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GASTRIC CANCER

Mechanisms inactivating the gene for E-cadherin in sporadic gastric carcinomas

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Abstract

AIM: To study the role of *CDH1/E-cadherin* (E-cad) gene alteration profiles including mutation, loss of heterozygosity (LOH), promoter polymorphism and hypermethylation in mechanisms of *CDH1* inactivation in gastric carcinoma (GC).

METHODS: Specimens were collected surgically from 70 patients with GC. Allelotyping PCR and detection of LOH, denaturing high pressure liquid chromatography and DNA sequencing, restriction fragment length polymorphism analysis, methylation specific PCR, and immunohistochemical staining were used.

RESULTS: Promoter polymorphism was not a major mechanism of E-cad inactivation. Only one truncating mutation was found in a diffuse type tumor (3%). Both LOH and promoter hypermethylation were major mechanisms of E-cad inactivation, but interestingly, there was a negative association between the fraction of allelic loss (LOH) in tumors and hypermethylation of *CDH1*. Therefore LOH and hypermethylation were two different tumorigenic pathways involved in GC.

CONCLUSION: Given the findings that somatic mutation was extremely low and the relationship between LOH and hypermethylation was inverse, any two combinations of these three factors cannot fulfill the classical two-hit hypothesis of *CDH1* inactivation. Thus, other mechanisms operating at the transcriptional level or at the post-translational level might be required to induce E-cadherin inactivation.

Key words: Gastric carcinoma; E-cadherin gene alteration profiles; Inactivation of E-cadherin

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INTRODUCTION

CDH1/E-cadherin (E-cad) is a member of the family of transmembrane glycoproteins expressed on epithelial cells and is responsible for calcium-dependent cell-to-cell adhesion[1]. E-cad forms complexes and connects actin filaments with α-, β-, and γ-catenins[2,3], which are essential to neoplastic transformation and metastasis[4, 5]. Loss of cell adhesion may contribute to loss of contact inhibition of growth, which is an early step in the neoplastic process. Furthermore, loss of cadherin activity may result in cancer cell detachment and metastasis[6, 7].

Gastric carcinogenesis is a multi-step process with morphological progression involving multiple genetic and epigenetic events. E-cad gene (*CDH1)* is an important putative tumor suppressor gene. In gastric carcinomas (GCs), the reduction in E-cad expression activation of *E-cad* gene varies from 17% to 92%, and is more frequent in diffuse type than in intestinal type tumors[8-13]. Germline mutation of the *CDH1* gene is found in all familial GCs[14, 15]. Somatic mutations of *CDH1* are found in more than

50% of diffuse type GCs but are not found in intestinal type GCs in Caucasians and Japanese populations[16-19]. The rate of loss of heterozygosity (LOH) ranges from 2.8% to 60% in diffuse and intestinal type tumors[16-20]. In addition to the well-known 'two-hit'inactivation mechanism proposed by Knudson (1971), *CDH1* can be silenced in GC by epigenetic promoter hypermethylation[17, 21]. Besides, Li *et al*[22] reported that the-60C/A polymorphism has a direct effect on the transcriptional regulation of *CDH1*. All above previous studies of the inactivation of this gene in patients with GC have been limited in their analyses. In this study, we investigated a range of alterations in *CDH1* expression profiles, including genetic mutations, LOH, promoter polymorphism, promoter hypermethylation, and immunohistochemical stain of E-cad protein together to determine possible genetic and epigenetic mechanisms of *CDH1* inactivation.

MATERIALS AND METHODS

Patients and samples

Specimens were collected surgically from 70 Taiwanese patients with GC between July 1999 and July 2002 at the Division of General Surgery, Department of Surgery, Tri-Service General Hospital, Taipei, Taiwan. None of the subjects received preoperative anticancer therapy. Clinical information was obtained from medical records. Samples were taken from representative cancerous lesions and the adjacent non-cancerous epithelial parts of the tissues were flash frozen in liquid nitrogen and stored at -80℃. All tumor DNA samples were obtained by micro-dissection from 5-μm thick hematoxylin and eosin stained and paraffin embedded tissue sections[23]. Non-cancerous DNA was extracted from tissues which were flash-frozen in liquid nitrogen and stored at -80℃. All 70 samples were classified according to the Lauren's criteria[23]: 27 were intestinal and 43 were diffuse types. The tumors were staged at the time of surgery using the standard criteria by TNM staging, with the unified international gastric cancer staging classification[24].

Allelotyping PCR and detection of allelic loss or loss of heterozygosity (LOH) of CDH1

DNA samples from tumor and normal mucosal specimens were used for allelotyping PCR with fluorescent primers (markers). Three micro-satellite markers (D16S3043, D16S3050, and D16S3021) at 16q22.1 were used to detect LOH at the CDH1 locus. PCR amplification was carried out as previously described[26]. PCR products were separated electrophoretically on an ABI PRISM 377 DNA sequencer, and fluorescent signals from the differently sized alleles were recorded and analyzed using Genotyper version 2.1 and GeneScan version 3.1 software packages. A given informative marker was considered to display LOH when a threefold or greater difference was seen in the relative allele intensities of the tumor and normal DNA samples.

Denaturing high pressure liquid chromatography (DHPLC) analysis and DNA sequencing for CDH1 mutation analysis

We used DHPLC and direct sequencing to determine inactivating mutations responsible for the loss of *CDH1* expression. The promoter region and 16 exons including the exon-intron boundaries were analyzed using the previously described protocol and primer pairs[26]. The optimal conditions for DHPLC analysis of each amplicon were available as requested. All variants detected by DHPLC were re-amplified and the site of variation was identified by direct DNA sequencing using an ABI PRISM 377 DNA sequencer.

Restriction-fragment length polymorphism (RFLP) analysis to identify nucleotide changes at –160 of the CDH1 promoter

The -160 polymorphic site contained either a C or A residue. The tumor type was determined by *Bst*EII digestion of the PCR products amplified using the primer set 5′-TGATCCCAGGTCTTAGTGAG-3′ (upstream) and 5′-AGTCTGAACTGACTT CCGCA-3′ (downstream). The 318-bp PCR product was cut into two fragments (208 and 110 bp) if it contained the A residue. To ensure that the observed polymorphism was specific and not an experimental artifact, the results were confirmed by direct DNA sequencing.

Methylation-specific PCR (MSP) and bisulfite-modified genomic sequencing to detect promoter hypermethylation of CDH1

Genomic DNA was modified by bisulfite treatment, converting unmethylated cytosines to uracils and leaving methylated cytosines unchanged. MSP was performed on the treated DNA to detect all three CpG islands in the *CDH1* promoter region as previously described[27]. Each unmethylated–methylated primer pair set was engineered to assess the methylation status of 4-6 CpGs with at least one CpG dinucleotide positioned at the 3′ end of each primer to discriminate between methylated and unmethylated alleles following bisulfite modification. Hs578t cells, which contain a heterogeneously methylated CpG island 1 and methylated CpG islands 2 and 3, served as the positive control, and MCF7 cells were used as the negative control.

Immunohistochemical staining and evaluation of E-cad expression

Sections (5 μm thick) were treated with monoclonal anti-E-cad antibody (Cappel, Aurora, OH, USA), then with secondary antibody. The signal was detected using a kit containing avidin–biotin complex and diaminobenzidine (DAB; Vector Laboratories, Burlingame, CA, USA). DAB produced a yellowish brown staining if the sample was positive. If more than 90% of the tumor cells exhibited intense membranous staining similar to that of normal cells, the result was considered positive (++). If the staining intensity was demonstrably reduced relative to that of normal cells and/or the staining pattern was heterogeneous (10%-90% positive), the result was deemed to be weakly positive (+). If IHC expression was completely lost or positive in less than 10% of cells, the result was defined as negative (–).

Statistical analysis

Analyses were performed using S-Plus® 2000 for

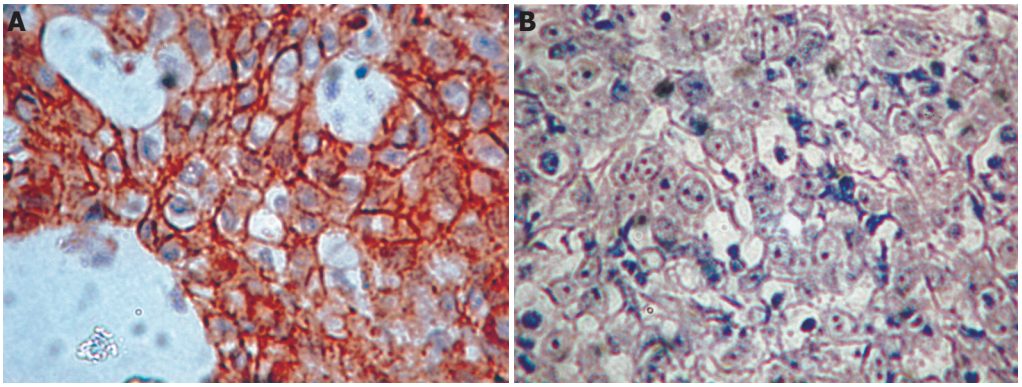

Figure 1 Immunohistochemical staining for positive (**A**) and negative (**B**) E-cad expression in diffuse type tumor.

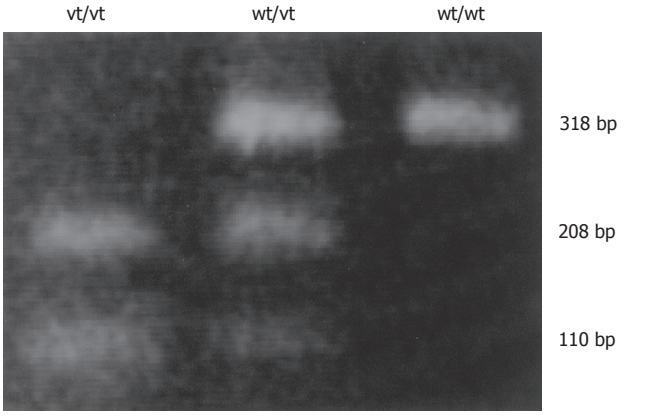

Figure 2 PCR-restriction fragment length polymorphism (RFLP) analysis of genetic polymorphism of the -160 site of the *E-cad* promoter. The C/A polymorphism was differentiated by *BstEII* digestion of PCR products homozygous for the wild-type (high-activity) allele (*wt/wt*, CC gentoype), heterozygous for the variant (low-activity) allele (*wt/vt*, CA genotype), and homozygous for the low-activity allele (*vt/vt*, AA genotype)

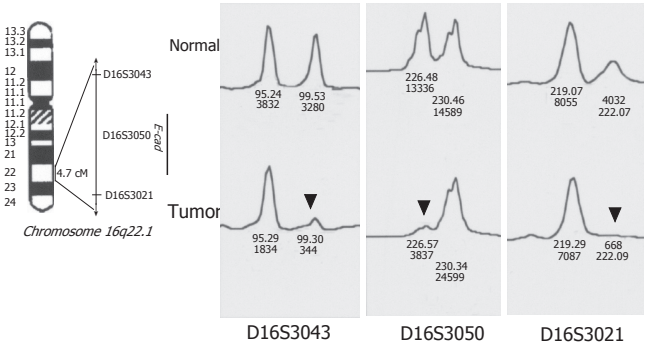

Figure 3 Allelic loss or loss of heterozygosity (LOH) of *CDH1*/*E-cad*. Left panel: *E-cad* detected by allelic loss or loss of heterozygosity (LOH) of the *E-cad* locus, reflected by three microsatellite markers (*D16S3043*, *D16SS3050* and *D16S3021*) at 16q22.1. *Right panel*: LOH in a representative GC. The locius of markers *D16S3043*, *D16S3050*, and *D16S3021* were considered to be informative when they were heterozygous in normal tissue (i.e. two alleles were seen), and showed LOH when a 3-fold or greater difference was seen in the relative allele intensity ratio between the tumor and normal DNA (arrow).

Windows statistical software (CANdiensten, Amsterdam, Netherlands). Significance was assumed at $P<0.05$ for all tests. Categorical variables were tested using Fisher's exact test.

RESULTS

Of the 70 patients, 52 were men and 18 were women. Their median age was 69.7 years (range 32-88 years). According to Lauren's classification, 27 and 43 tumors were intestinal and diffuse histotypes, respectively. Reduced gene expression was more frequent in diffuse type tumors (38/43, 88%) than in intestinal type tumors (13/27, 48%; $P=0.006$). Representative examples of immunohistochemical staining for E-cad expression in diffuse type tumors are shown in Figure 1.

Promoter polymorphism

Three of the 70 patients were omitted from our analysis of the -160C/A polymorphism due to insufficient samples. Among the other 67 patients, 29 were genotype C/C (43%), 24 were genotype A/C (36%) and 14 were genotype A/A (21%) (Figure 2). There was no significant difference in the frequency of the C/A + A/A genotypes between diffuse and intestinal type tumors (27/42, 64% *vs* 11/25, 44%). There was no significant difference in LOH between the C/C and C/A + A/A genotypes (10/25, 40% *vs* 13/33, 39%). There was also no significant difference in hypermethylation between C/C and C/A + A/A genotypes (20/29, 69% *vs* 24/37, 65%). There was no significant difference in the frequency of the C/A + A/A genotypes between tissues with reduced and normal E-cad expression (12/17, 71% *vs* 27/50, 54%).

Loss of heterozygosity

To detect allelic loss at *CDH1*, three micro-satellite markers (D16S3043, D16S3050, D16S3021) at 16q22.1 were used (Figure 3). The allelic status of this gene was reflected well by these three markers, because its locus was very close to the loci of these markers (LOD score > 4 estimated by linkage analysis). We considered the results for all three markers together and found heterozygosity in at least one. Of the 70 samples collected, 10 were omitted from the analysis or homozygous and could not be detected. A high frequency of allelic loss at *CDH1* was detected (23/60, 38%). The frequency of LOH at *CDH1* was similar between diffuse type tumors (15/38, 39%) and intestinal type tumors (8/22, 36%). Reduced E-cad expression was more frequent in LOH-positive tumors (21/23, 91%) than in LOH-negative tumors (24/37, 65%; $P=0.03$).

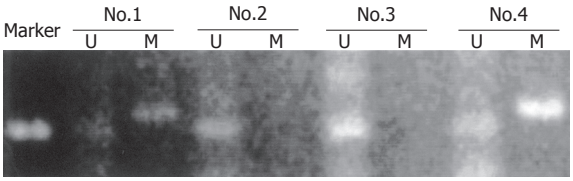

Figure 4 Promoter hypermethylation of the *CDH1/E-cad* detected by methylation-specific PCR (MSP). The presence of a visible PCR product in the lanes marked U indicates the presence of an unmethylated allele, while the presence of the product in the lanes marked M indicates the presence of a methylated allele. The intensity of each methylated band was further semi-quantitated, and as shown in the figure, cases 1 and 4 were defined as "hypermethylation"with "+"and "++", respectively, and cases 2 and 3 were defined as "unmethylation".

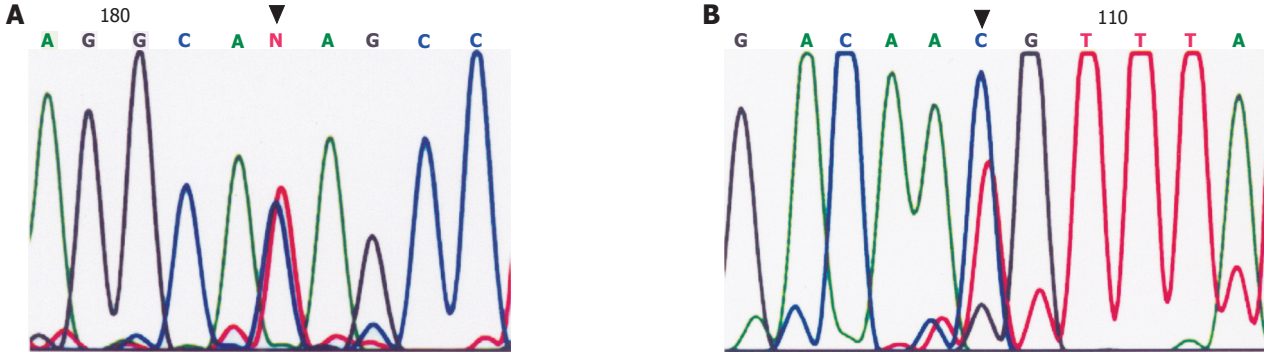

Figure 5 *CDH1*/E-cad mutation and polymorphism detected by direct DNA sequencing. Two tumors subjected to DNA sequencing were found to harbor C-to-T transversion in exon 13 (**A**), resulting in a truncated mutation (Gln to stop condon TAG) and C-to-T transversion in exon 14 (**B**), resulting in no amino acid change, which was considered to be polymorphism.

Promoter hypermethylation

The degree of hypermethylation estimated by MSP was defined as strongly detectable (+++, ++), detectable (+), or not detectable (–)(Figure 4). Three of the 70 samples were omitted from our analysis of hypermethylation due to insufficient samples. The *CDH1* promoter was hypermethylated in 45 of these 67 GCs (67%). Hypermethylation was more frequent in diffuse type tumors (31/41, 76%) than in intestinal type tumors (13/26, 50%; $P=0.03$ by Fisher's exact test). Furthermore, hypermethylation was more frequent in GCs with reduced E-cad expression than in those with normal levels (37/45, 82% *vs* 12/22, 55%; $P=0.02$). The fraction of allelic loss (FAL) of *CDH1*, calculated as the frequency of LOH at *CDH1* locus, was generally inverse to the degree of hypermethylation (Tables 1, 2).

Mutation

In these 70 patients, five diffuse type tumors (Case No. 15, 24, 29, 30, and 39) had a single-nucleotide polymorphism (SNP) at amino acid 692, and four diffuse type tumors (Case No. 35, 40, 59, and 63) had an SNP at position 755 . Case No.15 had a truncated mutation at position 699 (Figure 5). No *CDH1* mutation was found in intestinal type tumors.

DISCUSSION

In this study, 27 and 43 tumors were of the intestinal and diffuse histotypes, respectively. Inactivation of the *CDH1* gene and loss of normal E-cad expression were involved more frequently in diffuse type than in intestinal type tumors (88% *vs* 48%; $P=0.006$). However, the percentage of reduction in E-cad expression of GC varies from 17% to 92% in previous reports[8-13]

Promoter polymorphism

Li *et al*[22] reported that the A allele of the -160C/A promoter polymorphism alters transcriptional binding, resulting in a reduction in transcriptional efficiency of 68% relative to that of the C allele. In our study, there was no significant difference in the frequency of the C/A + A/A genotypes between diffuse and intestinal type tumors. There was no significant difference in LOH and hypermethylation between the C/C and C/A + A/A genotypes. There was also no significant difference in the frequency of the C/A + A/A genotypes between tumors with reduced and normal E-cad expression, suggesting that the A allele does not play a major role in the inactivation of *CDH1* and can not serve as the 'second hit'.

Mutations

Somatic mutations of *CDH1* are found in more than 50% of diffuse type GCs but not in intestinal type GCs in Caucasian and Japanese populations[16-19]. A review by Berx *et al*[28] noted that the predominant defects in diffuse type tumors are splice mutations causing skipping in exon 8 or 9, which account for in-frame deletions, whereas mis-sense and truncating mutations are less frequent in diffuse GCs. Moreover intragenic polymorphisms arise from changes in the third (wobble) position of the respective codons and are more frequent in codons 692 and 751. In the present study, five of the diffuse type tumors had a codon 692 polymorphism and four diffuse type tumors had a codon 755 polymorphism. Only one of 38 diffuse type tumors had a truncated codon 699 mutation. Because consistent findings have been obtained by repeated detection of the same specimens, we considered this finding to be valid. Therefore, this low rate of *CDH1* mutation in the Taiwanese GCs may suggest different tumorigenic mechanisms to inactivate this gene.

Table 1 Fraction of allelic loss (FAL) in tumors with different hypermethylation statuse

Promoter hypermethylation	FAL	
Yes (++,+++)	0.098	
Yes (+)	0.214	
No	0.377	$P=0.03$

FAL was estimated by allelic status at D16S3043, D16SS3050 and D16S3021. FAL (fraction of allelic loss) = number of loci showing LOH / number of informative loci in each tumor. The FALs of tumors with different hypermethylation status were calculated as the mean of FALs of individual tumors with the same hypermethylation status.

Table 2 Association between loss of heterozygosity and promoter hypermethylation of *CDH1*

	Loss of heterozygosity		
	Yes (%)	*n* (%)	Promoter
hypermethylation			
Yes (++,+++)	1 (9.1)	17 (34.7)	
Yes (+)	2 (18.2)	21 (42.9)	
No	8 (72.7)	11 (22.5)	$P=0.001$

LOH status is defined by D16S3043

Loss of heterozygosity

It was reported that the rate of LOH ranges from 2.8% to 60% in diffuse and intestinal type tumors [16-20]. A high frequency (38%) of allelic loss at *CDH1* was identified in our study. The frequency of LOH was similar between the diffuse and intestinal type tumors (39% *vs* 36%). Reduced E-cad expression demonstrated by immunohistochemical analysis was more frequent in LOH-positive tumors than in LOH-negative tumors (91% *vs* 65%; $P=0.03$), suggesting that LOH is a major mechanism for the inactivation of *CDH1*.

Promoter hypermethylation

Tamura *et al*[29] and Graziano *et al*[30] indicated that *CDH1* promoter methylation may play a major role together with mutations or deletions, in causing the inactivation of the *CDH1* gene in GCs, especially in diffuse type tumors. They also reported that *CDH1* promoter hypermethylation is associated with reduced E-cad expression detected immunohistochemically. In the present study, the *CDH1* promoter was hypermethylated in 67% of GCs. Hypermethylation was more frequent in diffuse type tumors than in intestinal type tumors ($P=0.03$). Furthermore, hypermethylation was more frequent in tumors with reduced E-cad expression than in normal E-cad expression (82% *vs* 55%; $P=0.02$), suggesting that *CDH1* promoter hypermethylation is a major mechanism for gene inactivation.

Methylation of the *CDH1* promoter has been documented as the 'second hit' responsible for the development of hereditary diffuse GCs[31] and sporadic diffuse GCs[17] among Caucasians. Because there was only one genetic mutation in diffuse type tumors and no mutation in intestinal type tumors in this series, we examined the hypermethylated status of tumors with or without LOH at the *CDH1* locus. We investigated the relationship between hypermethylation and FAL, which was estimated from the allelic status at D16S3043, D16S3050, and D16S3021. Hypermethylated tumors tended to have significantly lower FAL values (Table 1). This is contrary to the result predicted by the two-hit hypothesis. Further examination using individual markers to redefine the LOH status of tumors yielded similar results (Table 2). Therefore, cancers having lost one *CDH1* allele and those carrying hypermethylated *CDH1* alleles may be involved in two different tumorigenic pathways. Because the somatic mutation rate is extremely low, any two combination of these three factors cannot fulfill the classic 'two-hit' hypothesis. Other molecules involved in the E-cad-mediated cell-cell adhesion complex, such as the intracellular attachment proteins α, β, and γ-catenin, may be subjected to targeted inactivation[32-36]. Receptor tyrosine kinase (RTK), the main positive regulator of progression and tissue expansion, can repress E-cad function by transcriptional repression of *CDH1* via the transcription factor SNAI1[37,38], posttranscriptional repression via direct or indirect phosphorylation of adheren junction components such as β-catenin[39], or RTK-associated endocytosis and degradation of the E-cad protein[40]. This more flexible status achieved either by retaining an intact allele subsequent to LOH or by regulation via epigenetic mechanisms operating at the transcriptional or posttranslational levels, could provide an advantage in counteracting the changing microenvironment during tumor progression. Further investigation is needed at the transcriptional level and the post-translational level into E-cad inactivation of GC.

In conclusion, given the finding that somatic mutation was extremely low and the relationship between LOH and hypermethylation was inverse, any two combinations of these three factors can not fulfill the classical two-hit hypothesis of E-cadherin inactivation. Thus, other mechanisms operating at the transcriptional level or at the post-translational level, might be required to inactivate E-cadherin in GC.

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