of inheritance (27,28). Assuming that *CDHI* could be the target of a similar epigenetic phenomenon, we have analysed *CDHI* germline promoter hypermethylation in probands with high AI. The PBLs' DNA extracted from the Family 11 proband displayed *CDHI* hypermethylation with a striking pattern of clonal heterogeneity similar to that somatically found in sporadic carcinomas, cancer cell lines, and tumour foci from HDGC *CDHI* germline mutation carriers, leading to *CDHI* expression downregulation (7,29). We believe however that this proband harbours germline hypermethylation as the cloning experiment revealed that a large proportion of the sequences cloned were methylated. This result would not be expected if a small amount of cancer cells would be circulating in the blood, and is pointing to a germline defect rather than to somatic cancer-associated methylation reminiscent from cancer-circulating cells. The most interesting aspect in this case was that CpG methylation was occurring only at the rs16260\_A allele, raising the hypothesis that allele-specific methylation is generating germline-specific downregulation of the rs1801552\_C-containing allele. Further supporting this hypothesis is the fact that haplotype reconstruction by PHASE 2.02 software revealed that both alleles are indeed in phase. To assess *CDHI* germline hypermethylation in a larger series, we extended the methylation analysis but failed to identify this phenomenon in 56 additional GC family probands (data not shown); therefore, we believe this is a rare mechanism in this setting, according to previously reported findings (30).

Nevertheless, the overall frequency of *CDHI* germline methylation (1.47%—1/68) herein identified does not differ from that reported for h*MLH1* and h*MSH2* in HNPCC-like probands (0.6 and 3.2%, respectively) (27,28).

We also found that 2/12 (16.7%) high *CDHI* AI probands display large genomic deletions encompassing *CDHI*, so, in summary, we identified the potential mechanism underlying high AI in 25% (3/12) of the cases in this series, suggesting that AI most likely represents a marker for both germline genetic and epigenetic defects in *CDHI*.

Recently, germline heterozygous deletions of *TACSTD1* 3'-exons were found in 0.9% of HNPCC-suspected families, causing transcriptional read-through that resulted in h*MSH2* downregulation (31,32). In the light of our *CDHI* AI results, it is expected that the application of ASE analysis to h*MSH2*, in the aforementioned HNPCC families, would have spotted monoallelic expression of the gene. Conversely, it is also possible that a similar structural rearrangement, in the surroundings of *CDHI* gene, could generate AI in some probands of our series.

Recently it was shown that *TGFBR1* AI results in globally reduced expression of the gene, alters SMAD-mediated TGF $\beta$  signalling, is dominantly inherited, segregates in families and occurs in sporadic colorectal cancer (13). Two major *TGFBR1* haplotypes were found predominant among *TGFBR1* AI cases suggesting ancestral mutations; nevertheless, no causative germline change was identified (13).

As we failed to find a mechanism underlying the nine remaining cases displaying high *CDHI* AI, and given that the rs1801552\_C-containing allele was recurrently downregulated, we reasoned that this allele could be in linkage disequilibrium with a putative aetiological variant, either in *CDHI* or in its proximity. The rs1125557\_A allele (163+37235G>A) localized in *CDHI* intron 2 was recently claimed as a susceptibility variant for sporadic DGC, mainly in homozygous AA individuals (33). This SNP was studied in our series and contrarily to what is reported, the percentage of AA individuals was similar between probands (28%) and controls (33%). Moreover, our quest for high *CDHI* AI-related haplotypes generated no significant difference between haplotypic distribution in probands and in the HapMap reference/control group. As no haplotype is overrepresented within *CDHI*-negative probands, high *CDHI* AI is most probably being caused by haplotype independent mechanisms.

Another interesting hypothesis arises from a study describing that two *cis*-regulatory *FGFR2* SNPs are able to alter binding affinity for transcription factors *Oct-1/Runx2* and *C/EBP $\beta$*  (34), and that they synergize to augment *FGFR2* expression, thereby increasing the propensity for tumour formation (34). Whether a similar phenomenon is occurring in our patients, and another SNP exists that synergizes with *CDHI* rs1801552\_C allele, still needs to be addressed.

A last hypothesis to cause *CDHI* AI could be the existence of a *trans*-acting factor binding differentially to rs1801552\_C and rs1801552\_T alleles, as it was recently described for DEAF-1 (35). This protein was reported to allele-specific *trans*-regulate *GDF5*, through the differential binding to a *GDF5* sequence encompassing a common SNP (rs143383). In the context of *CDHI*-negative probands, individuals carrying the common rs1801552\_C allele would only be at risk of

developing cancer in a background with abnormal expression of a *trans*-acting factor, with differential binding affinity for the rs1801552\_C allele.

In summary, our results show that: (i) high *CDHI* AI may be used as a marker for increased susceptibility to familial aggregation of GC, namely HDGC, as it occurs specifically in *CDHI*-truncating mutation carriers (100%) and *CDHI*-negative probands (70.6%), but not in cancer-free individuals; (ii) germline allele-specific *CDHI* promoter methylation and deletion encompassing the *CDHI* locus underlie high *CDHI* AI in 25% of *CDHI*-negative probands; (iii) high *CDHI* AI determination provides a simple, cost-effective and efficient tool to perceive indirectly changes of *CDHI* expression that escape detection in gDNA-based screenings; and (iv) the remainder 75% of cases with high AI are not related to an extended susceptibility haplotype.

As AI detection does not vary upon the SNP used, any coding or even pre-mRNA SNP can be used to test ASE, and virtually every single individual can be analysed with this strategy. ASE quantification of gene transcripts opens the way to the detection of both *trans*- and *cis*-acting regulatory variations (35,36) and overcomes the difficulties resulting from non-genetic factors affecting mRNA levels and from mRNA instability, which can differ between samples.

In conclusion, we believe that most *CDHI*-negative probands, especially those presenting tumours with E-cadherin expression impairment, similar to that observed in *CDHI* germline alteration carriers, do have a germline *CDHI* expression defect caused by either direct or indirect mechanisms targeting the *CDHI* genomic sequence.

## MATERIALS AND METHODS

### Patients and samples

The collection of clinical data from patients and families and PBL samples from patients and controls was approved by the appropriate Ethics Committee from each of the centres participating in this work: University of British Columbia, Vancouver, Canada; Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal; and Hospital Geral de Santo António, Porto, Portugal. Family history of cancer was obtained with informed consent.

Thirty-two probands from families displaying aggregation of GC mainly of diffuse histology (HDGC) and early onset isolated cases (aged <45 years) with either DGC or LBC were selected for the study. Twenty-seven of the 32 probands tested negative for *CDHI* germline point mutations, whereas 5 have been described elsewhere to carry germline point mutations in the *CDHI* gene (26,37–40). A series of 50 cancer-free individuals was used as controls.

For germline *CDHI* hypermethylation analysis, we gathered 68 probands, including the 27 *CDHI*-negative probands from the aforementioned series and 41 other probands collected at IPATIMUP.

### Nucleic acid preparation

gDNA was extracted from PBLs from patients and controls using a standard protocol. Total RNA was isolated from

PBLs using the TriPure Isolation Agent (Roche Diagnostics, Basel, Switzerland) or the whole blood and bone marrow protocols—300 µl blood sample protocol from PURESCRIPT RNA Isolation Kit (GENTRA, MN, USA).

Approximately 300 ng of total RNA was used to synthesize first-strand cDNA with Superscript II Reverse Transcriptase and random hexamer primers (Invitrogen, CA, USA).

### *CDHI* SNP genotyping of cancer-free individuals and patients

Three *CDHI* SNPs were genotyped in all patients and controls: two *CDHI* coding SNPs rs1801552-2076C/T [minor allele frequency (MAF) T: 0.408 in HapMap phase II CEU panel from individuals with European ancestry] and rs33964119-2253C/T (MAF T: 0.045 in HapMap phase II European EGP CEPH panel); and a noncoding SNP rs1801026-g.\*+54C>T located at the 3'-UTR before the polyadenylation signal (MAF T: 0.205 in HapMap phase II European EGP CEPH panel) by PCR using standard conditions. MAFs were obtained from the dbSNP home page at <http://www.ncbi.nlm.nih.gov/projects/SNP/>. PCR products were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, CA, USA) and run in an ABI Prism 3130 DNA automated sequencer (Applied Biosystems). Primer sequences and amplicon sizes are listed in Supplementary Material, Table S1.

### ASE analysis

Primer extension analysis relies on the incorporation of a single dideoxynucleotide triphosphate (ddNTP) (complementary to the base of interest) that is selected to allow the differential extension of a primer annealed next to the polymorphic site (SBE primer). ASE products can be distinguished, and quantitatively measured, based on the presence of a specific fluorophore coupled to the incorporated ddNTP. PCR products encompassing the polymorphic site were used as templates for primer extension with fluorescently labelled dNTPs which are incorporated, causing chain termination in alleles having a C, G, A or T in this position. Purified PCR products were submitted to SNaPshot reaction using SNaPshot Multiplex Kit (Applied Biosystems) and an SBE-specific primer following manufacturer's instructions. Final products were analysed in a 310 Genetic Analyser (Applied Biosystems) using the size standard GeneScan 120 LIZ (Applied Biosystems). The peak area corresponding to each allele was quantified with the GeneScan results analyser software. The ratio of ddNTP incorporation by each allele was calculated by taking the ratio of the areas corresponding to T and C alleles. For samples presenting complete downregulation of one allele in germline RNA, a ratio value of 10 was attributed to characterize AI.

As a validation step, a PCR amplicon encompassing the same SNPs was generated from gDNA from patients and controls and submitted to the same analysis to demonstrate equivalent representation of each allele through equivalent ddNTP incorporation. Moreover, to exclude ASE bias mediated by a specific primer sequence, ASE results obtained from patients and control samples were confirmed using either different primer sequences for the primer extension assay or, whenever



possible, another SNP. Primer sequences and amplicon sizes are listed in Supplementary Material, Table S2.

### Promoter methylation analysis

DNA from probands' PBLs belonging to 68 *CDHI*-negative families mainly displaying GC aggregation, originating from Canada (10), Ireland (2), Spain (1), Brazil (1), China (1), Italy (1) and Portugal (52), was screened for hypermethylation at the CpG island 3 of the *CDHI* promoter. Approximately 50–100 ng of sodium bisulphite-modified DNA [EpiTect Bisulfite Kit (Qiagen, Hilden, Germany)—sodium bisulphite conversion of unmethylated cytosines in DNA from solutions with low concentrations of DNA] was used for PCR and sequencing to screen for methylation at 19 CpG sites (position –56 bp to position +115 bp from the *CDHI* TSS), with flanking primers not containing CpG sites. Samples displaying methylated CpG sites were resubmitted to an independent PCR that was cloned into pCR® 2.1-TOPO® vector (Invitrogen). Colony-PCR with Universal M13 primers was performed to determine the level and extent of *CDHI* promoter methylation. Primer sequences and amplicon sizes are listed in the Supplementary Material, Table S3.

### Amplification refractory mutation system-PCR

To analyse allele-specific *CDHI* hypermethylation, heterozygosity at the common *CDHI* promoter SNP rs16260 (–161C/A) was used to perform ARMS-PCR. Primers were designed to discriminate between templates which differed in a specific single nucleotide (41), thereby amplifying each allele separately from bisulphite-converted DNA. Briefly, primers flanking rs16260 SNP were used in a first-round PCR with 50 ng of bisulphite-converted DNA. Products were re-amplified with the same reverse primer and specific forward primers to the polymorphic A or C allele. Sequencing of each PCR product with the same reverse primer allowed analysis of 13 methylated CpG sites in independent alleles [the last three CpG sites had been previously analysed by cloning and sequencing (Fig. 3B and C)]. Primer sequences and amplicon sizes are listed in Supplementary Material, Table S4.

### Haplotype analysis

Twelve coding and noncoding SNPs with heterozygous frequencies ranging from 0.217 to 0.648 (HapMap phase II CEU panel from individuals with European ancestry, <http://www.ncbi.nlm.nih.gov/projects/SNP/>) scattered along *CDHI* and *CDH3* loci and covering a region of approximately 193 kb (67.227.572–67.420.442) were genotyped in a multiplex PCR, according to manufacturer's instructions (QIAGEN Multiplex PCR Kit, Quiagen). SNP-specific primers for primer extension assay were designed to be used in a SNaPshot multiplex reaction by adding different size tags to each primer (SBE primers). Purified PCR products and SBE primers were used for primer extension assay according to the SNaPshot Multiplex Kit (Applied Biosystems). Selected SNPs, primers (PCR primers and SBEs) and respective amplicon sizes are listed in Supplementary Material,

Table S5. The phased haplotypes from the CEU panel haplotypes from HapMap phase II database for European populations were used with our multilocus genotype data to statistically infer the probands' haplotypes, using the software PHASE 2.02 (16,17). Linkage disequilibrium statistic  $D'$  was calculated from the inferred haplotypes using the DNAsp program, version 5.0 (18).

### Statistical analysis

R statistical program was used for the construction of a standard boxplot, plotted with whiskers and outliers. Owing to the non-normal distribution of the data, a non-parametric test, in particular the Wilcoxon rank sum test was used to calculate significance. The  $P$ -values obtained were further corrected using the Bonferroni correction due to the multiple testing performed.

### SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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*Conflict of Interest statement.* None declared.

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