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Original Paper

Mechanisms and sequelae of E-cadherin silencing in hereditary diffuse gastric cancer

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Abstract

Around 25–40% of cases of hereditary diffuse gastric cancer (HDGC) are caused by heterozygous E-cadherin (*CDH1*) germline mutations. The mechanisms for loss of the second allele still remain unclear. The aims of this study were to elucidate mechanisms for somatic inactivation of the wild-type *CDH1* allele and to seek evidence for cadherin switching. Archival tumour material was analysed from 16 patients with *CDH1* germline mutations and seven patients fulfilling HDGC criteria without *CDH1* germline mutations. The 16 *CDH1* exons were sequenced. E-cadherin promoter methylation was analysed by bisulphite sequencing and pyrosequencing and allele specificity was determined using polymorphic loci. Loss of heterozygosity was analysed using microsatellite markers. Cadherin expression levels were determined by real-time RT–PCR and immunohistochemistry. Six of 16 individuals with germline mutations had at least one second hit mechanism. Two exonic mutations (exon 9 truncating, exon 3 missense) and four intronic mutations which may affect splicing were identified. Tumours from 4/16 individuals had promoter hypermethylation that was restricted to the A allele haplotype in three cases. E-cadherin loss (mRNA and protein) generally correlated with identification of a second hit. In cases without germline E-cadherin mutations there was no evidence for somatic mutation or significant promoter methylation. P-cadherin (>25% cells) was expressed in 7/13 (54%) and 4/5 (80%) with and without germline *CDH1* mutations, respectively, independent of complete E-cadherin loss. Overall, inactivation of the second *CDH1* allele occurs by mutation and methylation events. Methylation is commonly allele-specific and is uncommon without germline mutations. P-cadherin over-expression commonly occurs in individuals with diffuse type gastric cancer.

Keywords: second hit; methylation; CDH1; cadherin switch; P-cadherin; stomach; hereditary diffuse gastric cancer

Introduction

Gastric cancer remains a leading cause of cancer death worldwide and it has been estimated that up to 10% of gastric cancer cases have some kind of familial association [1,2]. Hereditary diffuse gastric cancer (HDGC) has been defined by the International Gastric Cancer Linkage Consortium (IGCLC) as: any family with two documented cases of diffuse gastric cancer in first- or second-degree relatives with one case under the age of 50 years or three or more documented cases of diffuse gastric cancer in first or second degree relatives at any age [3,4]. These families may also have an increased frequency of lobular breast cancer and signet ring cell carcinoma of the colon [5,6].

HDGC cases are caused by germline mutations of the E-cadherin gene (*CDH1*) in 25–40% cases, with a penetrance of 67–83% [3,7–11]. Approximately 75% of the mutations are truncating (including frameshift and nonsense mutations) and the remaining 25% are missense mutations which may have functional effects, through alteration of residues critical to protein structure or through alternative splicing [12,13]. Knudson's two-hit hypothesis predicts that both alleles of the E-cadherin gene must be inactivated in tumours [14,15]. Identified mechanisms of inactivation of the second *CDH1* allele include promoter methylation, somatic mutation and intragenic deletion, but only six cases from three families have been reported so far [16,17]. Loss of heterozygosity, a common mechanism of

inactivation of the wild-type allele in other hereditary cancer syndromes, has not been identified [5,6,16,17].

Loss and aberrant E-cadherin protein expression occurs in many different cancer phenotypes, including lobular breast, diffuse gastric and pancreatic carcinomas [18–20]. E-cadherin loss has been correlated with an increased expression of neural (N) cadherin which can alter cellular adhesive function and is known as cadherin switching [21]. Due to the mesenchymal nature of N-cadherin expressing cells, up-regulation of N-cadherin expression in epithelial cells may promote tumourigenesis and epithelial–mesenchymal transition (EMT) [22,23]. A switch from E-cadherin to P-cadherin expression has also been described in ovarian cancer, indicating that cadherin switching does not exclusively occur between E- and N-cadherin [24]. To date, although there is some evidence for EMT via activation of c-Src there have been no reports of cadherin switching in HDGC. [25]

In this study, we have performed the most comprehensive analysis to date of the second-hit mechanisms in HDGC patients with an identified *CDH1* germline mutation (16 patients from nine families) and with no germline mutation identified (seven individuals). In addition, we have sought evidence for cadherin switching by immunohistochemistry.

Materials and methods

Patient samples

Formalin-fixed paraffin-embedded tissue (FFPET) from therapeutic gastrectomies was available from the Cambridge-based Familial Gastric Cancer Study (Table 1). Ethical approval was obtained from the Eastern Multicentre Research Ethics Commmitte (Ref. 97/5/32) for collection and use of the samples. The code for individual cases is: M or N (M denotes *CDH1* germline mutation and N for no *CDH1* mutation identified); a family number; and a letter for each individual within that family. The germline mutation status was confirmed in a clinical reference laboratory and verified in our hands to be present in the heterozygous state for each case except M6A and M9A,B, for whom no blood was available. For the second-hit analysis, where possible, we used samples from the gastric tumour; however, in four cases (M2A, M2B, M2D, M5A) the primary was lobular breast or colon carcinoma and in three further cases (M1B, M4A, M4B) FFPET was not available from the primary site (Table 1). A consultant histopathologist from the referring hospital determined the original diagnosis, which was confirmed by an expert GI histopathologist (VS), who also determined tumour cellularity. A normal gastric biopsy specimen was obtained from a currently unaffected germline mutation carrier who has been undergoing endoscopic surveillance (patient M2E). Histopathologically verified normal gastric mucosa from three sporadic cancer cases, and three patients undergoing endoscopic surveillance for Barrett's oesophagus, served as controls for the expression analyses. Due to the limited availability of material, it was not possible to conduct every analysis on each patient. The numbers of patients used for each analysis is indicated in each relevant section of the results.

DNA extraction from FFPET

Five 10 μm sections were deparaffinized with xylene and washed with ethanol. DNA was incubated in 1 M NaCl, 0.5 M EDTA, 1.5 M Tris–HCl and 10% SDS, pH 8.0, containing 0.5 mg/ml proteinase K, and incubated for 3 days at 55 °C, followed by phenol/chloroform/isoamyl alcohol extraction (Invitrogen, Carlsbad, CA, USA; http://cc.ucsf.edu/people/waldman/Protocols/index.html). The DNA concentration was measured using the Nanodrop (Thermo Scientific) and a cut off value of 1.7 was used for the 260 : 280 ratio.

Sequencing of genomic DNA

Exon-specific PCR was carried out using AmpliTaq Gold (Applied Biosystems) for each of the 16 *CDH1* exons from published primer sequences [9,26] or as listed in Table S1 (see Supporting Information). If PCR reactions were unsuccessful, BioTaq kit (BioLine, London, UK) was used, utilizing the same PCR conditions: 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 55 °C, 58 °C or 60 °C for 30 s and 72 °C for 30 s, and finally 10 min at 72 °C. For exons 10 and 13 a touchdown programme was required. [27]

PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen). Sequencing reactions were performed using the BigDye Terminator v. 3.1 Cycle Sequencing Kit on the ABI3100 genetic analyser, using a 50 cm array and polymer POP6 (Applied Biosystems). Sequence data was collected using 3100 Data Collection Software v. 2 (Applied Biosystems) and analysed using Seqscape (version 1). The sequencing reaction itself was either run by a core service or contracted to Geneservice Ltd (www.geneservice.co.uk). The reference gene sequence was obtained from the Ensemble Genome Browser (www.Ensembl.org) and nucleotide position counted from the start of translation [12]. PCR reactions were repeated to confirm mutations. For tumour DNA sequence alterations, wherever possible, blood DNA was also analysed (Table 2).

In silico analysis of identified mutations

Common polymorphisms were identified using the Ensemble Genome Browser and other published sources. Sequence conservation was analysed using the LAGAN Alignment Toolkit [28]. The putative effects of mutations on splicing were assessed using RESCUE-ESE and the Alternative Splicing Database [29,30]. SIFT (sorting intolerant from tolerant) analysis was used to determine the likelihood

Table 1. Summary of the clinical details for HDGC patients with and without identified germline mutation

Patient	Sex	Age at diagnosis	Ethnicity	Country of residence	Time to death (months after diagnosis)	Primary tumour site	Histopathology	Stage	Origin of FFPET used in the study
M1A	M	22	Asian	Pakistan	7	Gastric	Poorly differentiated adenocarcinoma	T4	Gastric
M1B	F	40	Asian	Pakistan	4	Presumed gastric (metastatic to ovary)	Poorly differentiated adenocarcinoma	M1	Ovary metastasis
M1C	M	38	Asian	Pakistan	9	Gastric	Poorly differentiated diffuse type	N1M1	Gastric
M2A	F	80	Caucasian	UK	71	Breast	Poorly differentiated lobular type	N/A	Breast
M2B	F	74	Caucasian	UK	5	Caecum	Moderately differentiated adenocarcinoma	Duke's C	Colon
M2C	F	40	Caucasian	UK	3	Gastric	Signet ring/diffuse type	M1	Gastric
M2D	M	49	Caucasian	UK	N/A	Colon	Moderately differentiated adenocarcinoma	Duke's B	Colon
M3A	M	32	Caucasian	UK	1	Gastric	Poorly differentiated diffuse type	M1	Gastric
M4A	M	58	Caucasian	UK	2	Gastric	Poorly differentiated adenocarcinoma	M1	Lymph node
M4B	F	34	Caucasian	UK	N/A	Gastric	Poorly differentiated diffuse type	M1	Lymph node and omentum
M5A	M	70	N/A	UK	N/A	Colon	Well differentiated adenocarcinoma	N/A	Colon
M6A	F	42	Caucasian	UK	3	Gastric	Signet ring/diffuse type	N/A	Gastric
M7A	F	37	Caucasian	UK	3	Gastric	Signet ring/diffuse type	M1	Gastric
M8A	F	27	Caucasian	The Netherlands	N/A				Gastric
M9A	M	37	Caucasian	UK	9	Gastric	Signet ring/diffuse type	N/A	Gastric
M9B	M	11	Caucasian	UK	13	Gastric	Poorly differentiated diffuse type	T3N0M0	Gastric
—	8M:8F	43.2	—	—	Median: 4.5	—	—	—	
N1A	M	45	Caucasian	UK	2	Unknown origin	Poorly differentiated adenocarcinoma	N1	Lymph node
N2A	F	59	Caucasian	UK	4	Gastric	Poorly differentiated adenocarcinoma	N1M1	Gastric
N3A	F	48	Caucasian	UK	6	Gastric	Signet cell gastric adenocarcinoma	M1	Gastric
N4A	M	44	Caucasian	UK	<6	Gastric	Signet ring/diffuse type	N1M1	Gastric
N5A	M	49	Caucasian	UK	18	Gastric	Poorly differentiated adenocarcinoma	N1M1	Gastric
N6A	M	39	Caucasian	UK	6	Gastric	Poorly differentiated adenocarcinoma	M1	Gastric
N7A	F	44	N/A	Spain	N/A	Gastric	N/A	T3	Gastric
—	4M:3F	46.9	—	—	Median: 6	—	—	—	

ID codes are made up of germline mutation (M) or no germline mutation (N) identified, family (number) and individual (letter) identifiers. FFPET, formalin-fixed paraffin-embedded tissue; N/A, data not available and for missing parts of T, N, M data not shown. Nodal status is categorized as N0 or N1, as insufficient information was available to use the official gastric system.

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Table 2. Summary of sequence, methylation and expression findings for HDGC patients with and without identified germline mutation

Patient	Germline mutation identified	Tumour cellularity (%)	Blood available	Tumour DNA sequence c	Loss of heterozygosity	Methylation status	E-cadherin RNA expression	E-cadherin protein expression (%)	P-cadherin protein expression (%)
M1A	832 G > A	60		IVS5 − 14 C > T (ht) IVS9 − 19 C > T (ht)	ND	U	ND	25–50 M	25–50
M1B		95		260 G > A R87K (ht) IVS5 − 14 C > T (ht) IVS7 + 43 G > A (ht)	ND	M	Negative	Negative	25–50
M1C		25	Yes	None	ND	U	Reduced	>75 C/M	Negative
M2A	45insT	60		678 C > T A226A (ht)	ND	M	Greatly reduced	Negative	ND
M2B		95		1226 G > A W409STOP (ht)2520 C > T S840S (ht)	ND	M	Greatly reduced	Negative	>75
M2C		70		UTR-53 G > A (ht)	ND	M	ND	>75 C/M	<25
M2D		40	Yes	None	None	U	Reduced	>75, mainly C	25–50
M3A	1472insA	60	Yes	None	ND	U	ND	50–75 M	<25
M4A	1064insT	80		None	ND	U	Greatly reduced	Negative	Negative
M4B		70	Yes	None	None	U	Reduced	>75 C/M	ND
M5A	1565 + 1 G > T	40	Yes	None	None	U	Reduced	>75, mainly C	25–50
M6A	1466 insC	50		567 C > T F189F (ht)	Possible	U	ND	ND	<25
M7A	59 G > A	80		None	ND	U	ND	>75 C/M	>75
M8A	1134 del8, ins5	70	Yes	None	None	U	Reduced	50–75 M	<25
M9A	1792 C > T	75		None	ND	U	Strong	>75 C/M	50–75
M9B		80		None	ND	U	Reduced	>75 C/M	ND
—	—			6/16 Mutated (25%) (four likely pathogenic)	1/5 Possible	4/16 Methylated (25%)	10/11 Reduced/ negative (91%)	4/15 Negative (27%)	7/13 > 25 Cells (54%)
N1A	—	80		None	ND	U	Reduced	>75 M	<25
N2A	—	80		None	ND	U	Reduced	ND	ND
N3A	—	70		None	ND	U	ND	>75 M	>75
N4A	—	50		None	ND	U	Reduced	25–50 M	ND
N5A	—	80		None	ND	U	Reduced	>75, mainly C	50–75
N6A	—	90		None	ND	U	Normal	>75, mainly C	25–50
N7A	—	20		ND	ND	U	Reduced	ND	>75
	—			0/6 Mutated	ND	0/7 Methylated	5/6 Reduced (83%)	0/5 negative	4/5 > 25 cells (80%)

ID codes are made up of germline mutation (M) or no germline mutation identified (N), family (number) and individual (letter) identifiers.
ins, insertion; del, deletion; hm, homozygous; ht, heterozygous; M, membranous; C, cytoplasmic; ND, not done (no material available)

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of missense mutations being tolerated within the protein sequence [31]. The effects of mutations on 5′ UTR (untranslated) functional elements and transcription factor binding sites were analysed using UTRScan [32], TF Search version 1.3 and TRANSFAC [33]. Codon usage was obtained from the Codon Usage database [34].

Microsatellite LOH (loss of heterozygosity) analysis

PCR reactions were carried out using fluorochrome-conjugated primers for the microsatellites D16S3025, D16S496, D16S3067 and D16S3141 (for sequences, see Supporting Information, Table S2). PCR reactions used 1 U AmpliTaq Gold (Applied Biosystems), 0.2 mM dNTP mix and 20–25 pmol of both forward and reverse primer sequences in a total volume of 25 μl with a 55 °C annealing temperature. 2 μl PCR product were added to 0.5 μl GeneScan 500 LIZ Size Standard and 9.5 μl of Hi-Di Formamide and analysed using an ABI3100 genetic analyser, using polymer POP4 (Applied Biosystems). The data were analysed using GeneMapper v. 3.5 (Applied Biosystems). The ratio of the peak heights of the two alleles was calculated for the normal blood and tumour sample available from the same patient (blood sample from a sibling was used for patient 6A), using the formula:

$$\text{change in height} = \text{tumour height ratio} / \text{normal height ratio}$$

Bisulphite treatment

The MKN-1 gastric adenosquamous carcinoma cell line or CpGenome Universal Methylated DNA (Chemicon International, Temecula, CA, USA) were used as positive controls [35]. The MKN-45 diffuse gastric carcinoma cell line (a gift from Dr A. Ristimaki, University of Helsinki, Finland) and two histopathologically normal gastric samples were analysed as negative controls. For tissue and cell line material, 500 ng DNA was bisulphite-treated using the EZ Methylation-Gold Kit (Zymo Research, Orange, CA, USA).

Bisulphite sequencing

PCR primers were specific for *CDH1* promoter regions containing no CpG sites to enable amplification of both methylated and unmethylated DNA [forward, 5′-TAGTAATTTTAGGTTAGAGGG-3′; reverse, 5′-ACTAAAATCTAAACTAACTTC-3′); 2 μl bisulphite-treated DNA, 2.5 mM $MgCl_2$, 10× PCR mix, 1.25 U AmpliTaq Gold, 0.25 mM dNTP mix (GE Healthcare) and 0.8 μM primer mix]. A non-template control was included for each set of reactions. PCR conditions were as described for sequencing with a 57 °C annealing temperature and 35 cycles of amplification. PCR products were cloned using the TA Cloning Kit with One Shot TOP10 Chemically Competent *Escherichia coli* (Invitrogen) and at least five clones were sequenced for each patient sample. M13 primers were used (forward, 5′-TTGTAAAACGACGGCCAGTG-3′; reverse, 5′-CAGGAAACAGCTATGACCAT-3′) for amplification and sequencing of the cloned PCR products. The average percentage of the five clones was used to determine whether or not the allele was methylated.

Pyrosequencing

Four consecutive CpGs within the promoter region were selected due to their close proximity and the presence of a non-CpG cytosine residue, which was an internal control for the level of bisulphite conversion. Primary PCR reactions were carried out as for bisulphite sequencing, using a biotinylated reverse primer. This was followed by triplicate nested PCR reactions, which were combined, gel-purified and eluted into EB buffer, using the Qiagen Gel Extraction Kit. 10 μl purified PCR product were added to 30 μl Binding Buffer (Biotage), 2 μl streptavidin sepharose high-performance beads (GE Healthcare) and 30 μl sterile water, and single-stranded biotinylated templates were isolated using the PyroMark Vacuum Prep WorkStation (Biotage). The products were dispensed into PSQ 96 Plates containing 0.5 μl 10 μM sequencing primer (5′-AGGTGAATTTTTAGTTAATT-3′) and 11.5 μl Annealing Buffer (all Biotage) at 80 °C for 3 min, followed by room temperature for 15 min. Pyrosequencing reactions were carried out in the PyroMark MD machine using PyroGold Reagents and results analysed using pyro Q-CpG Software (all Biotage).

Allele-specific methylation analysis

A polymorphism at position −160 (C > A) (rs16260) was chosen for analysis of allele-specific methylation, as this has high heterozygosity (0.359 ± 0.225). This polymorphism was also used for genotyping allele-specific primers, to determine the nucleotide present at this locus on the allele containing the exon 1 germline mutation identified in family 2. Genotyping primer sequences were as follows: forward, 5′-ACTCCAGGCTAGAGGGCAC/A-3′, reverse, 5′-CAGGACCCGAACTTTCTTGGAAGAAG-3′. PCR conditions were the same as used for exon-specific PCR (60 °C annealing temperature for 45 cycles). PCR products were cloned as described above and five clones were analysed.

RNA extraction

Five 10 μm sections were deparaffinised in xylene and rehydrated using a decreasing ethanol series (100%, 90%, 70%). The samples were incubated in 10 mM Tris–HCl, 0.1 mM EDTA, 2% SDS and 0.5 mg proteinase K at 65 °C for 16 h. An additional 0.5 mg

Proteinase K was added and incubation continued for a further 3–4 h at 65 °C. The RNA was purified using a Qiagen RNeasy MiniKit (Qiagen, Crawley, UK) and DNAse treated using the Qiagen RNase-free DNase kit. The RNA concentration was measured using the Nanodrop (Thermo Scientific) and a cut-off value of 1.7 was used for the 260 : 280 ratio.

Real-time PCR

The High-capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used with 500 ng–1 μg RNA for each sample. Relative E-cadherin expression ($\Delta Ct = Ct_{\mathrm{GAPDH}} - Ct_{\mathrm{E-cadherin}}$) was determined by normalizing to GAPDH expression levels in the same sample. All reactions (including a non-template control) were run in triplicate, using SYBR Green Jumpstart Taq Ready Mix (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions for GAPDH (forward, 5′-CAAGATCATCAGCAATGCCT-3′; reverse, 5′-ATGAGTCCTTCCACGATACC-3′); and E-cadherin (forward, 5′-AAGGAGGCGGAGAAGAGGAC-3′; reverse, 5′-CGTCGTTACGAGTCACTTCAGG-3′). [36] PCR conditions were: 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, followed by a melt programme. The E-cadherin expression was considered reduced if it was up to three-fold lower than normal gastric specimens and greatly reduced if it was reduced by more than three-fold.

Immunohistochemistry

FFPET sections (4 μm) were immunostained for extracellular domain-specific E-cadherin antibody (1 : 40, clone 36B5; LabVision, Fremont, CA, USA), P-cadherin antibody (1 : 1000, clone 56; BD Biosciences, Franklin Lakes, NJ, USA) and N-cadherin antibody (1 : 500, clone 3B9; Invitrogen) as described previously. [37] Endogenous peroxidases were blocked using hydrogen peroxide and non-specific binding blocked by 10% BSA, 10% horse serum in TBS–Tween 0.05%. Primary antibodies were incubated overnight at 4 °C. Negative (no antibody addition) and positive (normal gastric) controls were included for each set of slides. Antibody visualization was achieved with biotinylated secondary antibodies followed by an avidin–horseradish peroxidase complex to break down 3′,3′-diaminobenzidine. Staining in tumour cells was compared to normal gastric tissue and described as membranous (M), cytoplasmic (C) or membranous/cytoplasmic (C/M), and the percentage of tumour cells expressing the protein was estimated (<5%, 5–25%, 25–50%, 50–75% and >75%) across each section (VS and FC).

Statistical analyses

Statistical analyses were undertaken using GraphPad Prism (GraphPad Software, San Diego, CA, USA). ANOVA (using the Kruskal–Wallis test), followed by Dunn's multiple comparison test, was used to determine statistical significance when more than two variables were being compared and the Mann–Whitney test was used to compare two variables. The χ^2 test was used to compare protein expression levels between patients with and without promoter methylation.

Results

Somatic sequence alterations in tumour tissue

Exons 1–16 were sequenced from tissue samples for each of the 16 patients with known germline mutations, with a complete success rate of 86%. The germline mutations were verified in all cases. In addition, heterozygous somatic mutations, which have not been previously detailed within the literature and are unlikely to be common polymorphisms, were identified in 5/16 individuals (Table 2, patients M1A, M1B, M2B, M2C and M6A). The mutations identified can be divided into three groups: exonic, intronic and 'silent' (see Suporting Information, Table S3, for a summary of their predicted pathogenicity based on the *in silico* analysis).

Exonic mutations (chromatograms are shown in Figure 1)

Patient M1B has a missense mutation in exon 3 (260 G > A R87K; heterozygous), resulting in an arginine to lysine substitution in a base pair which is conserved between mouse and human (LAGAN Alignment Toolkit). From *in silico* SIFT analysis, the amino acid change caused by this mutation is unlikely to be tolerated within the protein sequence. In addition, RESCUE-ESE software suggests that this mutation may activate a cryptic exonic splice site, which could be important since this mutation occurs within the propeptide domain. Patient M2B has a mutation in exon 9, which converts a tryptophan residue into a stop codon [1226 G > A W409STOP (ht)], resulting in truncation of the protein within the extracellular domain.

Intronic mutations

Four intronic mutations were identified in three patients (Table 2, patients M1A, M1B and M2C): the mutation present in the 5′ UTR [−53 G > A (ht)] of patient M2C did not affect transcription factor binding sequences or UTR functional elements and is therefore less likely to be pathogenic [32,38]. The other three mutations, IVS5 − 14 C > T (ht)-M1A, M1B, IVS9 − 19 C > T (ht)-M1A, and IVS7 + 43 G > A (ht)-M1B, occur in sequences that are evolutionarily conserved between human and mouse and one of these, IVS5 − 14 C > T (ht), affects a conserved nucleotide (LAGAN Alignment Toolkit). It is interesting that two first cousins (M1A, M1B) harboured this same intronic

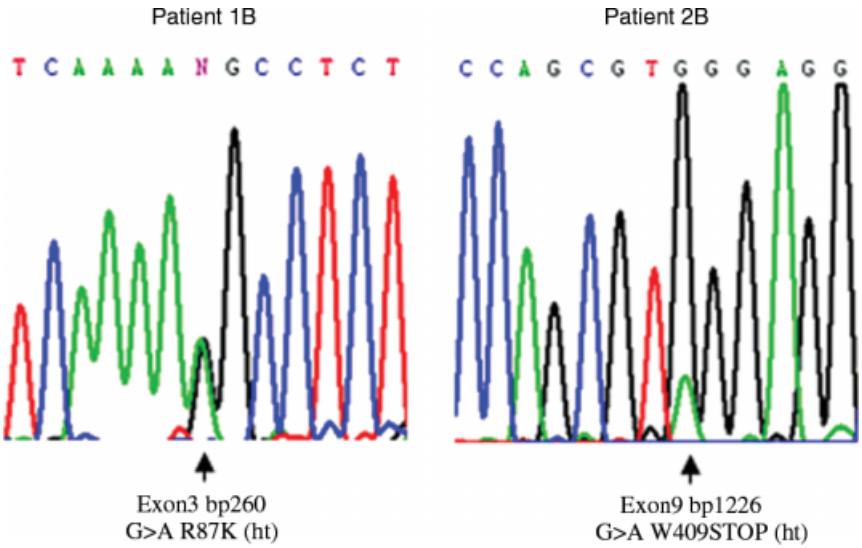

Figure 1. Chromatograms of potentially pathogenic exonic mutations. An arrow indicates the position of the mutation. ht, heterozygous

mutation [IVS5 − 14 C > T (ht)]. Splice factor binding sites may be affected by each of these intronic mutations (Alternative Splicing Database).

'Silent' mutations

Additionally, three synonymous exonic mutations that do not affect the amino acid sequence (567 C > T F189F (ht)-M6A, 678 C > T A226A (ht)-M2A and 2520 C > T S840S (ht)-M2B) were identified (Table 2, patients M2A, M2B and M6A). All three of these mutations were only identified in single individuals and have not been reported previously. *In silico* analysis suggests that these sequence alterations may affect splicing. For each of the sequence alterations identified in this study, the mutant codon usage was less common than the wild-type codon. The exon 5 mutation 678 C > T A226A from patient M2A resulted in a 13% reduction in codons used for incorporation of an alanine residue. This is similar to that hypothesized to affect protein conformation [39].

Six polymorphisms (IVS1 +6 T > C, exon 12 1896 C > T H632H, IVS12 −13 T > C, exon 13 2076 T > C A692A, exon 14 C > T D764D and exon 16 2634 C > T G878G) were found, which were also present in blood in many cases and have been previously reported in the literature. In contrast, apart from two known common polymorphisms, IVS1 +6 C > T (ht) and IVS4 C > G +10 (ht), no potentially pathogenic mutations were identified in tumour material from six HDGC patients without germline *CDH1* mutations [9,40–42].

Loss of heterozygosity analysis

Material was available for loss of heterozygosity (LOH) analysis from five individuals (M2D, M4B, M5A, M6A, M8A). No convincing evidence was found for LOH. In case M6A the patient was homozygous for the markers within dinucleotide repeats D16S3025 and D16S496 (data not shown; Table 2). However, the sequencing analysis did not confirm allelic loss, although this may not invalidate the microsatellite analysis, since the tumour cellularity was 50% and hence normal cells may have contaminated the sequencing result. LOH analysis was not performed for the non-mutation cases, since normal blood DNA was not available.

Methylation analysis

The methylation status of all patients with and without mutation was analysed. Bisulphite conversion prior to sequencing was almost complete [average 45.9/47 (97.6%) conversion rate]. The positive control (CpGenome Universal Methylated DNA) was heavily methylated, whereas the negative control cell line (MKN-45) and two normal gastric samples taken from members of HDGC families without germline mutations exhibited lack of methylation. In contrast, 4/16 (25%) patients with germline mutations, including three within the same family (M1B, M2A, M2B, M2C), had >25% methylation (measured by bisulphite sequencing), which is sufficient to cause a loss of E-cadherin expression [43], although this was never present in all of the clones analysed from an individual (Figure 2B, C). In contrast, bisulphite sequencing on endoscopic surveillance specimens of E-cadherin expressing histopathologically normal gastric tissue from an unaffected mutation carrier in family 2 (patient M2E) was negative for promoter methylation.

The possible explanations for the observed variation in methylation between patients include allele-specific methylation, polyclonal variation or normal cell contamination. The polymorphism at position −160 (C > A) enabled the identification of specific alleles in our cloned bisulphite sequences. In patients M1B, M2A and M2C, methylation was restricted to the A allele in each case (Figure 2B).

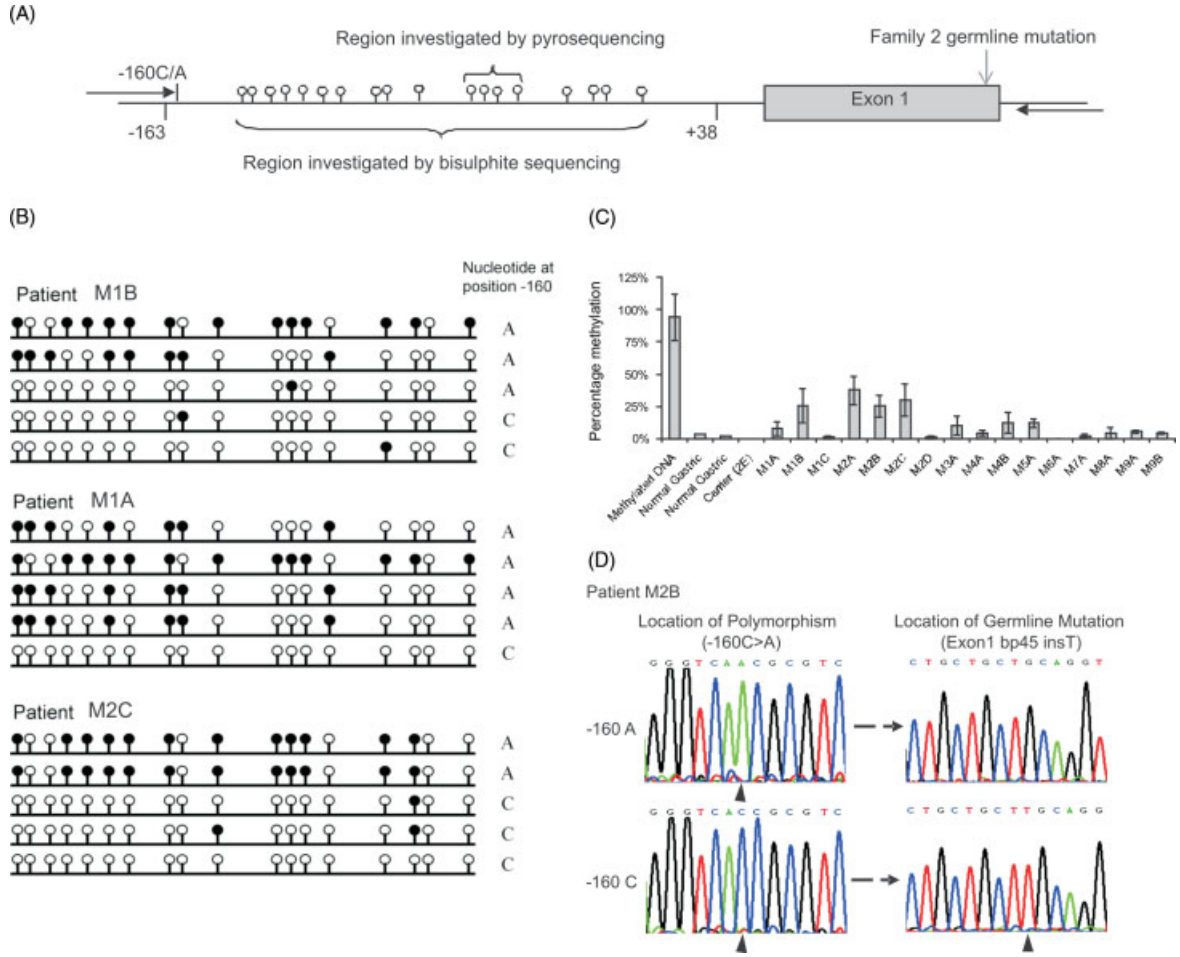

Figure 2. (A) E-cadherin promoter region under investigation. Bisulphite sequencing runs between nucleotides −163 and +38 and the four CpGs analysed by pyrosequencing are marked (each lollipop represents a CpG). The positions of the −160 C/A polymorphic locus and the germline mutation in family 2 (grey arrow) are also shown. Black arrows show the primers used for allele-specific PCR. (B) Methylation patterns within the E-cadherin promoter for three patients demonstrating allele-specific methylation. Black lollipops indicate methylation of each CpG. The nucleotide present at the −160 C/A polymorphic locus is given for each of the five clones for each patient. (C) Bisulphite sequencing results values are percentage methylation calculated for five clones (three clones for control subjects and the unaffected mutation carrier). Error bars are standard error of the mean. (D) Chromatograms demonstrating the germline mutation in patient M2B with respect to the 160C/A polymorphic locus. The germline mutation is present on the same allele as the C nucleotide at position −160 and not present when the A allele is present at this location

In order to further investigate the mono-allelic nature of this methylation, the −160 C > A polymorphism was used to design allele-specific primers that encompassed the polymorphic locus and the location of the exon 1 germline mutation (45 insT) found in family 2 (in family 1 the germline mutation is in exon 6 and hence this approach was not possible). Using this method for patient M2A, in whom there was sufficient material available for analysis, none of the clones containing the A nucleotide at the −160 C > A polymorphic locus contained the germline mutation. From patient M2B we had determined that the C nucleotide was on the same allele as the germline mutation (Figure 2D). Together these data suggest that, in family M2, allele-specific methylation is occurring to inactivate the allele which is not affected by germline mutation.

In order to obtain a quantitative estimate of methylation status in patients with and without germline mutations compared with normal gastric control tissue, pyrosequencing was performed (Figure 3). Pyrosequencing analysis was successful for 10 HDGC patients with germline mutations who had been analysed for somatic mutations. Despite a high degree of patient variability, the level of methylation was generally greater in the tumour samples from germline *CDH1* mutation cases compared to the seven non-germline mutation cases and normal gastric controls (Figure 3; p = not significant).

E-cadherin expression and relationship to N- and P-cadherin expression

E-cadherin mRNA expression was performed in 11/16 patients with germline mutation and six of seven patients without germline mutation (Table 2). The mean level of E-cadherin mRNA expression within the tumour material of diffuse gastric cancer patients

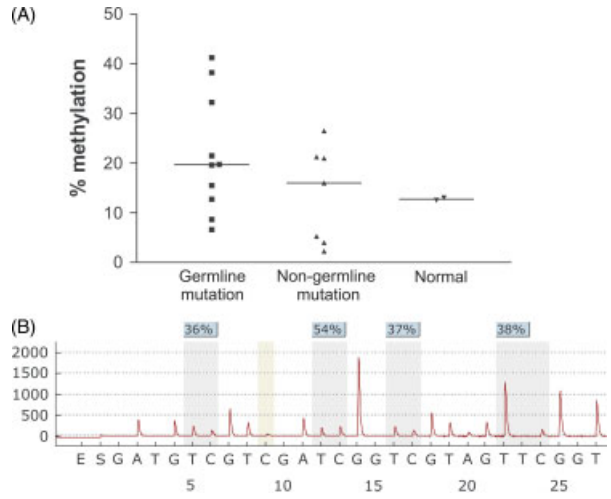

Figure 3. (A) Pyrosequencing analysis showing the level of methylation across the four CpGs in each sample (dot) and the mean level for the group (horizontal line). (B) Representative pyrosequencing trace from patient M2C. The percentage of PCR products with methylation is shown in blue for each of the four CpGs

(whether or not they harboured germline *CDH1* mutations) was lower than the expression found in the normal gastric controls ($n = 6$) and was absent in one individual (patient M1B), ($p < 0.05$; Figure 4A). In addition, the relative mRNA expression levels of those patients with promoter methylation (detected by bisulphite sequencing) and those patients without promoter methylation were compared (only germline mutation patients; Figure 4B). The patients harbouring promoter methylation had statistically significantly lower levels of relative mRNA expression levels ($p = 0.0242$). In contrast, the unaffected mutation carrier had strong E-cadherin expression.

The results of immunohistochemical analysis of E-cadherin protein expression levels are summarized in Table 2 and representative staining patterns are shown in Figure 4C. Immunohistochemistry for E-cadherin was performed on 15/16 patients with germline mutation and five of seven non-mutation patients (Table 2). Histopathologically normal tissue was used as a control and E-cadherin staining was considered to be normal when >75% epithelial cells had membranous expression without increased cytoplasmic expression. Using these criteria, 100% of patients with *CDH1* germline mutations for whom sections were available had abnormal staining. Staining was negative in four patients (M1B, M2A, M2B, M4A) and the remainder had reduced expression or protein aberrantly expressed within the cytoplasmic compartment.

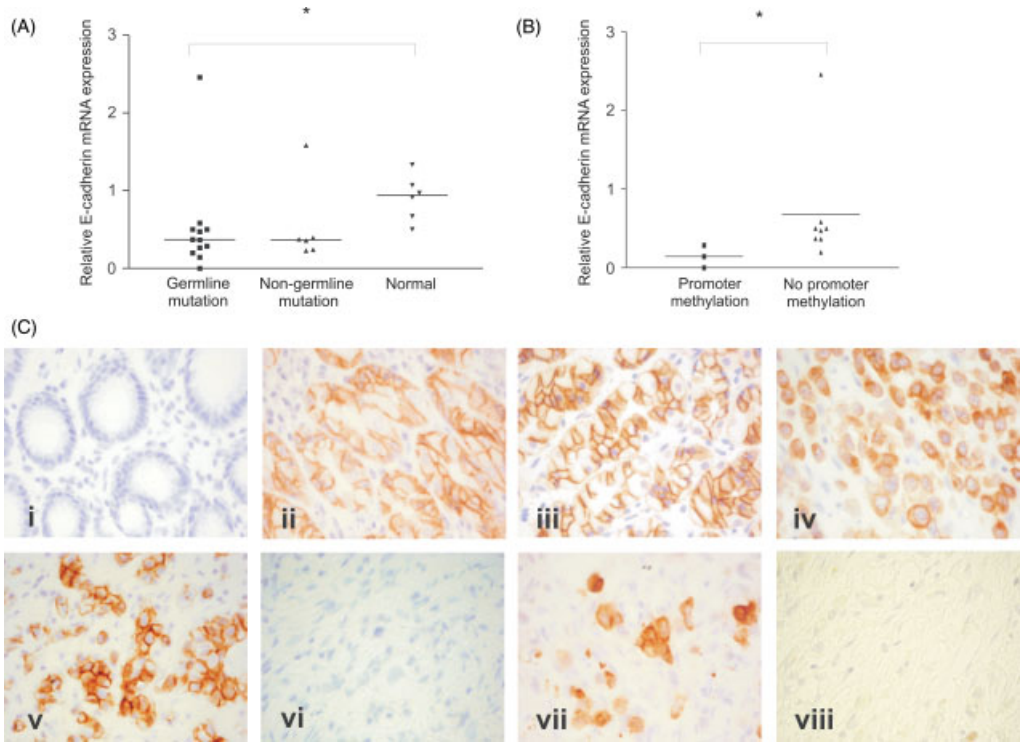

Figure 4. (A) The mean level of E-cadherin mRNA expression in tumour material from 10 mutation carriers is compared to six non-mutation carriers and six normal gastric samples ($p < 0.05$ for mutation cases compared with normal gastric). (B) A comparison of the mRNA expression levels in those patients with and without promoter methylation. Only patients with germline E-cadherin mutations are included ($^*p < 0.05$). (C) Examples of immunohistochemical staining patterns: i, negative control; ii, normal gastric tissue positive control (E-cadherin); iii, strong membranous E-cadherin in normal gastric tissue from unaffected germline mutation carrier; iv, membranous and cytoplasmic E-cadherin expression in tumour material from a patient without a germline mutation; v, membranous and cytoplasmic E-cadherin expression in tumour from a patient with a germline mutation; vi, lack of E-cadherin expression in tumour from a patient with a germline mutation; vii, tumour material demonstrating strong membranous and cytoplasmic P-cadherin expression; viii, tumour material demonstrating lack of P-cadherin expression. Magnification, ×400

Statistically significantly more patients with promoter methylation were negative for E-cadherin protein expression compared with patients without promoter methylation ($p = 0.012$, using χ^2 test). The unaffected mutation carrier (M2E) had normal membranous staining (Figure 4Ciii). In patients without germline mutation normal E-cadherin expression was seen in two of five patients, whilst three of five patients had abnormal expression.

Immunohistochemistry for P-cadherin was performed on 13/16 patients with germline mutation and five of seven non-mutation patients (Table 1). P-cadherin expression (Table 2, Figure 4C) was found in >25% of tumour cells in 7/13 (54%) patients with germline mutations and four of five (80%) patients without germline mutations. Two cases (M2B, M7A) with mutations and two cases (N13A, N17A) without mutations had expression in >75% cells. In patients M2B and M1B, P-cadherin expression was seen in the absence of E-cadherin, suggesting that cadherin switching had occurred. N-cadherin expression was not observed in any tumour material, despite positive staining of nerve cells as a positive internal control (data not shown).

Discussion

This study has demonstrated that somatic mutation and promoter methylation are both mechanisms of inactivation of the second allele in individuals with germline E-cadherin mutations. Interestingly, more than one mechanism may be involved in E-cadherin silencing of the second allele within the same patient. In contrast, tumour material from patients from families with HDGC without germline *CDH1* mutations have reduced E-cadherin expression but, in our series, did not have evidence of somatic mutations or hypermethylation of *CDH1*. For the first time we have shown that aberrant P-cadherin expression occurs commonly in HDGC tumours (11/18, 61%) whether or not a *CDH1* germline mutation is present. Although cadherin switching was observed in two patients, complete loss of E-cadherin was not necessary for expression of P-cadherin to occur.

There are a number of limitations to this study. All of the DNA and RNA were extracted from FFPET and therefore a significant level of degradation was present. Furthermore, the amount of material available was very limited and for this reason the study was conducted using whole sections rather than microdissected tumour material. The presence of stromal and non-cancerous epithelial cells in the whole sections may have reduced the signal from the tumour material, making the second hit more difficult to identify. The mean tumour cellularity of the samples used was 64%, which could have led to false-negative results. However, since most samples (13/16, Table 2) had a cellularity above 50%, we feel that this is unlikely. Further studies are required to determine the allele specificity of the DNA tumour sequence changes indentified. Extended sequencing analysis may help to achieve this but it will be very challenging when using DNA from FFPET material. Due to the rarity of HDGC, the number of samples studied is small and hence caution should be used when interpreting the significance of these results.

As well as somatic mutations, promoter methylation was identified as one mechanism of wild-type allele inactivation in four of 16 (25%) individuals, including three of four members from family M2. Three of these patients with a methylated promoter were negative for E-cadherin expression. This is in keeping with previous work reporting aberrant methylation in 50–80% of patients displaying lack of E-cadherin immunoreactivity [16,44]. Here we were able to extend previously published data and show in family M2 that there was no germline mutation in the methylated A allele. Thus, methylation is likely to be the second hit responsible for gene silencing.

In keeping with previously published data [44], there were individuals with a germline mutation who did not appear to exhibit any of the second-hit mechanisms under investigation (although LOH was not analysed in all cases). Where LOH was performed, although not conclusive, there was a possibility that this had occurred in patient M6A. This is interesting, since LOH has been previously described in sporadic diffuse gastric cancer as the second-hit mechanism [45–47]. Further investigation would be worthwhile in future cases using microdissected snap-frozen tumour material. It should also be borne in mind that alternative inactivation mechanisms, such as intragenic deletion, may have occurred [17]. Transcriptional repression or post-translational modifications, including ectoderm shedding, may also contribute to E-cadherin inactivation. It is also possible that mutation of only one E-cadherin allele is sufficient for tumour initiation, especially if it is acting in a dominant-negative fashion [48]. Other indirect mechanisms of silencing may also play a role, such as the miR-200 family of microRNA, which has been shown to indirectly regulate the expression of E-cadherin through the transcriptional repressors ZEB1 and ZEB2 [49,50].

In patients with gastric cancer without germline mutations a number of previous reports have described mutations, methylation and LOH as the main mechanisms for inactivation of E-cadherin [45–47]. However, the inverse correlation between LOH and promoter methylation, together with low rates of mutations reported by Liu *et al* [47], suggests that other mechanisms not fulfilling the classical two hit hypothesis are involved. Interestingly, methylation was identified in patients fulfilling the criteria for HDGC without any mutation. It may be possible that these patients without germline *CDH1* mutations behave more like sporadic cases than familial cases with regard to mechanisms for E-cadherin silencing. However, it would be interesting to investigate LOH, which was not possible with the material available for this study [45–47].

The finding that P-cadherin was frequently expressed in the presence of E-cadherin was interesting, since it may suggest that cadherin switching is occurring in these tumours. Other studies have demonstrated that even when E-cadherin continues to be expressed, aberrant expression of N-cadherin can also have a direct and dominant influence on the phenotype of epithelial cells [51]. P-cadherin was expressed to similar levels in HDGC patients and sporadic gastric cancer cases [52,53], suggesting that both tumour types may become more invasive through similar mechanisms, despite their different primary aetiology. Functional studies would be required in order to further investigate the significance of increased P-cadherin in this context.

Overall, this study has yielded useful data, despite the limitations imposed by the small quantity and archival nature of the patient material. Although E-cadherin loss is a common phenomenon in diffuse type gastric cancer, somatic alterations, such as mutation and methylation, were restricted to those patients with germline *CDH1* mutations. Furthermore, since the data suggest that there are a variety of second-hit mechanisms, this may make the future application of chemopreventive options, such as demethylating agents, more limited than previously hoped [16]. More research is required to understand the key molecular genetic events that lead to the development of cancer in HDGC patients, so that alternatives to prophylactic surgery can be offered.

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Supporting information

Supporting information may be found in the online version of this article.

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