The E-cadherin gene (*CDH1*) variants T340A and L599V in gastric and colorectal cancer patients in Korea

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Abstract

Introduction—Germline mutations in E-cadherin (*CDH1*) have been reported in families with early onset, diffuse gastric cancer. More recently, mutations in *CDH1* have been described in colorectal cancer cell lines.

Aims—We have investigated if germline mutations in *CDH1* occur among different groups of Korean gastric and colorectal cancer patients, with and without a positive family history.

Methods—We studied 131 patients and 168 normal controls (88 Korean and 80 non-Korean). Patients were divided into five groups: group I, 20 gastric cancer patients with a family history; group II, 26 colorectal cancer patients with a family history of gastric cancer (those from familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) kindred were excluded); group III, 16 HNPCC patients without identified germline mutations in *hMLH1* and *hMSH2*; group IV, 35 gastric cancer patients without a family history; and group V, 34 colorectal cancer patients without a family history. Polymerase chain reaction, single strand conformational polymorphism analysis, direct sequencing, and genotyping for identified variants were performed.

Results—Several germline changes in *CDH1* were found. In addition to previously described polymorphisms, we found three novel changes, two of which were missense changes (T340A and L599V). T340A was present in one patient in group III and one in group V. L599V was present in one patient in group II, in two in group III, and in one in group IV. T340A was not found in normal controls while L599V was present in two of 88 Korean controls. Patients with these variants may appear to have a tendency to early onset cancer with a positive family history, although differences in frequencies did not reach statistical significance. Genotyping results suggest that these variants might have a common origin, particularly T340A.

Conclusion—We have described two new missense germline variants in *CDH1* in various groups of Korean gastrointestinal cancer patients. Further work is required to assess if these variants increase the risk of gastrointestinal cancer.

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In sporadic cancers, mutations are acquired somatically while in most hereditary cancers one important mutation is already present in the germline. The genetic basis of gastrointestinal cancer is not completely understood and indeed in colorectal cancer, mutations in the known cancer related genes do not explain all cancers that are thought to have a hereditary component.¹ In gastric cancer, the genetic events associated with tumorigenesis are less well elucidated than those for colorectal cancer. Recently, however, two groups have reported germline mutations of the E-cadherin gene (*CDH1*) in families with early onset diffuse gastric cancer.^{2,3} At the somatic level, frequent inactivating mutations in *CDH1* have been described in diffuse gastric cancers, and the majority of mutations in these cancers are exon skipings which cause inframe deletions.⁴ Although E-cadherin expression is known to be reduced in colorectal cancer, it is only recently that *CDH1* mutations have been described. Frameshift mutations in exon 3 of *CDH1* were reported in 30% of RER+ colorectal cancer cell lines, and transfection of full length *CDH1* cDNA into one of these cell lines enhanced intercellular adhesion, induced differentiation, retarded proliferation, and inhibited tumorigenicity.⁵ These data suggest that mutations in *CDH1* are an important step in the carcinogenesis sequence of gastric and colorectal cancer.

E-cadherin, a member of the cadherin family of cell surface glycoproteins, is a Ca²⁺ dependent cell-cell adhesion molecule found mainly in epithelial tissues. It is thought to be related to embryogenesis, polarisation, differentiation, and cell migration in inflamed tissue.⁶⁻⁸ E-cadherin carries out its adhesive function by homotypic interaction of five extracellular repeated domains.^{9,10} Its intracellular portion complexes with several distinct undercoat proteins, including α -, β - and γ -catenin/placoglobin, through which it is linked to the actin cytoskeleton.^{11,12} The cadherin-catenin complex appears to be essential for normal epithelial cell-cell adhesion. E-cadherin is also intimately related to malignancy which is characterised by uncontrolled proliferation, dedifferentiation, invasion, and metastasis. There is overwhelming evidence that loss of E-cadherin function is associated with invasiveness, lymph node metastasis, distant metastasis, and other poor prognostic factors.¹³⁻¹⁵

Abbreviations used in this paper: HNPCC, hereditary non-polyposis colorectal cancer; FAP, familial adenomatous polyposis; APC, adenomatous polyposis coli.

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Inactivation of E-cadherin function may be achieved by a variety of mechanisms. Mutations in *CDH1* may alter its adhesive function and have been reported in a number of cancers.^{16–25} The highest frequencies of *CDH1* mutation have been reported in lobular breast cancer and diffuse-type gastric cancer. Histologically these tumours are characterised by loss of glandular architecture and infiltration of tumour cells into the stroma. Genetic alterations in any component of the cadherin-catenin complex seem to induce loss of adhesion function.^{26–27} Furthermore, epigenetic changes such as promoter region hypermethylation, post-transcriptional alteration, and aberrant phosphorylation of members of the cadherin-catenin complex can deregulate E-cadherin function.^{24–28–30} It has been shown that E-cadherin expression is reduced by hypermethylation of the *CDH1* promoter region in a number of tumours, including breast, prostate, hepatocellular, and thyroid carcinomas.^{24–28–29–31} A study in transgenic mice demonstrated that loss of E-cadherin mediated cell adhesion is one of the rate limiting steps in the progression from adenoma to carcinoma.³² Thus it seems that inactivation of the E-cadherin complex by various mechanisms can play a significant role in both the early and late stages of carcinogenesis.

It remains to be ascertained whether germline mutations in *CDH1* are involved in gastric cancer patients with a weaker family history, or even without a family history when a germline variant may have low penetrance. Recently, a new class of subpolymorphic missense variants has been described in the adenomatous polyposis coli (APC) gene³³ which are associated with colorectal cancer but not always with a family history. In this study, we have searched for germline mutations in *CDH1* in several groups of Korean gastric and colorectal cancer patients with and without a positive family history. We report two germline missense variants that have not been previously described but which could play some role in tumour development through altered E-cadherin function.

Methods

SUBJECTS

Peripheral blood samples were obtained from 131 Korean patients with diffuse gastric or colorectal cancer who were selected according to their family history (Gastrointestinal Tumour Registry, Asan Medical Centre, Seoul, Korea).

Group I (n=20) consisted of patients with gastric cancer and a positive family history (more than one first degree relative with gastric cancer). Group II (n=26) comprised patients with colorectal cancer and a positive family history of gastric cancer. Patients with familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) were excluded. Group III (n=16) consisted of patients with a family pedigree suggestive of HNPCC but without identified germline mutations in hMLH1 and hMSH2: seven of these satisfied the Amsterdam criteria³⁴ while nine met less stringent criteria.³⁵ Group IV (n=35) comprised patients who had gastric

cancer without a family history of malignancy, and group V (n=34) those who had colorectal cancer without a family history of malignancy. In the case of sequence variants in patients, whenever possible, blood samples from relatives were obtained. The DNA from leucocytes of 88 healthy Korean and 80 healthy non-Korean subjects (collected originally for a HLA allele frequency study, and supplied by Dr J Bodmer, Oxford, UK) was analysed for the frequencies of variants in the general population. Family history was obtained by interview and confirmed by hospital records.

POLYMERASE CHAIN REACTION AND SINGLE STRAND CONFORMATIONAL POLYMORPHISM (PCR-SSCP) ANALYSIS

Genomic DNA was extracted using standard techniques. PCR reactions for amplification of E-cadherin were performed using previously published primers.¹⁶ All reactions were carried out in a total volume of 50 µl with a final reaction concentration of 1×PCR buffer (Promega, Madison, Wisconsin, USA), 200 µM dNTPs, 1.5 mM MgCl₂, 0.2 µM of each primer, and 1 U of Taq polymerase. Because of the high content of GC nucleotides in exon 1, 5% DMSO was added. To reduce the size of amplicon spanning exon 4–5 and render it suitable for SSCP, the PCR product was digested by adding 2 U of *RsaI* enzyme to the PCR mixture and incubating at 37°C for two hours. PCR product (3 µl) was mixed with 4 µl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured, and immediately placed on ice. The mixture was run overnight at 20 mA on a 6% non-denaturing polyacrylamide gel containing 10% glycerol. The gel was fixed in a solution containing 10% ethanol and 0.5% acetic acid and stained by soaking for 15 minutes in a 0.1% solution of silver nitrate. After quick washes in distilled water, the gel was incubated in a solution of 1.5% sodium hydroxide and 0.1% formaldehyde to visualise the bands.

DIRECT SEQUENCING

For all patients with abnormal band mobility of PCR products on SSCP analysis, the nucleotide sequence was determined by direct sequencing using the same primer as used for PCR-SSCP, and the ABI Ready Reaction Dye Terminator, with products being analysed on an ABI Prism 377 semiautomated sequencer (Perkin-Elmer, Norwalk, Connecticut, USA). Sequences obtained by forward and reverse sequencing, respectively, were compared with published sequences (Accession ID, GDB:120484) using Sequencer 3.0 software. All changes were confirmed in duplicate.

GENOTYPING

Patients carrying the T340A or L599V variants and 48 Korean normal controls were genotyped at three CA repeat markers flanking *CDH1*: *D16S3107* (Accession ID, GDB:611622), *D16S398* (GDB:186392), and *D16S520* (GDB:200145). PCR reactions were carried out with fluorescent dye labelled PCR

Table 1 Summary of sequence changes in *CDH1*

Codon	Reference	Nucleotide change	Consequence	Frequency*	
				Patients (n=131)	Korean controls (n=88)
Intron 4	16, 20	gaaag→gaaac	—	4	ND
340		ACG→GCG	Thr→Ala	2	0
599		CTA→GTA	Leu→Val	4	1
Intron 12	2,20	attgc→actgc	—	5	ND
692	2,16,20–22	GCC→GCT	Ala→Ala	43	ND
751	16,17, 20	AAC→AAT	Asn→Asn	10	ND
782		GTG→GTA	Val→Val	1	0

ND, not determined

Neither alleles T340A nor L599V were found in tumour specimens of 40 non-Korean sporadic colorectal cancer patients or in the germline of 80 non-Korean controls.

*Based on a diploid chromosome number.

primers. PCR product (2 µl) was mixed with 0.5 µl of Rox350 size standard (ABI) and 1 µl of formamide loading buffer, heat denatured, and electrophoresed on a 6% acrylamide sequencing gel on an ABI Prism 377 semiautomated sequencer for 2–4 hours. Data were analysed using Genescan 2.02 software (Perkin-Elmer).

STATISTICAL ANALYSIS

Comparison of sex, age, and frequency of variants among each group was performed using Fisher's exact test, *t* test, or ANOVA using SAS software (version 6.11, SAS Institute Inc, North Carolina, USA).

Results

Among each group, there were no differences in sex and age distributions. When we compared those groups with and without a positive family history, there were no significant differences in sex or age distributions, but the group with a positive family history had a tendency for early onset tumours.

We found a number of germline changes in *CDH1* which included three novel changes, two of which were missense changes (table 1) and four were previously described polymorphisms. The three new germline changes were in exon 8 at codon 340 (ACG > GCG; Thr > Ala) (fig 1A, C), in exon 12 at codon 599 (CTA > GTA; Leu > Val) (fig 1B, D), and in exon 15 at codon 782 (GTG > GTA; Val > Val). The T340A and L599V changes were all in heterozygotes. Their frequencies in the 131 cancer patients were 0.008 and 0.015, respectively, based on a diploid chromosome number. The T340A change was present in one patient in group III (ascending colon, poorly differentiated) and in one patient in group V (sigmoid colon, moderately differentiated). The L599V change was present in one patient in group II (ascending colon, poorly differentiated), in two patients in group III (rectum and ascending colon, moderately differentiated; sigmoid colon, moderately differentiated), and in one patient in group IV (diffuse gastric, antrum). The polymorphisms described previously included: intron 4 change (gagaag > gagaac), intron 12 change (attgc > atcgc), and silent changes in exon 13 at codon 692 (GCT > GCC; Ala > Ala) and exon 14 at codon 751 (AAC > AAT; Asn > Asn).^{16–18 20 21 25} While the frequency of the t > c change in intron 12 was

Figure 1 Silver staining of polymerase chain reaction (PCR) products following electrophoresis on an acrylamide gel. The T340A (A) and L599V (B) heterozygote band shifts are arrowed. Sequence chromatograms of the single base substitutions of T340A (C) and L599V (D). The wild-type sequence is shown for comparison.

lower in Korean patients than in Western populations, the frequencies of the other polymorphisms were similar to those previously described.

We subdivided all patients into those with a family history of gastric or colorectal cancer and those without a family history, and into those ≤50 years of age and those >50. For T340A, one patient was in the ≤50 age group and had a family history and the other was in the >50 age group and had no family history. For L599V, three of four patients were in the ≤50 age group and had a family history of gastric or colorectal cancer (table 2). However, these differences did not reach statistical significance.

DNA from family members of patients with the T340A and L599V changes were examined to identify if they had the same variants. We obtained DNA from two family members of one T340A carrier proband. Both were siblings of the proband, healthy, and less than 30 years of age. Both had the same variant. In the family of one L599V carrier proband, DNA samples of six first degree relatives were obtained. One who was 45 years old and healthy had the same variant. The T340A change was not found in any controls (Korean and non-Korean) but two of 88 Korean controls had the L599V change. We could not obtain tumour samples from the patients and thus could not examine the somatic status of *CDH1*. In a collection of 40 sporadic colorectal cancers of non-Korean origin, these variants were not identified (data not shown).

Haplotype analysis using CA markers flanking *CDH1* suggested that each of these variants

Table 2 Distributions of *CDH1* variants T340A and L599V in patients and controls according to age and family history

	T340A (%)	L599V (%)
Normal controls (n=168)		
Non-Korean (n=88)	0 (0)	0 (0)
Korean (n=88)	0 (0)	2 (2.2)
Patients		
All patients with tumour (n=131)	2 (1.5)	4 (3.1)
Patients ≤50 y (n=44)	1 (4.5)	3 (6.8)
Patients >50 y (n=87)	1 (1.1)	1 (1.1)
Positive family history (n=62)	1 (1.6)	3 (4.8)
Negative family history (n=69)	1 (1.4)	1 (1.4)

may have a common origin (table 3). The possibility of a common origin was much stronger for T340A, given the frequencies of these markers in normal Koreans (table 4).

Discussion

It has been suggested that there is a familial component to gastric cancer and the increased risk of gastric cancer in HNPCC families is well known.³⁶ Recently, germline mutations of the tumour suppressor gene *CDH1* have been reported in large families with gastric cancer, all of which were consistent with the criteria applied to HNPCC, except for one family in which the pedigree was not described.^{2,3} The described gastric cancers were the diffuse-type with early onset, as expected in familial cancer syndromes.

On the premise that *CDH1* may play a role in familial gastrointestinal cancer syndrome, we searched for germline mutations in *CDH1* in several groups of Korean cancer patients and found several germline changes. Among the variants that we found in this study, there were two missense changes (T340A and L599V). There is some evidence that this type of missense variant could have mild but significant effects on protein function, leading to an increased risk of colorectal or other tumours. The role of subpolymorphic missense variants has recently received increased attention, with two missense variants (I1307K and E1307Q)

of *APC* having been described which are associated with an increased cancer risk, but not always a family history, in the carriers of these variants.^{33,37,38}

With regard to *CDH1*, expression of a truncated protein might influence cadherin mediated adhesion even before the wild-type allele is lost. Soluble fragments can disrupt cell-cell adhesion in cultured epithelial cells when added to epithelial cell monolayers.³⁹ This dominant negative action is provoked by direct involvement of this molecule in the adhesive process, allowing the cadherin fragment to compete with native molecules at the cell surface. A dominant negative effect has also been found in an N-cadherin embryonic cell adhesion model. In this model, N-cadherin with an extracellular deletion blocked cell adhesion.⁴⁰ Both variants that we have reported could produce mild changes in protein structure, in extracellular domain 2 for T340A and extracellular domain 4 for L599V of E-cadherin, both regions where homophilic adhesion interactions occur. In T340A, the uncharged polar amino acid threonine is substituted by a relatively hydrophilic non-polar amino acid, alanine. In L599V, both leucine and valine are categorised as uncharged polar amino acids. These substitutions could produce a subtle change in E-cadherin function but are perhaps unlikely to confer a significant selective advantage on the potential tumour cells. This is why they may only be of significance when they arise in the germline.

Although both missense variants described in this study encode for extracellular regions of the E-cadherin protein and may alter the ability of the protein to produce homophilic adhesion, there are no data available on the precise functional effect that these two amino acid changes will generate. In a recent review⁴ of mutations in the *CDH1* gene, only 11 missense mutations were identified (six diffuse gastric cancers, one lobular breast cancer, two en-

Table 3 Results of genotypes of T340A and L599V carriers with CA repeated markers flanking *CDH1*

Locus*	T340A			L599V				
	Case 1	Case 2	Shared	Case 3	Case 4	Case 5	Case 6	Shared
D16S3107	290/298	298/300	298	288/296	296/298	296 /	296/300	296
D16S398	174/186	176/186	186	174/186	188/198	188/200	182/198	—
<i>CDH1</i>		T340A				L599V		
D16S520	188/192	178/188	188	184/190	186/	184/186	186/194	—

*Listed centromeric (D16S3170) to telomeric (D16S520). Alleles given as sizes in bp.

Table 4 Results of genotypes of 48 normal Korean controls with CA repeated markers flanking *CDH1*

D16S3107 (n=96*)		D16S398 (n=96*)		D16S520 (n=96*)	
Size (bp)	Number (%)	Size (bp)	Number (%)	Size (bp)	Number (%)
280	1 (1.0)	174	1 (1.0)	176	5 (5.1)
282	1 (1.0)	176,178	0 (0)	178	6 (6.1)
286	5 (5.1)	180	17 (17.3)	180	7 (7.1)
288	14 (14.3)	182	12 (12.2)	182	5 (5.1)
290	11 (11.2)	184	19 (19.4)	184	20 (20.4)
292	16 (16.3)	186	33 (33.7)	186	22 (22.4)
294	14 (14.3)	188	12 (12.2)	188	15 (15.3)
296	17 (17.3)	190–194	0 (0)	190	9 (9.2)
298	13 (13.3)	196	2 (2.0)	192	6 (6.1)
300	4 (4.1)			194	1 (1.0)

*Based on a diploid chromosome number. Cases in homozygotes were counted as duplicates.

dometrial cancers, one ovarian cancer, and one papillary thyroid cancer). Interestingly, one of these missense mutations (R598Q) is next door to our L599V variant and was described in a diffuse gastric cancer as in our series (one diffuse gastric cancer patient and three colorectal cancer patients). Furthermore, a study of in vitro created missense mutations in the region of a calcium binding site abolished the adhesiveness of E-cadherin protein.⁴¹ However, it is beyond the scope of this study to examine the function of our two missense variants at the protein level.

These germline variants in every cell in the body can elevate the possibility of tumorigenesis even if each has only a relatively small selective advantage. In our study, we could not find T340A in any of the random control group but L599V was found in two of the Korean random controls. The difference between patients and controls did not, however, reach statistical significance. Before reaching a conclusion, we should consider several issues. Firstly, there are factors that could have led to bias, such as not including enough information about patient history, unknown cause of death of family members, asymptomatic disease, and lack of endoscopic screening. Secondly, the scale of the study was quite small for population genetics. Given a low incidence and imperfect penetrance of the alleles, the probability of several carriers of the variant all having gastrointestinal carcinomas will be small. Low penetrant alleles have no or a mild selective advantage and random genetic drift can allow such variants to increase by chance, especially in one population group relative to another.

We did not find any of the two missense variants in non-Korean cancer patients and controls. It is also notable that four of six of the variants were in young onset cancer cases. It will be necessary to perform long term follow up on the relatives of these cases. In the future, large population studies which include cancer patients (particularly with gastrointestinal tumours) and a large number of controls will be required to confirm whether these variants increase the risk of cancer in Korean and other ethnic groups. Other issues to address include examining the somatic status of *CDH1* in tumour specimens of cancer patients who have these variants and performing functional studies of these variants. Without further work we cannot dismiss these variants as being innocuous.

It has recently been reported that *CDH1* mutations may predispose to both familial gastric cancer and colorectal cancer,⁴² with novel truncating mutations in *CDH1* being described in patients with gastric cancer and one patient with early onset colorectal cancer. Others have failed to detect *CDH1* mutations in familial colorectal cancer (HNPCC was excluded), and feel that *CDH1* is unlikely to be a common "low penetrance" gene for colorectal cancer.⁴³ Both of these studies involved small number of families.

We did not find definite inactivating mutations in *CDH1*. This failure may be attributable

to the small numbers in each group and also to the relatively generous criteria used to select the cases. If similarly strict criteria for HNPCC had been applied or the spectrum of cases had been narrowed to those with early onset and diffuse-type gastric cancer, the possibility of finding mutations may have been increased.

In conclusion, we have described novel germline missense variants in *CDH1* in several groups of Korean gastrointestinal cancer patients. The existence of subpolymorphic variants that have a small but significant influence in increasing the risk of cancers could explain a group of gastrointestinal cancers that are considered to have a genetic background but which is not clearly defined. Common "polymorphisms" in other cancer predisposing genes have been noted and some of these polymorphisms may not be innocent. Identification of such a mechanism could offer a better understanding of the development of malignancy.

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