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E-cadherin Methylation and Protein Decrease are Related to Infiltrative Growth and Peritoneal Dissemination in Gastric Cancer

Kazushige ITO, Kazuhiko KASUYA, Tatsuya AOKI, Yu TAKAGI, So KATAYANAGI, Akihiko TSUCHIDA and Yasuhisa KOYANAGI

Department of Surgery, Tokyo Medical University, Tokyo, Japan

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Summary. **Background: The first step of cancer metastasis requires an abnormality of adhesive factors, especially E-cadherin. The causes of abnormal E-cadherin expression consist of gene mutation and methylation of its promoter. An abnormality of E-cadherin protein expression has been frequently found in infiltratively growing tumors such as breast cancers rather than in highly differentiated tumors such as colorectal cancers. However, there have been few reports about abnormal E-cadherin methylation in gastric cancer. In the present study, we examined the relationships between the abnormal expression of E-cadherin, methylation of E-cadherin, abnormal expression of α-catenin and pathological prognostic factors, tumor growth type, and distant metastasis.**

Materials and Methods: We evaluated the expression of E-cadherin andα-catenin immunohistochemically and the mutational status of E-cadherin methylation in 38 patients with gastric cancer.

Results: Abnormal expressions of E-cadherin and α-catenin were observed in 20 cases (52.6%) and 11 cases (28.9%), respectively. The E-cadherin expression was significantly correlated with tumor growth type, histological type, depth of invasion and size of tumor. Fifteen cases were positive for E-cadherin methylation (39.5%), which was seen in cases with dissemination or tumors localized in the upper stomach (in particular the fundic gland zone).

Conclusion: A decrease of either E-cadherin expression or methylation has is closely related to infiltrative tumor growth and dissemination.

Key words—E-cadherin, methylation, α-catenin, gastric cancer.

INTRODUCTION

The first step of cancer metastasis requires an abnormality of adhesive factors, such as E-cadherin or catenin, in the primary tumor[1]. In particular, E-cadherin plays an important role in not only an adhesive function but also the invasion of gastric cancer[2]. Causes of abnormal E-cadherin expression include gene mutation, loss of heterogeneity, abnormal transcription, or methylation of the promoter domain regulating the gene. Methylation is a reversible function that controls gene expressions for each step of maturation and the growth of organisms[3,4], which the demethylated gene initially expresses, followed by methylation causing inactivation of the gene expression. Yoshiura et al[5]. treated E-cadherin-negative carcinoma cells with the demethylating agent 5-azacytidine, resulting in a reexpression of the gene and reversion of scattered spindle shaped cells to cells with an epithelial morphology.

An other important factor for adhesion is that each cell is connected by E-cadherin in a homophilic manner with the support of various proteins such as α-catenin which combine actin proteins in the cytoskeleton, and the α-catenin protein which controls adhesion between β-catenin and E-cadherin[6]. Abnormalities of E-cadherin protein expression have been frequently found in infiltrating tumors such as breast cancers rather than in highly differentiated tumors such as colorectal cancers[2]. In addition, in highly differentiated gastric cancer, E-cadherin expression is normally maintained, but undifferentiated gastric

Correspondence: Kazushige Ito, MD, Department of Surgery, 6-7-1 Nishi-Shinjuku, Shinjuku-ku, Tokyo 160-0023, Japan.

Abbreviations—M or M-band, Methylated specific band; U or U-band, Unmethylated specific band.

cancers have shown a high incidence of the absence or loss of E-cadherin[7-9]. However, there are only a few reports about abnormality of E-cadherin, including methylation in gastric cancer. In the present study, we examined the relation between adhesive molecules such as E-cadherin, E-cadherin methylation, or α-catenin and pathological prognostic factors, tumor growth type, and distant metastasis.

MATERIALS AND METHODS

Thirty-eight gastric cancers resected in the Department of Surgery, Tokyo Medical University Hospital from June 1997 to August 1999 were used. They were obtained from 26 men and 12 women with a mean age of 64.6 years (range: 39–80). Resected specimens were fixed in formalin followed by measurement of tumor diameter. Cancers were macroscopically divided into an expansive growth type (n=16) or infiltrative growth type (n=22), and were histologically classified into 17 differentiated type cases (well and moderately tubular adenocarcinoma, and papillary adenocarcinoma) and 21 undifferentiated type cases (solid and non-solid poorly differentiated adenocarcinoma, signet-ring cell carcinoma and mucinous adenocarcinoma) according to the Japanese Research Society for Gastric Cancer[10]. Primary lesions were located in the upper third region (n=10), middle third region (n=13), lower third region (n=13) and the entire stomach (n=2). Depth of invasion[10] was T1 (submucosa) in 3 cases, T2 in 15 (2 muscularis propria, 13 subserosa), T3 (penetration of the serosa) in 16, and T4 (invasion of adjacent structures) in 4. Lymph node metastasis was found in 30 cases (78.9%). Peritoneal recurrence occurred in 9 cases and liver metastasis in 5 cases during the follow-up period from June 1997 to March 2000.

Immunostaining of E-cadherin and α-catenin

Surgical materials including cancer tissues without the necrotic sites were immediately fixed in cold acetone. Primary antibodies used were: anti-human E-cadherin mouse monoclonal antibody HECD-1 (Takara Shuzo Co. Ltd., Otsu, Japan) and anti-α-catenin antibody (Transduction Laboratories, Lexington, KY, USA). Staining was carried out using the avidin-biotinylated peroxidase complex (ABC) method. According to the immunohistochemical results reported by Akimoto et al.,[11] our criteria for E-cadherin and α-catenin expression were defined as follows: the expressions were judged normal when more than 80 % of the cancer cells were stained and as abnormal when fewer than 80 % of cancer cells were stained.

Methylation specific polymerase chain reaction (PCR)

DNA was extracted from the frozen specimens of tumors using Sepa Gene (Sanko Junyaku Co. Ltd., Tokyo, Japan). Before extraction, DNA was modified using a CpG E-cadherin Modification Kit (Intergen Co., New York, NY, USA) followed by methylation-specific PCR using either the CpG E-cadherin Amplification Kit (Intergen) or a Premix Taq Kit (Takara). We used a template DNA of 10 ng and 4 primers as follows.
methylated specific primer:
sense primer; 5'-TTAGGTTAGAGGTTATCGCGT-3'
anti-sense primer; 5'-TAACTAAAAATTCACCTA CCGAC-3'
unmethylated specific primer:
sense primer; 5'-TAATTTTAGGTTAGAGGGTTA TTGT-3'
anti-sense primer; 5'-CACAACCAATCAACAACAC A-3'
PCR was carried out using a thermal cycler (DNA Engine, PTC200; MJ Research Inc., Watertown, MA, USA), and the denaturing was conducted at 95℃ for 12 min., followed by 95℃ for 45 sec., 60℃ for 45 sec., and 72℃ for 60 sec., performing 40 cycles using the hot start method. PCR products were applied to 2% agarose gel for electrophoresis, then stained by ethidium bromide. Methylation was judged as positive when the methylated specific band showed either the same or stronger staining than the unmethylated specific band. It was judged as negative when the methylated specific band was clearly weaker than the unmethylated one (Fig. 1).

Statistical analysis

Results were compared between the groups using the chi-squared test, Fisher's exact probability test, and the Mann-Whitney U test. A p value of less than 0.05 was considered to indicate a statistically significant difference.

RESULTS

Immunostaining of E-cadherin expression was shown at the cell membrane in either epithelial cells or cancer cells. In addition, the cells with invasion into the stroma (Fig. 2a) and with construction of clusters in the vessel or lymphatic duct showed a normal

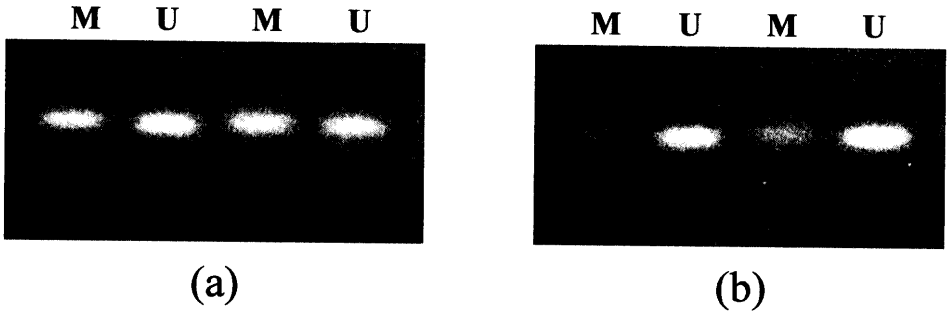

Fig. 1. Methylation specific PCR analysis. M (band): Methylated specific band. U (band): Unmethylated specific band. Cells were judged as methylation positive when the intensity of the M-band was of an intensity equal or more than that of the U-band (**a**), and as unmethylated when the intensity of the M-band was less than that of the U-band (**b**).

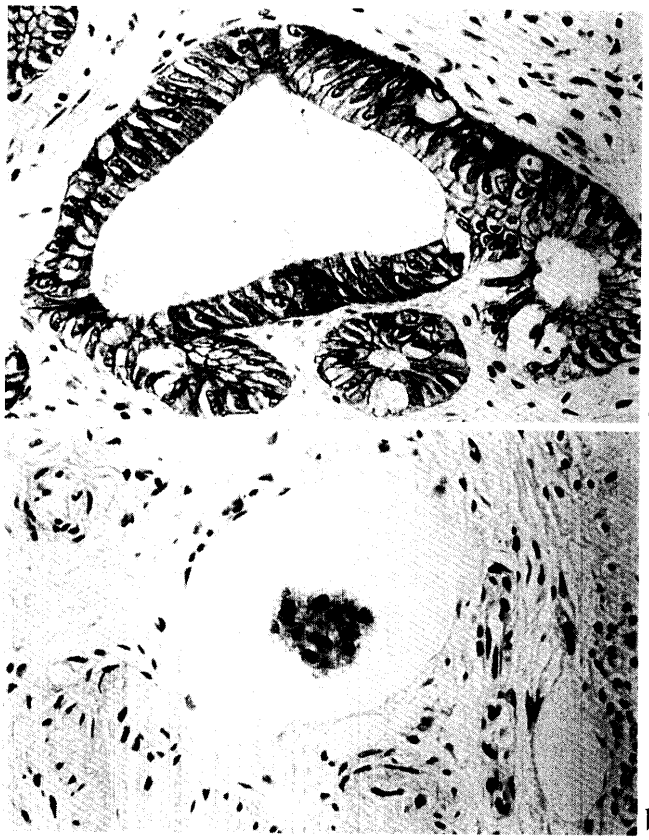

Fig. 2. **a.** Membrane of those cells are diffusely immunoreactive (expressed) for E-cadherin staining. This case is judged as normal E-cadherin expression (×400) **b.** The cytoplasm of cancer cells constructing a cluster in the vessel or lymphatic duct is also diffusely immunoreactive for E-cadherin expression, while the membrane of cancer cells is obscurely stained. (×400)

expression of the cell membrane, while the membrane of cancer cells was obscurely stained. (Fig. 2b)

Abnormal staining of E-cadherin or α-catenin was shown in 20 (52.6%) or 11 (28.9%) out of 38 cases, respectively. However, all three cases with early cancer exhibited a normal expression. Methylation of E-cadherin was positive in 15 (39.5%) of 38 cases, whereas all negative cases showed a very weak intensity of the methylation specific band. All of 15 methylation positive cases were advanced gastric cancers, while all three early cases were methylation negative (Table 1). There was a significant correlation between E-cadherin expression and α-catenin expression ($p=0.0037$); however, there was no correlation between E-cadherin expression and E-cadherin methylation ($p=0.1983$) (Table 2).

Abnormal expression of E-cadherin was significantly elevated in cases with infiltrative growth ($p=0.0076$), undifferentiated type ($p=0.021$), deeper invasion ($p=0.0084$), larger tumor diameter ($p=0.0083$) and peritoneal dissemination ($p=0.0206$). However, there was no significant correlation between the abnormal expression of E-cadherin and lymph node metastasis, liver metastasis, and tumor location. There was no correlation between the abnormal expression of α-catenin and any factors except for a deeper invasion ($p=0.0037$). There was a significant correlation significantly elevated between E-cadherin methylation positive status in tumors located in the upper portion of the stomach ($p=0.0287$), infiltrating growth type ($p=0.0435$) and peritoneal dissemination ($p=0.0159$), while there was no correlation with other factors (Table 3).

DISCUSSION

Carcinomas usually change their histological features and infiltrative morphology according to their growth and proliferation, particularly due to changes in adhesive activity among tumor cells. In general, differentiated cancers have strong cell adhesion, resulting in a low tendency towards infiltration. E-cadherin expression in these cases is similar to that in normal cells. E-cadherin abnormality plays an important role in cell adhesion, especially in undifferentiated or infiltrative growing gastric cancers[12-14]. E-cadherin is a cell-cell adhesion molecule connecting in a homophilic manner. An abnormality of the cell-cell adhesion system can cause the migration of cancer cells from the primary tumor.

We investigated whether abnormally expressed E-cadherin protein increased in large or deeply invading tumors. As advanced gastric cancer is generally accompanied by a reduction of the E-cadherin protein, the invading cancer cells tend to indicate an infiltrative feature. Shino et al.[7] reported that an abnormal expression of E-cadherin protein was shown in 32.2% of gastric cancers in early stages, which was lower than our result of 52.6%. Mayer et al[14]., reporting only advanced gastric cancer with lymph node metastasis or distant metastasis in the liver or omentum, described most of the investigated primary gastric carcinomas (92%) exhibited a reduced E-cadherin expression. These results suggest that the incidence of abnormal E-cadherin increases in relation to the rate of advanced cancer.

We performed methylation specific PCR to detect E-cadherin methylation. Our series revealed methylation-positive cases in 39.5% (15/38), which was lower than the results from Machado et al[16]. with 47.5%, and Tamura et al[17]. with 69%, possibly due to differences in evaluatory methods. Although very weak methylation specific bands were detected in our 38 cases, including the three early gastric cancers or adjacent normal mucosa of these cases, it was insufficient to judge them as positive. Suzuki et al[18]. also suggested that normal mucosa had methylation of E-cadherin. Gene methylation was found not only in tumor cells but in normal cells by Hirohashi[19], which was consistent with our results. In contrast, Kanai et al[19]. supposed that methylation was shown in chronic hepatitis as a precancerous lesion. Laird et al[21]. also pointed out that DNA methylation have brought a greater awareness of the role in epigenetics to overlook alternative or complementary mechanisms for mutational events in oncogenesis. Consequently, we speculated that methylation was already present in normal mucosa and does not always arise in advance of neoplastic change. No cases showed methylated specific bands in the same studies of Machado et al[16]. and Tamura et al[17].

We confirmed that the cancer lesion located in the upper or the entire stomach contained many cells with methylated E-cadherin, compared with those of the lower stomach. It is well known that gastric cancer generated from the fundic gland zone assumes an infiltrative form. These findings suggest that the cancer cells generated from the upper part of the stomach (the fundic gland zone) tend to be accompanied by methylated E-cadherin and to be infiltrative.

Regarding peritoneal dissemination, the adhesion of the cancer cells disappeared and E-cadherin expression of cell in pleural effusion or ascites decreased[22]. Yonemura et al[9]. have also reported that peritoneal dissemination results from a transformation of cancer cells into isolated single cells with an

Table 1. E-cadherin, α-catenin, mutation and E-cadherin methylation status in a series of 38 cases of gastric cancers

Case	Stage	E-cadherin		α-catenin
		Protein expression**	Methylation***	Protein expression**
1	E	−	−	−
2	E	−	−	−
3	E	−	−	−
4	A	−	+	−
5	A	−	−	−
6	A	−	−	−
7	A	−	+	−
8	A	−	−	−
9	A	−	−	−
10	A	−	−	−
11	A	+	+	+
12	A	−	+	−
13	A	+	+	+
14	A	+	+	−
15	A	+	−	−
16	A	−	−	+
17	A	+	−	+
18	A	−	+	−
19	A	+	−	+
20	A	+	+	−
21	A	+	+	−
22	A	+	−	+
23	A	−	−	−
24	A	+	+	+
25	A	+	+	−
26	A	+	+	−
27	A	−	−	−
28	A	+	+	+
29	A	+	+	+
30	A	−	−	−
31	A	−	+	−
32	A	+	−	−
33	A	+	−	−
34	A	+	−	+
35	A	+	−	+
36	A	−	−	−
37	A	+	−	−
38	A	+	−	−

*A, advanced; E, early; **+, abnormal (loss or decrease) expression of E-cadherin or α-catenin protein; −, normal expression of E-cadherin or α-catenin protein; ***+, methylated E-cadherin gene; −, unmethylated E-cadherin gene.

abnormal expression of E-cadherin. Our results support their opinion on peritoneal dissemination being caused by isolated cancer cells with inactivated E-cadherin. On the other hand, there was no correlation between invasion of the lymph vessel or vein and abnormal E-cadherin, no correlation with liver metastasis or lymph node metastasis. Cells must be non-adhesive to separate from the main tumor, and they

Table 2. Correlation between E-cadherin protein expression and α-catenin protein expression, E-cadherin methylation

		E-cadherin protein expression					
		+*	−**			+*	−*
α-catenin protein expression	+	10	1	(p=0.0037)	E-cadherin methylation	10	5	(p=0.1983)
	−	10	17			10	13	

*+: abnormal(loss or decrease) expression of E-cadherin or α-cadherin protein, **−: normal expression of E-cadherin or α-catenin protein, *+: methylated E-cadherin gene, *−: unmethylated E-cadherin gene.

Table 3. Correlation between E-cadherin, α-catenin, expression, methylation and histological features

		E-cadherin			Methylation**			α-catenin		
	n	*+	**−	P value	†+	‡−	P value	*+	**−	P value
Growth type										
Expansive growth	16	4	12	0.0076	3	13	0.0435	3	13	0.296
Infiltrative growth	22	16	6		12	10		8	14	
Histological type										
Differentiated	17	5	12	0.0210	6	11	0.6353	4	13	0.721
Undifferentiated	21	15	6		9	12		7	14	
Location										
Upper third	10	6	4		6	4		2	8	
Middle third	13	7	6	0.2972+	5	8	0.0287+	4	9	0.9227+
Lower third	13	5	8		2	11		3	10	
Entire stomach	(2)	(2)	(0)		(2)	(0)		(2)	(0)	
Depth of invasion										
T1, T2	18	5	13	0.0084	7	11	0.9442	1	17	0.0037
T3, T4	20	15	5		8	12		10	10	
Tumor size										
Mean(cm)	8.132	5.450	8.173	0.0083+	8.300	6.157	0.0749+	8.173	6.526	
(Range)	(2～16)	(2～10)	(4～13)		(4～16)	(2～10)		(4～13)	(2～16)	
Lymph node metastasis										
Absence	8	4	4	>0.9999	3	5	>0.9999	2	6	>0.9999
Presence	30	16	14		12	18		9	21	
Peritoneal dissemination										
Negative	29	12	17	0.0206	8	21	0.0159	6	23	0.0875
Positive	9	8	1		7	2		5	4	
Liver metastasis										
Negative	33	18	15	0.6525	12	21	0.3649	11	22	0.2949
Positive	5	2	3					3	2	

*, abnormal (loss or decrease) expression of E-cadherin or α-catenin protein; **, normal expression of E-cadherin or α-catenin protein; †, methylated E-cadherin gene; ‡, unmethylated E-cadherin gene (chi-squared, Fisher's exact probability test; +Mann-Whitney U test)

must invade vessels or lymphatic ducts. In fact, we showed that clustered cancer cells rather than isolated single cancer cells were frequently present in vessels or lymphatic ducts on tissue specimens. This may be due to the fact that cancer cells persisting to form a mass rather than single cells easily metastase in the lymph node. We found that the cancer cell-constructed clusters in vessel or lymphatic duct had normal expressions of adhesive protein in the cytoplasm.

In conclusion, gastric cancer cells with abnormalities of E-cadherin methylation or protein expression result in peritoneal dissemination rather than lymphatic or hematogenous metastasis.

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