

Characterization of a Recurrent Germ Line Mutation of the *E-Cadherin* Gene: Implications for Genetic Testing and Clinical Management

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Abstract Purpose: To identify germ line *CDH1* mutations in hereditary diffuse gastric cancer (HDGC) families and develop guidelines for management of at risk individuals.

Experimental Design: We ascertained 31 HDGC previously unreported families, including 10 isolated early-onset diffuse gastric cancer (DGC) cases. Screening for *CDH1* germ line mutations was done by denaturing high-performance liquid chromatography and automated DNA sequencing.

Results: We identified eight inactivating and one missense *CDH1* germ line mutation. The missense mutation conferred *in vitro* loss of protein function. Two families had the previously described 1003C>T nonsense mutation. Haplotype analysis revealed this to be a recurrent and not a founder mutation. Thirty-six percent (5 of 14) of the families with a documented DGC diagnosed before the age of 50 and other cases of gastric cancer carried *CDH1* germ line mutations. Two of 10 isolated cases of DGC in individuals ages <35 years harbored *CDH1* germ line mutations. One mutation positive family was ascertained through a family history of lobular breast cancer (LBC) and another through an individual with both DGC and LBC. Occult DGC was identified in five of six prophylactic gastrectomies done on asymptomatic, endoscopically negative 1003C>T mutation carriers.

Conclusions: In addition to families with a strong history of early-onset DGC, *CDH1* mutation screening should be offered to isolated cases of DGC in individuals ages <35 years and for families with multiple cases of LBC, with any history of DGC or unspecified GI malignancies. Prophylactic gastrectomy is potentially a lifesaving procedure and clinical breast screening is recommended for asymptomatic mutation carriers.

Gastric cancer is one of the three leading causes of cancer death worldwide (1). Although the incidence of gastric cancer in older patients is decreasing, in younger patients as well as in

cases with familial clustering it remains stable, suggesting that genetic predisposition is an increasingly important risk factor for gastric cancer (2). In this respect, as few as 1% to 3% of all

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gastric carcinomas have an autosomal dominant gastric cancer susceptibility, for which highly penetrant mutations account for the increased risk (3).

E-cadherin (*CDH1*; MIM 192090) germ line inactivating mutations have been shown to underlie about 30% of hereditary diffuse gastric cancer (HDGC) families of various ethnic backgrounds (4). As many as 50% of these mutations are expected to be found in families with both a documented diffuse gastric cancer (DGC) case diagnosed at age 50 or younger and a family history of gastric cancer (5). To date, 45 distinct *CDH1* germ line mutations have been reported in HDGC, the majority of which result in truncated, nonactive proteins (6). Germ line missense mutations are also found, but clinical management of these families remains controversial. Functional assays can be used to determine the potential pathogenic role of *CDH1* missense variants, which assists in the clinical counseling of mutation carriers (7, 8).

CDH1 germ line mutations have also been identified in a small portion (2 of 104) of DGC patients without a family history (7, 9). Recently, associations between *CDH1* germ line mutations and both lobular breast cancer (LBC; refs. 5, 10, 11) and signet ring carcinoma of the colon (5) have been reported in DGC families; the latter association being confined to two families with germ line missense mutations. The association of germ line *CDH1* mutations with both DGC and LBC is not surprising as somatic *CDH1* mutations have been described both in DGC and infiltrative LBC (12, 13). Although somatic *CDH1* mutations cluster in the exon 7 to 9 hotspot region of the extracellular domain of the protein, germ line mutations are dispersed throughout the gene (6).

In this study, we ascertained 31 previously unreported HDGC families and screened them for *CDH1* germ line mutations. These included 10 isolated early-onset DGC cases and one case of synchronous DGC and LBC. Two of the families under analysis shared the same germ line change, an exon 7 nonsense mutation previously reported in a Swedish HDGC family (14). We used haplotype analysis on the three families to rule out the possibility of founder mutation. Six asymptomatic *CDH1* mutation carriers from a family with the recurrent mutation underwent prophylactic gastrectomy.

Materials and methods

Families and patients. The 31 families were collected over a period of 18 months between April 2003 and October 2004. Families were mainly ascertained through genetics clinics although a few were referred directly by oncologists. All clinical data, including pathology reports and pedigrees, was collected by the genetic counselor at each separate site. This data was then sent to the study investigators for verification of eligibility into the study.

The 31 families included 20 families with a positive history of gastric cancer, 10 isolated early-onset DGC cases, and one case diagnosed with both DGC and LBC. All were North Americans: seven were from Canada and 24 were from the United States. Ethnicity was known in all. Eleven were of Northern European descent, whereas the others were of Spanish ($n = 3$), German ($n = 5$), Italian ($n = 3$), Chinese ($n = 3$), Afro-American ($n = 3$), Indian ($n = 1$), and Native American ($n = 2$) descent. These findings clearly exemplify significant racial/ethnic diversity with respect to DGC.

For both ascertainment and testing, families were subdivided according to six published inclusion criteria (5): (1) two or more documented cases of DGC in first-degree relatives, with at least one diagnosed before age 50; (1A) two or more cases of gastric cancer, with

at least one DGC diagnosed before age 50; (2) three or more documented cases of DGC in first-degree relatives, diagnosed at any age; three or more cases of gastric cancer, diagnosed at any age, with at least one documented case of DGC; (2A) isolated individual diagnosed with DGC at <45 years of age; (3) isolated individual diagnosed with both DGC and LBC (no other criteria met); (4) One family member diagnosed with DGC and another with LBC (no other criteria met); (5) One family member diagnosed with DGC and another with colon cancer (no other criteria met).

In families where a mutation was identified, the proband was informed of results by the referring site's genetic counselor. Family members were informed by the proband and then referred to their local genetic counselor for counseling and carrier testing.

With the exception of three cases (C5, C6, and C7; Table 2A), where only paraffin tissue blocks were available, constitutional genomic DNA was extracted from a blood sample of one affected individual of each family, usually the proband.

This study was done after approval by the Clinical Research Ethics Board of the University of British Columbia. Informed consent was obtained from each subject, or next of kin in the case of a deceased affected individual.

DNA extraction. Genomic DNA was purified from peripheral blood leukocytes, using the Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions. Paraffin embedded sections from normal tissue were deparaffinized with xylene then treated with 100%, 70%, and 50% ethanol for rehydration. Pellets were incubated with Proteinase K [10 mg/mL Proteinase K, 1 mol/L KCl, 1 mol/L Tris (pH 8.0), 1 mol/L MgCl₂] at 56°C overnight and then boiled and centrifuged at 14,000 rpm. The supernatant was purified by phenol/chloroform extraction. DNA was precipitated with 100% ethanol, dried at room temperature, and resuspended with double-distilled H₂O.

PCR amplification and denaturing high-performance liquid chromatography analysis. The complete coding sequence of the *E-cadherin* gene including splice junctions was amplified by PCR and screened for mutations using denaturing high performance liquid chromatography (Transgenomic, Inc., Omaha, NB). Primer sequences, PCR conditions, and denaturing high-performance liquid chromatography column conditions are shown in Table 1A and B. PCR reactions were carried out in a volume of 20 μ L containing 100 ng genomic DNA template, 1.5 mmol/L MgSO₄, 0.5 mmol/L of each PCR primer, 2 mmol/L deoxynucleotide triphosphates, and 1.25 units Optimase polymerase 1 \times PCR buffer. Samples were initially denatured at 95°C for 2 minutes followed by 15 touchdown cycles with a 0.5°C decrease of the annealing temperature at each cycle, and 20 cycles of 30 seconds at 94°C, 30 seconds at a primer pair-specific annealing temperature, and 20 to 50 seconds of extension at 72°C. Cycling was done in a programmable thermocycler (MJ Research PTC-225 or Tetrad, Waltham, MA). The PCR products were denatured for 4 minutes at 94°C and cooled to room temperature at a rate of 1°C/min. Three to 15 μ L of PCR products were applied to a preheated reverse phase column (DNA-Sep, Transgenomic, Omaha, NB). Elution of the DNA was done in a linear acetonitrile gradient. The optimal temperatures for resolution of heteroduplex and homoduplex DNA for the *CDH1* exons were established by analyzing the melting behavior of the PCR fragments in the temperature range corresponding to the calculation of the Wavemaker software. Fragments with more than one melting domain were analyzed at additional temperatures.

DNA sequence analysis. PCR products with an aberrant denaturing high-performance liquid chromatography chromatogram were directly sequenced in both directions starting from a new PCR product. Purification of the PCR product was done by AmPure magnetic beads (Agencourt Bioscience, Beverly, MA), according to the manufacturer's instructions. Cycle sequencing with fluorescent-labeled dye terminators (Big Dye Terminator Mix V.3, Applied Biosystems, Foster City, CA) and separation with an ABI 3700 capillary sequencers were done as previously described (5). Samples showing a putative pathogenic

Table 1.

A. Primers used for denaturing high-performance liquid chromatography analysis

Exon	Forward 5'-3'	Reverse 5'-3'
1	CAGCCAATCAGCGGTACGGG	CGGGCGGGGGCGGGCGGGCCGCGTCCCTCGCAAGTCAGG
2	GCGGCCCGCCGCCCGCCGAGGGAACCTCCGAGTCACC	CTACTCCGCCAGGGACAC
3	GCGGCCCGCCGCCCGCCGGAATGCTCTTGCTTTAATCTGTCC	CCTGGATTAGACAGCGCACTA
4	CCTGAAGTATCCGTCTTGAATTG	CTCCTTGGTACTTCTGTGCCA
5	ATTGGCAGAAGTACCAA	CCCATCACTTCTCCTTAGCA
6	AGCCTAGGAAGGTGTGGCAG	CGGGCGGGGGCGGGCGGGCCGCGGGTCCAAAGAACCTAAGAGTC
7	ACCCAGTCCCAAAGTGCAGC	GGGATTGAGCTAATACACATTTGTCC
8	GCTAGTGTCTCTGGTCTGACT	CCATGAGCAGTGGTGACACTTAG
9	CCAGCTGGTGACAGTGAGAT	CAGCTGTGAGGATGCCAGTTTC
10	GCGGCCCGCCGCCCGCCGCGGTGGCAGAAACCACAGTTACTT	CGGGCGGGGGGTCTTGACAGACAAATGAC
11	GTTGTTTGTCTGTCTTATC	GAAGTAGCTAGGAGGTCGAG
12	GCGGCCCGCCGCCCGCCGCTTACTGTTGCCAAGCTGCCACAT	GGCAGTTGGAGCAAAGTTGCCA
13	GCGGCCCGCCGCCCGCCGCGGGTGTCTTTAGTTCAGTAGCA	CCAGGAAATAAACCTCCTCCATT
14	CTCTAACACTTGTCTGTCTGTC	AGAGATCACCAGTGTGAGCTAC
15	TCTATAAACTGAACATAGCCCTG	CGGGCGGGGGCGGGCGGGCCGCTGAGCTTAGAGATGAGCC
16	GCGGCCCGCCGCCCGCCGAGGTGTGCCCTTCTTTTAC	CGGGCGGGGGCGGGCGGGCCGCTGCATCACTAACAGTCTCT

B. Conditions used for denaturing high-performance liquid chromatography analysis

Exon	Initial T touchdown (15 cycles, 0.5°C/cycle)	Final T (20 cycles)	Extension time	% DMSO	Column temperature
1	67.3	60.3	30	5	66, 68, 68.6
2	67.3	60.3	20	5	66.2, 67.8
3	63.4	56.4	40	10	60, 60.5
4	63	56	30		58.1, 59.3
5	64.5	57.5	30		57.8, 59.3
6	64.6	57.6	40		60, 60.5, 64.1
7	65.2	58.2	30		61.1
8	65.9	58.9	30		58.1, 60.5
9	65.7	58.7	40		58.7, 61
10	59.7	52.7	50		54.5, 56.8, 59.8
11	61.3	54.3	30		61, 62
12	65	58	40	5	59.9
13	63.4	56.4	40	5	57.8, 59, 60.5, 62.4
14	61.2	54.2	30		60, 62
15	60.7	53.7	30		58.5, 62.7
16	62.5	55.5	40	5	59.7, 61, 61.7

mutation were confirmed by another independent PCR and sequencing reaction.

For the criterion 4 case (C7), because the mutation c.3G>C removes the *NcoI* restriction site, the presence of the mutation was also confirmed by RFLP analysis. RFLP analysis was carried out by digesting 5 µL of PCR product with 5 units of *NcoI* restriction enzyme (Fermentas, Burlington, ON) at 37°C for 1 hour. A 10-µL portion of the reactant was run on 3.0% agarose gel and visualized under UV illumination.

Construction of the plasmid encoding the E-cadherin mutant Pro⁴²⁹Ser. The mutant plasmid was obtained by nested PCR using specific primers (C1285T: forward 5'-CCACAAATTCAGTGAACAACG-3', reverse 5'-CGTTGTTCACTGAATTTGTGG-3') and wild-type E-cadherin cloned in pcDNA3 as DNA template. Chinese hamster ovary (CHO-K1) cells stably expressing the E-cadherin mutant Pro⁴²⁹Ser were established by electroporation as previously described (7, 8). Cells were grown at 37°C under 5% CO₂ in humidified air, in α-MEM medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 5% fetal bovine serum, 2 mmol/L L-glutamine, 1% penicillin/

streptomycin, and 1 mg/mL geneticin. Single cell clones were selected and analyzed for E-cadherin expression by Western blotting, using the human E-cadherin monoclonal antibody HEC1D1 (R&D Systems, Minneapolis, MN; 1:3,500 dilution) for protein staining. At least two independent clones were used in each experiment to exclude clonal dependence of the results.

Cell aggregation assay. As previously reported (7, 8), the ability of cells expressing the mutant Pro⁴²⁹Ser to aggregate was assessed on soft agar, using cells expressing the wild-type protein as control. Briefly, cells were first trypsinized and then transferred to an agar gel (0.66%, w/v) in a 96-well plate and then incubated at 37°C under 5% CO₂ in humidified air. Aggregates formation was evaluated after 24 to 48 hours using an inverted microscope.

Matrigel invasion assay. Matrigel invasion chambers were purchased from BD Biosciences (Mississauga, ON) and the assay done following the manufacturer's instructions. Briefly, 1 × 10⁵ cells were seeded on top of the Matrigel and incubated for 22 hours at 37°C. Invasion indices (%) were expressed as ratios between the number of invasive cells through

the Matrigel membrane and the number of cells migrating through the control insert membrane, as counted in at least 12 independent areas. Cells expressing wild-type E-cadherin were used as control.

Haplotype analysis. Haplotype analysis was done on each sample using the following microsatellite markers: D16S503, D16S3025, D16S496, D16S3095, D16S742, and D16S512. Amplification of each marker was achieved using 50 ng of genomic DNA, 1 unit AmpliTaq DNA polymerase (Roche, Laval, PA), 800 nmol/L deoxynucleotide triphosphates (200 nmol/L each; Invitrogen, Carlsbad, CA), 5 μ L (1 \times) amplification Buffer (Sigma-Aldrich, St. Louis, MO), and 500 nmol/L each of the forward and reverse amplification primers (custom synthesized by Invitrogen) in a total volume of 25 μ L. Amplification was done with a DNA engine Tetrad (MJ Research, Waltham, MA). The cycling conditions were as follows: initial denaturation at 96°C for 3 minutes followed by 35 cycles of 95°C for 30 seconds, 53°C for 20 seconds, and 72°C for 30 seconds. Final extension of the amplification products was for 10 minutes at 72°C. The individual reaction products were then combined and resolved using an ABI 3100 genetic analyzer (Applied Biosystems). Individual alleles were then analyzed and sized using the GenScan genotyping software associated with the analyzer. Individual amplification primers were as listed in the Genome database (<http://www.gdb.org>) and labeled as follows: D16S503(F)forward 5-

HEX-AGTGTCTCGGAATGATGTG-3; D16S503(R)reverse 5-TTGCTAGG-TAGTTGTCTCCC-3; D16S3025F 5-HEX-TCCATTGGACTTATAAC-CATG-3; D16S3025R 5-AGCTGAGAGACATCTGGG-3; D16S496F 5-6FAM-GAAAGGCTACTTCATAGATGGCAAT-3; D16S496R 5-ATAAGC-CACTGCGCCCAT-3; D16S3095F 5-HEX-TCAGTTGGAAGAT-GAGTTGG-3; D16S3095R 5-TATAGTTTGTGTCCCCCGAC-3; D16S752F 5-6FAM-AATTGACGGTATATCTATCTGTCTG-3; D16S752R 5-GATTGGAGGAGGGTGATTCT-3; D16S512F 5-6FAM-GCCAAAACG-CAAGTTTCAA-3; D16S512R 5-TACTTGCAAGCATTTTCTCC-3.

Results

E-cadherin variant detection. Of the 20 families with a positive history of gastric cancer (clinical criteria 1, 1A, 2, 2A, 5, and 6), six (30%) harbored *CDH1* germ line mutations (Table 2A). Of the 10 isolated early-onset DGC cases (clinical criterion 3), two (20%) tested positive for *CDH1* germ line mutation (Table 2A). In addition, the single criterion 4 case under analysis (C7) tested positive for a *CDH1* germ line mutation. In this case, only paraffin-embedded tissue was available. This archival paraffin tissue block was pathologically

Table 2.

A. Study criteria and E-cadherin mutation status

Clinical criteria	No. families	No. affected families	Type of E-cadherin mutation	Predicted effect
1. Two or more documented cases of DGC diagnosed before age 50	6	2	1 substitution 1 deletion	Premature stop-codon Frameshift
1A. Two or more cases of GC, with at least one DGC before age 50	8	3	2 substitution 1 substitution	Premature stop-codon Alternative Splice site
2A. Three or more cases of gastric cancer, diagnosed at any age, with at least one documented case of DGC	4	1	1 substitution	Premature stop codon
3. Isolated individual diagnosed with DGC at <45 y	10	2	1 substitution 1 deletion	Missense change Frameshift
4. Isolated individual diagnosed with both DGC and LBC	1	1	1 substitution	Loss of start codon
5. One family member diagnosed with DGC and another with LBC	1	0	—	—
6. One family member diagnosed with DGC and another with colon cancer	1	0	—	—

B. Details of the gastric cancer families in the study and mutations detected

Case/criterion	Members reported with gastric cancer/confirmed DGC	Age range	Members reported with breast cancer/confirmed LBC	Gene location	Nucleotide change
F1/1	8/2	25-67	0	Exon 7	c.1003C>T
F2/1	10/2	35-87	2/1	Exon 14	c.2276del
F3/1A	5/1	31-55	0	Exon 7	c.1003C>T
F4/1A	4/1	21-75	0	Exon 13	c.2161C>G
F5/1A	4/1	30-54	2/0	Exon 12	c.1792C>T
F6/2A	3/1	24-52	3/2*	Exon 3	c.187C>T
C5/3	1/1	27	0	Exon 8	c.1063del
C6/3	1/1	27	0	Exon 9	c.1285C>T
C7/4	1/1	73	1/1	Exon 1	c.3G>C

*One individual had mixed lobular and ductal carcinoma of the breast with signet ring cells.

verified to contain normal and not cancer tissue. The mutation was seen by direct sequencing in all of four confirmatory independent PCR reactions from the initial DNA extraction and confirmed by RFLP analysis of another independent PCR reaction. However, we were unable to confirm this mutation in DNA extracted from a small amount of residual material contained in the same tissue block and believe that this represents a preferential amplification of the wild-type allele in the second extraction. No other material was available for further analysis.

In all, nine inactivating mutations were identified in this study, including four nonsense mutations, two deletions, one splice site substitution, a single nucleotide change affecting the start codon, and one missense mutation (Table 2A and B). The missense mutation was characterized for functional consequences as described below. All mutation carriers were heterozygous for the mutation, consistent with the autosomal dominant inheritance of HDGC in these families. With the exception of the nonsense mutation 1003C>T, which was previously identified in a HDGC family from Sweden (14), all other mutations herein reported are novel. In this study, the 1003C>T mutation (Fig. 1A and B) was shared by two apparently unrelated families, F1 and F3 (Table 2B). The germ line mutation found in family F4 is a C-to-G transition of the fourth nucleotide upstream of the exon/intron 13 boundary, where the consensus donor sequence for the splicing of that intron is located. This substitution affects a nucleotide that is highly conserved in human, mouse, rat, *Xenopus*, and chicken, suggesting that it is functionally important. Because of a lack of material, we used a theoretical approach to predict the effect of this mutation on the splicing. Netgene2 (<http://www.cbs.dtu.dk/services/netgene2>) uses the scoring system developed by Brunak & colleagues (1991) a Netgene2 to calculate the score of a donor or acceptor splice site and the

change in the score associated with a point mutation of this site. The program predicted that a potential cryptic splice site could be created by the mutation, with a splice score of 0.83 versus 0.81 obtained for the original splice site of the wild-type sequence.

The G-to-C transition at position 3 in the criterion 4 case C7 (Table 2B) disrupts the ATG start codon, with a predicted deleterious effect on the initiation of protein translation.

Functional characterization of the E-cadherin (CDH1) missense mutation. The early-onset gastric cancer case C6 harbors a C-to-T transition at nucleotide 1285 in exon 9, responsible for a proline-to-serine change at codon 429. This amino acid residue belongs to a region of the protein highly conserved among species. This third extracellular repeat of the protein contains a calcium-binding motif essential for the adhesion process (15). In the absence of a three-dimensional structure, only a theoretical prediction of mutation effect is possible. In this respect, because conserved proline or glycine residues are suggestive of the presence of a β -turn in the protein structure, we could speculate that the change Pro⁴²⁹Ser is likely to disrupt a functionally important domain. To confirm this prediction, we characterized *in vitro* the effect of this variant on the E-cadherin ability to mediate cell-cell adhesion and suppress cell invasion. As described in previous studies (7, 8), Chinese hamster ovary E-cadherin-negative cells were used as the cell model system and transfected to express the E-cadherin variant Pro⁴²⁹Ser (Fig. 2A). Cell-cell adhesion on soft agar and Matrigel invasion assay were done using mock cells and cells expressing the wild-type protein as control. In contrast to the behavior of Chinese hamster ovary cells expressing wild-type E-cadherin, Chinese hamster ovary cells expressing the Pro⁴²⁹Ser variant fail to produce compact cellular aggregates, suggesting that the ability of E-cadherin to mediate cell to cell adhesion may be impaired by the

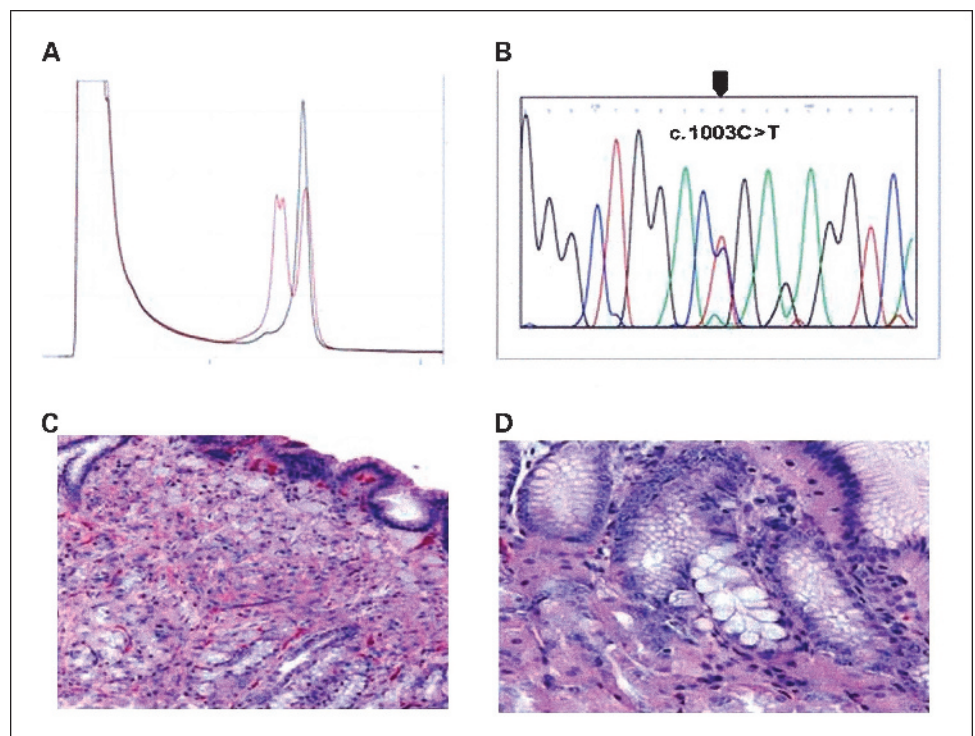


Fig. 1. A and B, denaturing high-performance liquid chromatography and sequencing plots for the CDH1 mutation c.1003C>T. C and D, representative early diffuse gastric cancer and *in situ* carcinoma, respectively, from a prophylactic gastrectomy from family F1. In the invasive carcinoma, the superficial infiltrate of signet ring cells neither distorts the surface mucosa nor invades the muscularis propria, both factors account for its invisibility during gross examination or endoscopy.

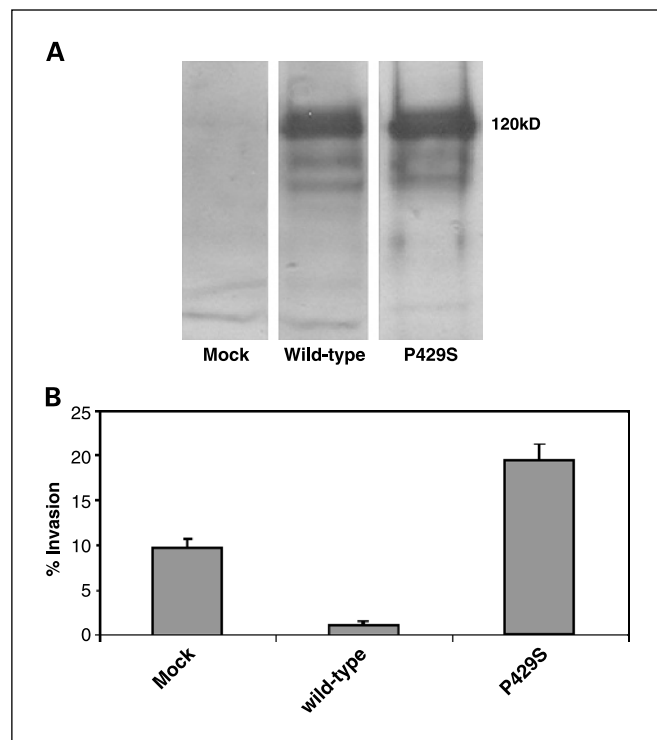


Fig. 2. A, Western blot analysis of the E-cadherin-transfected cell lines; equal amount of protein was loaded in each lane. B, Matrigel invasion assay. Columns, percentage of invasion for each experimental condition.

mutation. When seeded on top of Matrigel, the same mutant expressing cells showed a 20% increased cell invasion over the wild-type expressing cells (Fig. 2B), confirming the theoretical prediction and supporting the pathogenic role of this mutation in gastric cancer.

Haplotype analysis. As described above, for the HDGC families F1 and F3, the same 1003C>T nonsense alteration was identified in exon 7. Jonsson et al. (14) had previously reported this same truncating germ line mutation in a HDGC Scandinavian family, indicating either that these families share a common ancestral haplotype or that they represent independent mutation events. To rule out the possibility of a common genetic origin of the mutation, haplotype analysis was done. Six microsatellite markers were chosen around the *E-cadherin* gene and haplotypes were determined in several members of all three families with the C1003T nonsense alteration (the Swedish family and families F1 and F3). As shown in Table 3, no common haplotype was shared by the mutation carriers in the three families, excluding the possibility that it is a *CDH1* founder mutation.

Clinical follow-up (family F1). After genetic counseling, 20 unaffected adults from family F1 (Fig. 3A) underwent predictive testing for the 1003C>T mutation. Eleven tested positive. In the year since the mutation was identified, six asymptomatic mutation carriers have undergone prophylactic total gastrectomy. All procedures were preceded within 6 months by negative endoscopy and done after extensive counseling with a surgeon, genetic counselor, and dietician. The gastrectomy specimens were processed using a published protocol (16). Five of the six prophylactic gastrectomy specimens had multifocal DGC (Fig. 1C and D).

Discussion

Hereditary gastric cancer is a rare cancer susceptibility syndrome. In 1998, Guilford et al. (17) described for the first time the germ line truncating *CDH1* mutations in three Maori families with autosomal dominant DGC. Since then, similar mutations have been described in DGC patients of various ethnic origins (7, 10, 18–22). The syndrome of HDGC was defined by the International Gastric Cancer Linkage Consortium (23), as any family with (a) two or more documented cases of DGC in first/second-degree relatives, with at least one diagnosed before the age of 50, or (b) three or more cases of documented DGC in first/second-degree relatives, independent of age. We recently revised these criteria in a comprehensive study, which included the ascertainment of 42 apparently HDGC families. Six different inclusion criteria were assessed (5). *CDH1* germ line mutations were identified in close to 50% of families with both a documented DGC case diagnosed at the age of 50 or younger and a family history of gastric cancer (criteria 1 and 1A), representing the optimal criteria for *CDH1* mutation screening.

In keeping with these observations, among the 14 HDGC families ascertained in this study, that fulfilled criteria 1 or the less stringent 1A (5), five proved positive for *CDH1* germ line mutations (5 of 14, 36%).

In family F1 (Fig. 3A), eight gastric cancer cases occurred in proven carriers of the 1003C>T *CDH1* germ line mutation or in their first-degree relatives. The mutation analysis was extended to other unaffected at-risk members of the family, and of the 20 individuals screened, 11 were shown to be carriers of the germ line mutation. Six carriers chose to undergo prophylactic total gastrectomy and for five of them, pathology showed early (intramucosal) DGCs. All were asymptomatic and had had negative endoscopies with random biopsies within 6 months of surgery. Including these and previously published cases, 17 of 18 prophylactic gastrectomy specimens from asymptomatic mutation carriers without endoscopic evidence of disease have contained early DGCs (24–26). Based on these results and taking into account the disorder's high penetrance in the range of 70% to 80% (23), our data reinforce the lifesaving potential of prophylactic total gastrectomy. The procedure should

Table 3. Haplotype linked to the mutated allele at the *CDH1* locus in the three HDGC families harboring the c.1003C>T germ line mutation

Microsatellite	Swedish family (4/5)*	F1 (6/8)*	F3 (1/3)*
D16S503 (17213228) †	228	233	220
D16S3025 (22180595)	92	87	101
CDH1 (22385518)	+	+	+
D16S496 (22562909)	220	217	217
D16S3095 (23560429)	150	150	152
D16S752 (24949389)	102	114	110
D16S512 (27681791)	257	262	260

*Number of affected members versus number of members analyzed.
†Microsatellite marker position.

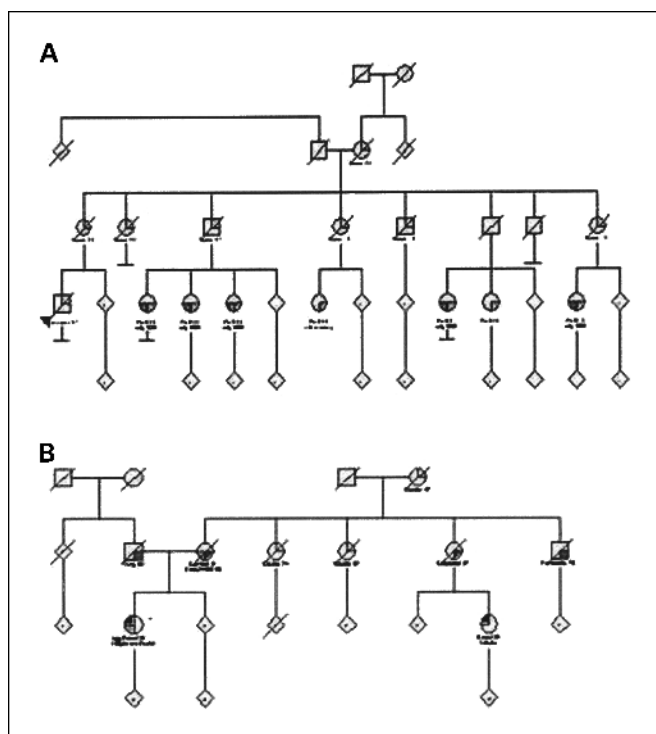


Fig. 3. A, family F1 showing the autosomal dominant inheritance of gastric cancer. Unaffected mutation carriers and negative mutation tests are not shown to preserve identity. B, family F6 showing multiple breast cancer.

therefore be seriously considered as an option for *CDH1* germ line mutation carriers. During the counseling of asymptomatic mutation carriers, an in-depth discussion of both the morbidity and mortality of this operation is required. It is also important to stress the fact that perhaps 20% to 30% of such high-risk individuals with the deleterious mutation will, due to the penetrance phenomenon, never develop advanced gastric cancer.

In family F1, a first-degree relative of an affected individual underwent prophylactic gastrectomy before the availability of *CDH1* mutation screening. Her gastrectomy specimen was normal and she later tested negative for the mutation. This case along with a similar, previously reported one (18), highlights the use of genetic testing in aiding decision making.

Recently, in a single case report, van Kouwen et al. reported on the potential of [¹⁸F]fluoro-2-deoxy-D-glucose positron emission tomography in early detection of HDGC (27). In that study, one of 40 random gastric biopsies done in an asymptomatic germ line *CDH1* mutation carrier showed early DGC. [¹⁸F]fluoro-2-deoxy-D-glucose accumulated at two sites, one corresponding to an intramucosal DGC in the antrum and the second to a DGC in the gastric cardia. Further evaluation of the use of [¹⁸F]fluoro-2-deoxy-D-glucose positron emission tomography in *CDH1* mutation carriers is warranted, as it could potentially enhance the sensitivity of clinical screening for carriers who choose not to undergo prophylactic gastrectomy for medical or social reasons.

In a previous study (5), all criteria 2 and 2A HDGC families seemed negative for *CDH1* germ line mutations. Here a criterion 2A HDGC family (F6) harbored a *CDH1* germ line truncating mutation in exon 3. This indicates that families

lacking an individual with early-onset disease but with a strong history of DGC (criteria 2 and 2A) could carry *CDH1* mutations. However, it should be noted that in this family, three cases of gastric cancer in first-degree relatives were present, with the age of onset ranging between 24 and 52 years. The histologic classification of the tumor was confirmed as a DGC in the 52-year-old individual. No pathology was available for the other two cases, making classification difficult. Of note, the family had been ascertained through a family history of breast cancer (Fig. 3B). Thus, *CDH1* mutation families with multiple cases of late onset DGC and no other reason to consider testing are likely very rare.

We also screened 10 isolated early-onset DGC (criterion 3) cases, two of which harbored germ line *CDH1* mutations (2 of 10, 20%). One mutation was a truncating mutation and the other a missense mutation, which seems pathogenic by both *in vitro* and *in silico* analysis. These two *CDH1* mutation positive, isolated early-onset gastric cancer cases in this study C6 and C7, were both diagnosed at the age of 27 years (for the remaining negative cases, the age of onset ranged between 36 and 44 years; mean, 40). For the two early-onset gastric cancer patients previously identified (7, 9), an age of onset of 30 and 29 years, respectively, was reported. Based on this data, we propose that criterion 3 be revised, with the cutoff for the age of diagnosis decreased from 45 to 35 years.

Our finding of *CDH1* mutations in 20% (2 of 10) of the early-onset DGC cases analyzed contrasts with previous studies suggesting that germ line mutations of *CDH1* are very rare in this subset of DGCs. Of the 104 isolated early-onset cases previously screened for E-cadherin germ line mutations, only two had proven positive for mutations (6). The specific geographic origin of the HDGC cases in our study could at least partially account for this discrepancy, as early-onset DGC in regions with high incidence of DGC are likely caused by environmental factors and gene-environment interactions rather than autosomal dominant *CDH1* mutations. In this regard, similar differences can be observed when the frequencies of *CDH1* germ line mutations in DGC families from countries with high (i.e., Japan, Portugal, Germany, etc.; refs. 9, 22) or low (United States, Canada, England, etc.; refs. 5, 7), incidence of gastric cancer are compared, with almost all reported mutations to date coming from low incidence populations (6), suggestive of etiopathologic differences. Other genetic or environmental factors likely contribute to familial clustering of DGC in high incident populations. It could also be speculated that when combined with a high-risk environment, *CDH1* germ line mutations would lead to cancers at such a young age that they approach genetic lethality, thus explaining the paucity of mutations from Japanese and Korean studies (28–30).

Increasing evidence suggests an association between germ line *CDH1* mutations and the development of LBC (10, 31, 32). In sporadic LBCs, loss of E-cadherin expression is commonly seen, usually as the result of somatic mutation of one allele and loss of heterozygosity at the *CDH1* locus (12, 13). In our previous study (5), we identified three LBC cases in three HDGC families carrying germ line mutations of *CDH1* supporting but not proving an association between LBC and E-cadherin germ line mutations. In the present study, LBC was observed in two *CDH1* germ line mutation carriers, one of which had synchronous cancers of DGC and LBC (C7,

Table 4. Criteria for CDH1 mutation testing modified to reflect current data

Modified testing criteria	<ol style="list-style-type: none"> 1. Family with two or more cases of gastric cancer, with at least one DGC diagnosed before the age of 50. (>30%)* 2. Family with multiple LBC with or without DGC in first-degree relatives (unknown)* 3. Isolated individual diagnosed with DGC at <35 y from a low-incidence population (>10%)* 4. Isolated personal history of both DGC and LBC (unknown)*
Potential additional criteria	<ol style="list-style-type: none"> 5. Family with three or more cases of gastric cancer diagnosed at any age one or more of which is a documented case of DGC; no other criteria met (such families are extremely rare) 6. Family with one or more cases of both DGC and signet ring colon cancer (this association is unproven)

*Percentage of expected positive results.

Table 2B). Two other cases of LBC occurred in relatives of mutation carriers. Another four cases of breast cancer, without available pathology records, occurred in relatives of mutation carriers. The proband of family F6 (Fig. 3B) had a mixed LBC and ductal breast cancer with signet ring cells at age 39. Her mother had breast cancer (pathologic subtype unknown) at age 56 and a cousin had LBC at age 39. As neither the proband nor her mother had gastric cancer, this case highlights the possibility of identifying HDGC families through breast cancer histories. It also reinforces the association between LBC and *CDH1* germ line mutations. On this basis, any family with a positive history of LBC and gastric cancer should also be considered for *CDH1* mutation screening. The risk of developing breast cancer for female carriers of germ line truncating *CDH1* mutations was previously estimated to be close to 40% (31). All female carriers of such mutations should be regularly screened for breast cancer by expert manual examination, mammography, and magnetic resonance imaging. Magnetic resonance imaging with mammography may be more sensitive for the detection of early breast cancers in high-risk women than mammography alone (33). Although further penetrance analysis will be required for the development of clinical screening guidelines, a recommendation of screening from age 35 or 5 years before the youngest age of onset seems prudent based on these current data.

In all, we identified nine *CDH1* germ line mutations. Of these, only one was a missense mutation, confirming that the majority of HDGC-associated *CDH1* germ line mutations are inactivating (nonsense, splice site, or frameshift). Functional characterization of the missense variant supports its pathogenic nature, stressing the importance of *in vitro* studies as an adjunct for determining the pathogenicity of missense *CDH1* mutations.

The nonsense mutation 1003C>T was identified in two distinct families in this study and was also previously described in a third family of Scandinavian origin (14). Distinct haplotypes associated with the mutated allele in the

three families, suggesting that this is a recurrent mutation resulting from independent mutation events. This finding is somehow unexpected, because the *CDH1* germ line mutations reported to date are evenly distributed along the gene, excluding the presence of a hotspot for mutations. However, in sporadic DGC a clustering is observed in exon 7 to 9, which could indicate that this specific region of the gene is more prone to mutation. A 1792C>T nonsense mutation in exon 12 has been identified by independent groups in apparently unrelated families, but no further analysis has been done to exclude a common ancestral origin for this mutation. The 1003C>T mutation is the first reported recurrent HDGC-associated *CDH1* germ line mutation.

A total of 14 HDGC families and eight early-onset gastric cancer cases did not harbor germ line E-cadherin mutations. We can not exclude the possibility that specific *CDH1* mutations such as deletions might have been missed because of insufficient sensitivity of our method. Nevertheless, it seems more likely that most of these families carry mutations in other yet unidentified HDGC susceptibility genes.

In conclusion, our results confirm that approximately one third of families in which there is both a documented DGC diagnosed before the age of 50 and a family history of other gastric cancers will carry germ line *CDH1* mutations. We also showed that a proportion of early-onset DGC cases without a family history of gastric cancer harbor mutations and we strengthened the association between *CDH1* mutations and LBC. These data have been used to modify our testing criteria for potential HDGC families (Table 4). In addition, we have described the first clear example of recurrent *CDH1* germ line mutation, affecting a region of the gene in which somatic *CDH1* mutations cluster. The finding of occult DGCs in five of six prophylactic gastrectomies done in carriers of this mutation reinforces the idea that prophylactic total gastrectomy is a valuable and potentially life saving option for asymptomatic *CDH1* mutation carriers and should thus be strongly considered during counseling.

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