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ORIGINAL ARTICLE

수술 전 위암조직에서의 Cadherin과 혈관내피 성장 인자 농도의 의의

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Significance of Preoperative Tissue Levels of Vascular-endothelial Cadherin, Liver-intestine Cadherin and Vascular Endothelial Growth Factor in Gastric Cancer

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Background/Aims: The aims of this study were to examine the expressions of endothelium specific VE-cadherin, intestine specific LI-cadherin, and vascular endothelial growth factor (VEGF), and to determine their relationships with the clinicopathological parameters of gastric cancer.
Methods: A total 47 patients with gastric cancer who underwent surgery were enrolled. Endoscopic biopsies were obtained from the cancer and normal mucosa, respectively. Using semiquantitative RT-PCR, the mRNA expression levels of VE-cadherin, LI-cadherin and VEGF were measured by tumor/normal (T/N) ratios. The protein expressions of VE-cadherin, LI-cadherin and VEGF were examined by Western blot and immunohistochemical stain in surgically resected tissues. The clinicopathological variables were reviewed and analyzed, retrospectively.
Results: Twenty two cases (46.8%) of VE-cadherin, 25 cases (53.2%) of LI-cadherin and 27 cases (51.1%) of VEGF mRNA expressions were overexpressed in gastric cancer compared to normal tissue. There was a tendency for T/N ratio of VE-cadherin mRNA to correlate with the lymphatic invasion (p=0.07) and the lymph node metastasis (p=0.099) in advanced gastric cancer. The T/N ratio of LI-cadherin mRNA showed significant association with distant metastasis (p=0.031) and lymphatic invasion especially in advanced gastric cancer (p=0.023). There was a tendency for the T/N ratio of VEGF mRNA to correlate with the distant metastasis (p=0.073) in advanced gastric cancer.
Conclusions: As increased mRNA expression of LI-cadherin was associated with distant metastasis and lymphatic invasion especially in the biopsy specimen of advanced gastric cancer before surgery, it may provide useful preoperative information on tumor aggressiveness. **(Korean J Gastroenterol 2012;60:229-241)**

Key Words: Cadherin; Vascular endothelial growth factor; Stomach neoplasms

INTRODUCTION

Gastric cancer is the most common cancer and the third most common cause of cancer-related death in Korea, accounting for 26,253 new cases in 2007 and 10,779 deaths in 2006.[1] The overall gastric cancer incidence is decreasing, possibly due to the decrease in *Helicobacter pylori* prevalence associated with improved living standards. However, there are still many patients diagnosed with advanced stage disease, for which the prognosis is poor. In spite of intense in-

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terest and extensive investigations, the prognosis for such cases has not improved significantly in the past several years.[2] Therefore, early detection and curative resection of gastric cancer is still the most important issue.

The most frequently used tumor marker, for gastric cancer, is the carcinoembryonic antigen (CEA). However, this marker is elevated only in a modest proportion of advanced gastric cancer. It is essential to evaluate preoperative clinical stages, and the depth of tumor invasion to determine appropriate treatment modality and predict the prognosis of gastric cancer, but no reliable methods or biologic markers that can predict metastasis and vessel invasion have been found until now.[2-4]

Adhesion molecules such as the cadherins are considered important for the invasion and/or metastasis of tumors. Cadherins are calcium dependent homophilic adhesion molecules frequently associated with specific junctional structures referred to as adherens junctions. Cadherins are expressed in several types of tissues with some specificity: E-cadherin is mostly present in epithelial cells, N-cadherin in the nervous system, smooth muscle cells, fibroblasts and endothelial cells, vascular-endothelial cadherin (VE-cadherin) is specific for the endothelium and related with vasculogenesis, and liver-intestine cadherin (LI-cadherin) is found in the intestinal epithelium.[5]

The major transmembrane component of endothelial adherens junctions is VE-cadherin. VE-cadherin has been implicated in cell growth, migration, vasculogenesis and vascular remodeling.[6,7] Angiogenesis is important in tumorigenesis, so the assessment of angiogenesis may provide significant information of cancer behavior.

LI-cadherin is solely expressed in the liver and intestine of the rat. Human LI-cadherin is exclusively found in the intestinal epithelium and the stomach is also negative for LI-cadherin.[8] LI-cadherin was recently found to be overexpressed in gastric cancer and the expression of LI-cadherin was associated with intestinal metaplasia.[3,8,9] Gastric cancer is generally believed to develop from a multistep progression from gastritis, intestinal metaplasia, dysplasia and subsequently to cancer.[4] The expression of LI-cadherin may therefore occur during the steps of gastric carcinogenesis. Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis and induces proliferation of the endothelium along with the formation of new capillary vessels that loosen the tight adherens junctions and induce proliferation of the endothelium contributing to tubulogenesis and increased vascular permeability.[10,11]

Since there are few reports of VE-cadherin and LI-cadherin which can predict the characteristics of gastric cancer, we performed this study to examine the expression of VE-cadherin, LI-cadherin and VEGF in gastric cancer and to evaluate the correlation between their expression and clinicopathological parameters.

SUBJECTS AND METHODS

1. Materials

Tissue specimens from 47 patients with gastric cancer who were diagnosed and underwent a surgery at Ewha Womens University Mokdong Hospital, Seoul, Korea, from 2005 to 2008, were obtained by means of endoscopic biopsy. At least 4 pieces of endoscopic biopsies were taken from the cancer and macroscopically normal mucosa, at least 5 cm away from the cancer, respectively before definitive treatment. The collected specimens were frozen immediately and stored at −70°C until extraction of mRNA to determine the levels of VE-cadherin, LI-cadherin and VEGF. Patients had underwent a gastrectomy procedure in the department of surgery. During the surgery, tissues of the cancer and normal mucosa were obtained. The collected tissues were frozen immediately and stored at −70°C for the protein analysis. The pathologic diagnoses for endoscopically biopsied specimens were matched with those of the surgically resected tissues, and a consultant pathologist determined the pathologic staging. Preoperatively the stage was determined by endoscopic and computed tomographic findings. We determined the postoperative pathologic stage using the 6th edition of the International Union against Cancer (UICC)/American Joint Committee on Cancer (AJCC) TNM classification system. The clinicopathological data from 47 patients who underwent a surgical procedure were reviewed. Informed consents were obtained from all patients and the study was approved by the Institutional Review Board of Ewha Womans University Mokdong Hospital.

The patients were consisted of 28 men and 19 women ranging in age from 35 to 88 years (65±13 years [mean±SD]). Histologically, 4 cases (8.5%) were well differentiated, 15 (31.9%) were moderately differentiated, 13 (27.7%) were

poorly differentiated, 11 (23.4%) were signet ring cell carcinoma, and 4 (8.5%) were mucinous type. Twenty six cases had intestinal type of differentiation and 16 (34.1%) were diffuse type according to Lauren's classification. Twenty cases (42.6%) had venous invasion and 29 cases (61.7%) had lymphatic invasion. Of the 47 cancer cases, 12 (25.5%) were classified as T1, 7 (14.9%) were T2, 26 (55.3%) were T3, and 2 (4.3%) were T4; 16 cases (34.0%) were N0, 17 (36.2%) were N1, 2 (4.3%) were N2 and 12 (25.5%) were N3; 40 cases (85.1%) were M0 and 7 (14.9%) of M1. If we consider the TNM stage on the whole, 14 cases (29.8%) were in stage I, 6 (12.8%) were stage II, 10 (21.3%) were stage III and 17 cases (36.2%) were in stage IV (Table 1).

2. Semi-quantitative reverse transcript-polymerase chain reaction

1) RNA extraction

Total RNA was extracted from biopsy tissue using the easy-BLUE™ total RNA extraction kit (Intron Biotechnology, Gyeonggido, Korea). Preperation of fresh tissues were added to 800 μL easy-BLUE™ reagent and homogenized using a homogenizer or equivalent. Vigorous vortex was applied in room temperature for 3 minutes. Then, 200 μL of chloroform was added and vortex was applied. After centrifuge the solution at 12,000 rpm (4°C) for 15 minutes, 400 μL of supernatant was transferred to an empty 1.5 mL tube. Then, 400 μL isopropanol (2-propanol) was added, and mixed it well by inverting the tube 5-6 times. It was left for 5 minutes at room temperature. After centrifugating the solution at 12,000 rpm (4°C) for 10 minutes, remove the supernatant to obtain RNA pellet. Then, 1 mL of 75 % ethanol was added and the solution mixed well by inverting the tube 2-3 times. The mixtures were centrifuged for 5 minutes at 12,000 rpm (4°C). The supernatant discarded, and the remaining RNA pellet was dried. RNA was dissolved using 20-50 μL of RNase, Dnase free water for storage at −70°C. The amount and purity of the extracted RNA was quantitated by spectrophotometry.

2) cDNA synthesis (reverse transcription)

cDNA was synthesized with 2 μg of total RNA, oligodT primer. In a sterile RNase-free microcentrifuge tube, add 0.5 μg of oligodT primer and 2 μg RNA sample. The tube was heated at 70°C for 5 minutes, and cooled immediately on ice. Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Promega, Fitchburg, WI, USA) 200 unit, rRNasin Ribonuclease inhibitor (Promega) 40 unit, 5×RT buffer and dNTP were added to the tube. The tube was gently mixed, incubated for 40 minutes at 42°C and heated for 5 minutes at 95°C. The cDNA was stored at −20°C.

3) Polymerase chain reaction amplification

The primer sequences for amplification which were used are shown in Table 2. All primers were synthesized by Cosmo GeneTech (Seoul, Korea). The amplification reaction was carried out in a 20 μL of PCR mixture containing 20 μL of the syn-

Table 1. Characteristics of the Patients

Variable	Data
Age (yr)	65±13
Gender	
Male	28 (59.6)
Female	19 (40.4)
Differentiation by WHO classification	
Well	4 (8.5)
Moderately	15 (31.9)
Poorly	13 (27.7)
Signet ring cell carcinoma	11 (23.4)
Mucinous	4 (8.5)
Differentiation by Lauren's classification	
Intestine	26 (55.3)
Diffuse	16 (34.1)
Mixed	5 (10.6)
Venous invasion	
Negative	27 (57.4)
Positive	20 (42.6)
Lymphatic invasion	
Negative	18 (38.3)
Positive	29 (61.7)
Depth of invasion	
T1	12 (25.5)
T2	7 (14.9)
T3	26 (55.3)
T4	2 (4.3)
Nodal status	
N0	16 (34.0)
N1	17 (36.2)
N2	2 (4.3)
N3	12 (25.5)
Distant metastasis	
M0	40 (85.1)
M1	7 (14.9)
Stage	
I	14 (29.7)
II	6 (12.8)
III	10 (21.3)
IV	17 (36.2)

Values are presented as mean±SD or n (%).
WHO, World Health Organization. Stages are determined by the 6th edition of International Union against Cancer/American Joint Committee on Cancer TNM classification System.

Table 2. Primers of the Genes

Gene	Primer (sense/antisense)	Annealing temperature (°C)	Size (bp)
GAPDH	5'-GCCTCAAGATCATCAGCAAT-3' (530-549 bp) 5'-TCCAGCTCAGGGATGACCTT-3' (757-776 bp)	55	245
VE-cadherin	5'-ACCGGATGATGACCAAGTACAGC-3' (1,025-1,044 bp) 5'-ACACACTTTGGTCTGGTAGG-3' (1,601-1,620 bp)	60	596
LI-cadherin	5'-ACAGACCCACGTTTCTC-3' (482-501 bp) 5'-ATATTGTGCACCGGGATCAT-3' (914-933 bp)	55	452
VEGF	5'-GCAGAATCACGACGAAGTGG-3' (1,133-1,152 bp) 5'-GCATGGTGTTGGACTCC-3' (1,325-1,344 bp)	58	212

GAPDH, glyceradlehyde 3-phosphate dehydrogenase; VE-cadherin, vascular-endothelial cadherin; LI-cadherin, liver-intestine cadherin; VEGF, vascular endothelial growth factor.

thesized cDNA solution, 4 μL of 5×polymerase reaction buffer, 200 μM of dNTP, 0.5 μM of each primer (sense and antisense) and 0.5 unit Taq polymerase (Promega). The PCR mixure was amplified using a GeneAmp PCR System 9600 (PERKIN-ELMER, Wellesley, MA, USA). Amplified products (10 μL) were identified by electrophoresis of PCR on 2 % agarose gel containing ethidium bromide and ultraviolet illumination. Forty seven endoscopic biopsy tissues were analyzed for mRNA expression. The expression of VE-cadherin, LI-cadherin and VEGF was evaluated using the tumor: normal (T/N) ratio for VE-cadheirn, LI-cadherin and VEGF. The results are expressed as the mean±SD for gastric cancer tissues.

3. Western blot analysis

Twenty four sets of cancerous and normal gastric tissue samples which were obtained by the surgical procedure for protein analysis were frozen immediately and kept at −70°C until use. The tissues were washed with 300 μL ice-cold phosphate-buffered saline solution (PBS) and then washed into lysis buffer (Proprep™, protein extraction solution®, Intron Biotechnology, Gyunggido, Korea). After incubation for 20 minutes on ice, tissue lysates centrifuged at 12,000 rpm for 20 minutes at 4°C. The resulting supernatant was assayed for the protein concentration to ensure that a consistent amount of protein assay (BCA protein assay reagent A and B® (50 : 1); Pierce, Rockford, IL, USA)

After determination of protein concentrations, protein extracts 50 μg were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 1.5 hours at 100 V room temperature. The fractionated proteins were transferred overnight at 4°C onto the polyvinyl difluride membrane at 80 mA. Nonspecific binding of proteins was blocked by incubating the membranes in 5% skim milk in PBS containing 0.1% Tween 20 (PBSCT) for 120 minutes. The membranes were incubated with goat anti-VE-cadherin antibody, goat anti LI-cadherin and rabbit anti-VEGF antibody with the appropriate dilution for overnight at 4°C, and then the membranes were rinsed three times with PBSCT at 5 minute intervals. The membranes were incubated with seondary antibody for 2 hours at room temperature. After the membranes were rinsed three times with PBST at 5 minute intervals, the blots were developed using the ECL western blotting detection solution (ECL™ Western blotting detection reagents; Amersham Biosciences, Buckinghashire, UK). The blots were detected by LAS system (LAS-3000, Fugifilm, Seoul, Korea). The results of western blotting were standardized with the density of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

4. Immunohistochemical study

Paraffin embedded 18 samples of gastric cancer tissue which were obtained by the surgical procedure were used for immunohistochemical study. Immunohistochemistry was performed through the biotin-streptavidin (B-SA) method using the labelled streptavidin biotin (LSAB) kit (Dakocytomation Inc., Carpinteria, CA, USA). The paraffinized tissues were sectioned 4 μm thickness and deparaffinized with xylene twice for 5 minutes. The tissues were rehydrated serially with 100%, 90%, 80% and 70% alcohol for 5 minutes and washed with distilled water. Antigen retrival condition included heat-induced epitope retrival in 10 nm/L citrate buffer (pH 6.0) for 7 minutes.

The endogenous peroxidase activity was blocked by incubation in 3 % hydrogen peroxide solution for 5 minutes. The tissue was incubated with the monoclonal anti-VE-cadhrin (SA-6452, dilution 1 : 200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-LI-cadherin (SC-6974, dilution 1 : 200;

Santa Cruz Biotechnology), and anti-VEGF (SC-152, dilution 1 : 1,000; Santa Cruz Biotechnology) in a moist chamber for 2 hours at the room temperature. The negative control was incubated with diluent with background reducing components (Ready-to-use, Dakocytomation Inc.) instead of the primary antibody. Subsequently, the tissues were incubated for 30 minutes with the biotinylated secondary antibody solution. The tissues were then incubated with streptavidin peroxidase solution for 30 minutes, and liquid DAB substrate chromogen solution was used as a substrate to yield the brown-colored reaction products. Hematoxylin staining was performed for counterstaining. The stained tissues were then photographed using the Olympus microscope (Olympus, Tokyo, Japan) with ColorView3 digital camera (Soft Imaging System, Gmbh, Münster, Germany). A cancer was regarded as positive if any cancer cells showed immunostaining, whereas it was classified as negative if there was a complete absence of immunostaining in cancer cells.

5. Statistical analysis

STATA 10 (StataCorp, College Station, TX, USA) was used for the statistical analysis. Continuous variables were expressed as means±SD. The association between clinicopathological features and mRNA expressions of VE-cadherin, LI-cadherin and VEGF in cancer were studied by a nonparametric test. The correlations of the expression of VE-cadherin, LI-cadherin and VEGF were analyzed by the Pearson correlation test. The data were considered significant if the p-value was less than 0.05.

RESULTS

1. Expression of VE-cadherin

Overexpression of mRNA of VE-cadherin was detected in 22 of 47 (46.8%) gastric cancer lesions. The mRNA expression of VE-cadherin in gastric cancer and normal tissue were shown in Fig. 1. In western blot analysis, VE-cadherin

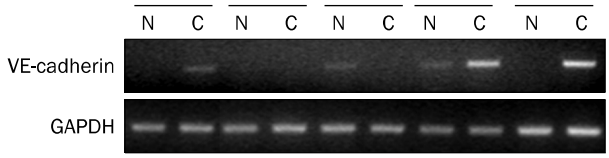

Fig. 1. RT-PCR analysis of vascular-endothelial cadherin (VE-cadherin) in gastric cancer tissue (C) and normal tissue (N). Representative RT-PCR cases were shown. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

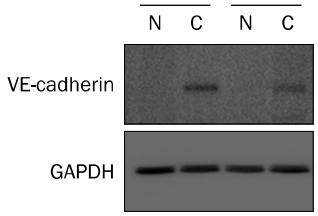

Fig. 2. Western blot analysis of vascular-endothelial cadherin (VE-cadherin) in gastric cancer tissue (C) and normal tissue (N). Representative cases were shown. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

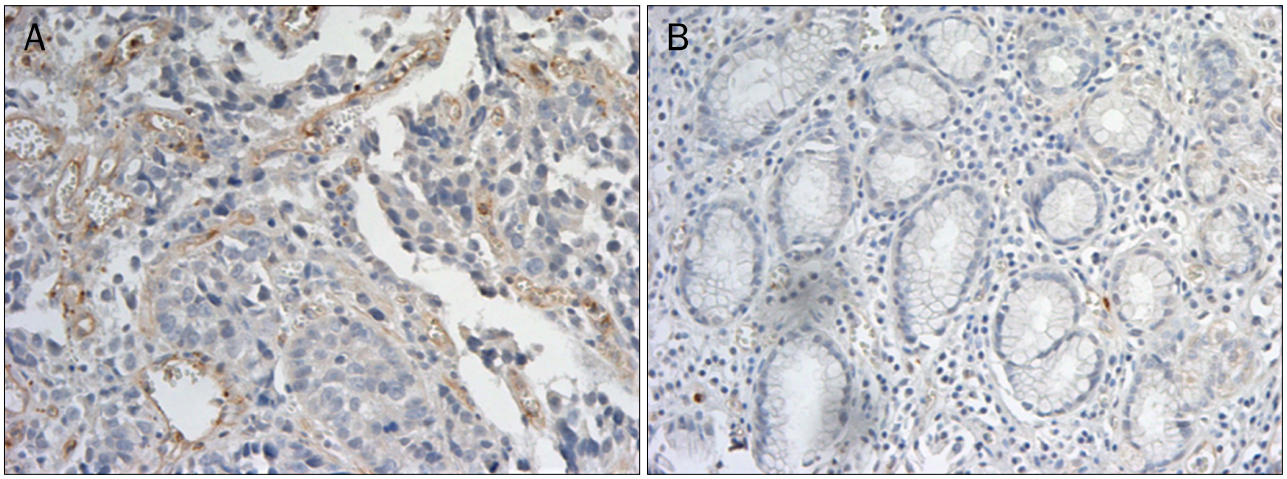

Fig. 3. Immunohistochemical staining with vascular-endothelial cadherin (VE-cadherin) (×200). (A) Immune staining was evident on vascular endothelial cells in gastric cancer tissues. (B) Immune reactivity of VE-cadherin was not detected in normal gastric tissues.

proteins were expressed in 12 of 24 (50%) gastric cancer cases (Fig. 2). In immunohistochemical study, 12 of 18 (66.7%) cases showed positive immunostaining. Immunostaining was evident on the vascular endothelial cells in the gastric cancer tissues (Fig. 3).

There were no associations between the T/N ratio of VE-cadherin mRNA with differentiations by World Health Organization (WHO) classification and by Lauren's classification, venous invasion, depth of invasion, nodal status, and distant metastasis (Table 3).

There was a tendency for the T/N ratio of VE-cadherin mRNA to correlate with the lymphatic invasion (p=0.070) and lymph node metastasis (p=0.099) in advanced gastric cancer (AGC).

2. Expression of LI-cadherin

LI-cadherin mRNA was overexpressed in 25 of 47 (53.2%) gastric cancer cases (Fig. 4). LI-cadherin proteins were ex-

Table 3. Relationships between VE-cadherin mRNA Expression and Clinicopathological Characteristics

Variable	T/N ratio of VE-cadherin mRNA	p-value
Differentiation by WHO classification		
Well	0.88±0.11	NS
Moderately	0.79±0.63	
Poorly	1.20±0.74	
Signet ring cell carcinoma	1.31±0.60	
Mucinous	1.20±0.61	
Differentiation by Lauren's classification		
Intestine	0.96±0.52	NS
Diffuse	1.14±0.84	
Mixed	1.36±0.49	
Venous invasion		
Negative	1.12±0.79	NS
Positive	1.02±0.53	
Lymphatic invasion		
Negative	1.17±0.72	NS
Positive	0.89±0.48	
Depth of invasion		
T1	0.97±0.44	NS
T2	1.05±0.49	
T3	1.11±0.78	
T4	1.06±0.20	
Nodal status		
N0	0.81±0.50	NS
N1	1.27±0.76	
N2	1.06±0.20	
N3	1.12±0.62	
Distant metastasis		
M0	1.06±0.65	NS
M1	1.08±0.69	
Stage		
I	0.94±0.43	NS
II	0.71±0.64	
III	1.35±0.90	
IV	1.13±0.59	

Values are presented as mean±SD.
WHO, World Health Organization; VE-cadherin, vascular-endothelial cadherin; WHO, World Health Organization; NS, not significant. Stages are determined by the 6th edition of International Union against cancer/American Joint Committee on Cancer TNM classification System.

Table 4. Relationships between LI-cadherin mRNA Expression and Clinicopathological Characteristics

Variable	T/N ratio of LI-cadherin mRNA	p-value
Differentiation by WHO classification		
Well	1.34±0.73	NS
Moderately	1.00±0.38	
Poorly	1.19±0.61	
Signet ring cell carcinoma	1.45±1.04	
Mucinous	1.17±0.65	
Differentiation by Lauren's classification		
Intestine	1.14±0.51	NS
Diffuse	1.07±0.44	
Mixed	1.92±1.51	
Venous invasion		
Negative	1.18±0.46	NS
Positive	1.22±0.82	
Lymphatic invasion		
Negative	1.17±0.79	NS
Positive	1.03±0.44	
Depth of invasion		
T1	1.13±0.51	NS
T2	1.01±0.24	
T3	1.25±0.83	
T4	1.65±0.60	
Nodal status		
N0	1.05±0.46	0.082
N1	1.04±0.53	
N2	1.65±0.60	
N3	1.55±1.00	
Distant metastasis		
M0	1.13±0.69	0.031
M1	1.58±0.53	
Stage		
I	0.94±0.43	NS
II	0.71±0.64	
III	1.35±0.90	
IV	1.13±0.59	

Values are presented as mean±SD.
LI-cadherin, liver-intestine cadherin; WHO, World Health Organization; NS, not significant. Stages are determined by the 6th edition of International Union against cancer/American Joint Committee on Cancer TNM classification System.

Table 5. Relationships between VEGF mRNA Expression and Clinicopathological Characteristics

Variable	T/N ratio of VEGF mRNA	p-value
Differentiation by WHO classification		
Well	0.78±0.15	0.041
Moderately	1.40±0.56	
Poorly	0.99±0.36	
Signet ring cell carcinoma	1.02±0.31	
Mucinous	1.23±0.26	
Differentiation by Lauren's classification		
Intestine	1.17±0.45	NS
Diffuse	1.06±0.50	
Mixed	1.19±0.25	
Venous invasion		
Negative	1.14±0.55	NS
Positive	1.13±0.37	
Lymphatic invasion		
Negative	1.12±0.47	NS
Positive	1.15±0.41	
Depth of invasion		
T1	1.08±0.49	NS
T2	1.27±0.33	
T3	1.09±0.47	
T4	1.44±0.31	
Nodal status		
N0	1.11±0.52	NS
N1	1.12±0.37	
N2	1.44±0.31	
N3	1.12±0.49	
Distant metastasis		
M0	1.07±0.40	0.073
M1	1.47±0.58	
Stage		
I	1.04±0.46	NS
II	1.30±0.53	
III	0.99±0.37	
IV	1.13±0.45	

Values are presented as mean±SD.
VEGF, vascular endothelial growth factor; WHO, World Health Organization; NS, not significant. Stages are determined by the 6th edition of International Union against Cancer/American Joint Committee on Cancer TNM classification System.

pressed in 14 of 24 (58.3%) gastric cancer cases. Representative examples of the Western blotting are shown in Fig. 5. In immunohistochemical study, 13 of 18 (72.2%) cases showed positive immunostaining. Immune reactivity of LI-cadherin in the mucosa of gastric cancer tissue was noted (Fig. 6).

There were no associations between the T/N ratio of LI-cadherin mRNA with differentiations by WHO classification and by Lauren's classification, venous invasion, and depth of invasion (Table 4).

There was a tendency for the T/N ratio of LI-cadherin mRNA to correlate with the nodal status (p=0.082). When nodal status was divided into two subgroups (N0/1 *vs.* N2/3), a significant difference was noted (1.05±0.49 *vs.* 1.56±0.93 [mean±SD]; p=0.013).

There was a significant relationship between the T/N ratio of LI-cadherin mRNA and distant metastasis (p=0.031) (Table 4). A significant relationship was also noted between the T/N ratio of LI-cadherin mRNA and lymphatic invasion especially in AGC (p=0.023) (Table 6).

3. Expression of VEGF

Overexpression of the mRNA of VEGF in gastric cancer was observed in 27 of 47 cases (51.1%). Fig. 7 shows the mRNA expression of VEGF in gastric cancer and normal tissue. VEGF proteins were expressed in 14 of 24 (58.3%) gastric cancer specimens (Fig. 8). In immunohistochemical study, 10 of 18 (55.6%) cases showed positive immunostaining. Immunostaining was evident in the vascular endothelial cells of the gastric cancer tissues (Fig. 9).

There were no associations between the T/N ratio of VEGF mRNA with differentiations by Lauren's classification, venous invasion, lymphatic invasion, depth of invasion and nodal status (Table 5).

There was a tendency for the T/N ratio of VEGF mRNA to

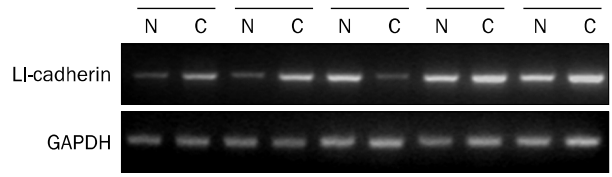

Fig. 4. RT-PCR analysis of liver-intestine cadherin (LI-cadherin) in gastric cancer tissue (C) and normal tissue (N). LI-cadherin mRNA was detected in gastric cancer. Representative RT-PCR cases were shown. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

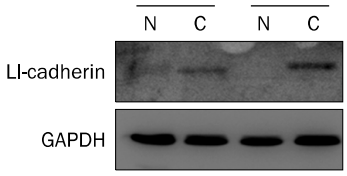

Fig. 5. Western blot analysis of liver-intestine cadherin (LI-cadherin) in gastric cancer tissue (C) and normal tissue (N). LI-cadherin protein was detected in gastric cancer. Representative cases were shown. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

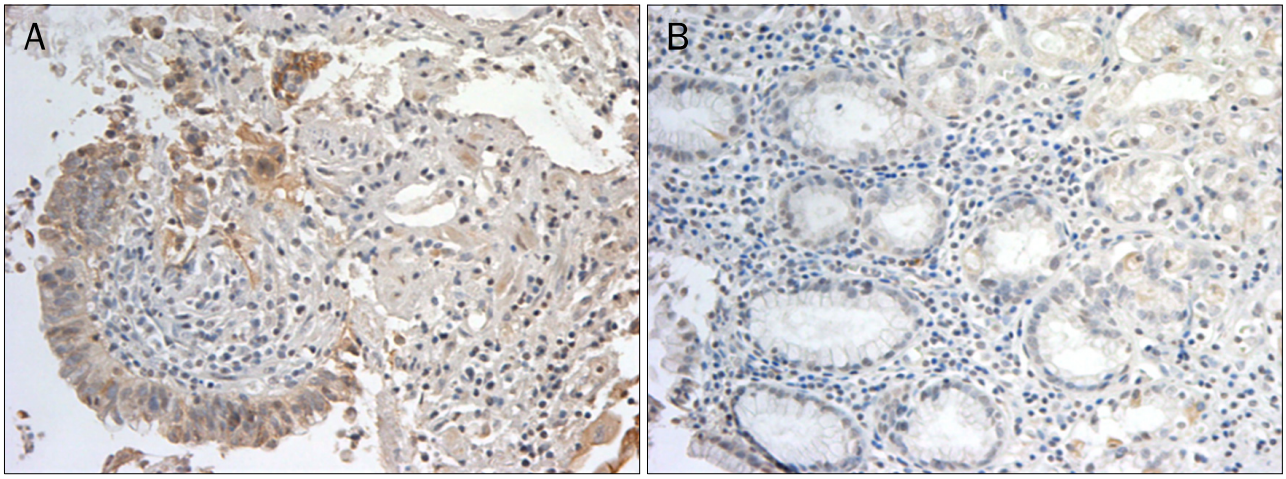

Fig. 6. Immunohistochemical staining with liver-intestine cadherin (LI-cadherin). (A) Immune staining was evident on the mucosal cells in gastric cancer tissues (×100). (B) Immune reactivity of LI-cadherin was not detected in normal gastric tissues (×200).

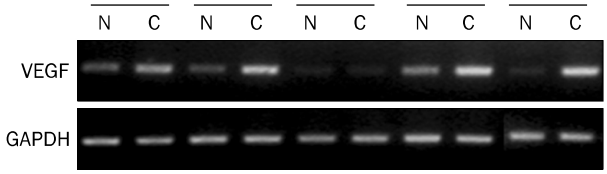

Fig. 7. RT-PCR analysis of vascular endothelial growth factor (VEGF) in gastric cancer tissue (C) and normal tissue (N). VEGF mRNA was detected in gastric cancer. Representative RT-PCR cases were shown. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

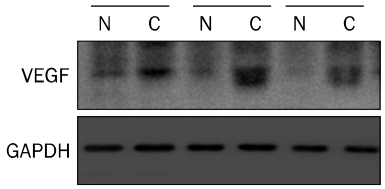

Fig. 8. Western blot analysis of vascular endothelial growth factor (VEGF) in gastric cancer tissue (C) and normal tissue (N). VEGF protein was detected in gastric cancer. Representative cases were shown. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Table 6. Relationships between VE-cadherin, LI-cadherin and VEGF mRNA Expression and Clinicopahtological Characteristices in Advanced Gastric Cancer

Variable (n)	VE-cadherin		LI-cadherin		VEGF	
	T/N ratio	p-value	T/N ratio	p-value	T/N ratio	p-value
Venous invasion						
Negative (16)	1.05±0.59	NS	1.25±0.99	NS	1.14±0.26	NS
Positive (19)	1.14±0.80		1.02±0.46		1.16±0.56	
Lymphatic invasion						
Negative (7)	0.73±0.50	0.070	0.80±0.15	0.023	1.23±0.25	NS
Positive (28)	1.19±0.73		1.33±0.80		1.13±0.48	
Lymph node metastasis						
Negative (5)	0.54±0.58	0.099	0.88±0.15	NS	1.25±0.60	NS
Positive (30)	1.19±0.69		1.28±0.78		1.13±0.42	
Distant metastasis						
Negative (28)	1.10±0.72	NS	1.14±0.76	0.035	1.07±0.36	0.070
Positive (7)	1.08±0.70		1.58±0.53		1.47±0.59	

Values are presented as mean±SD.
VE-cadherin, vascular-endothelial cadherin; LI-cadherin, liver-intestine cadherin; VEGF, vascular endothelial growth factor; NS, not significant.

correlate with distant metastasis (p=0.073) (Table 5). Also there was a tendency for the T/N ratio of VEGF mRNA to correlate with distant metastasis in AGC (p=0.070) (Table 6).

4. Correlations with cadherins and VEGF

There were no significant correlations between the T/N ratios of VE-cadherin and VEGF (r=−0.187, p=0.207) (Fig. 10),

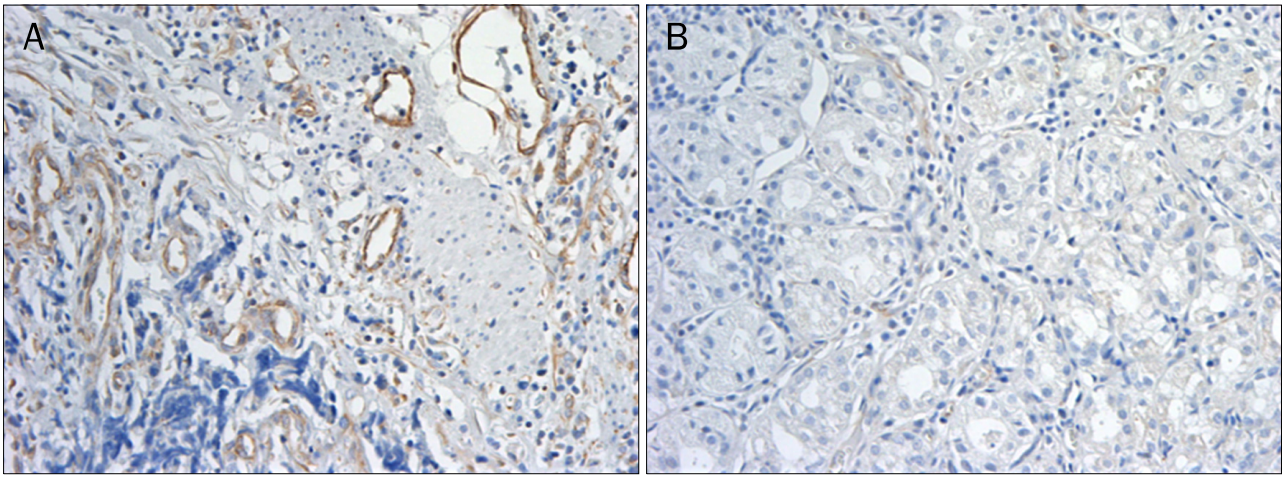

Fig. 9. Immunohistochemical staining with vascular endothelial growth factor (VEGF) (×200). (A) Immune staining was evident on vascular endothelial cells in gastric cancer tissues. (B) Immune reactivity of VEGF was not detected in normal gastric tissues.

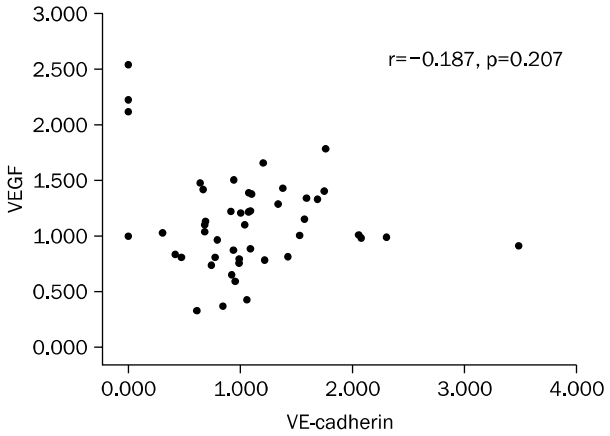

Fig. 10. Correlation between vascular-endothelial cadherin (VE-cadherin) and vascular endothelial growth factor (VEGF) mRNA expression. There was no significant relationship between VE-cadherin and VEGF.

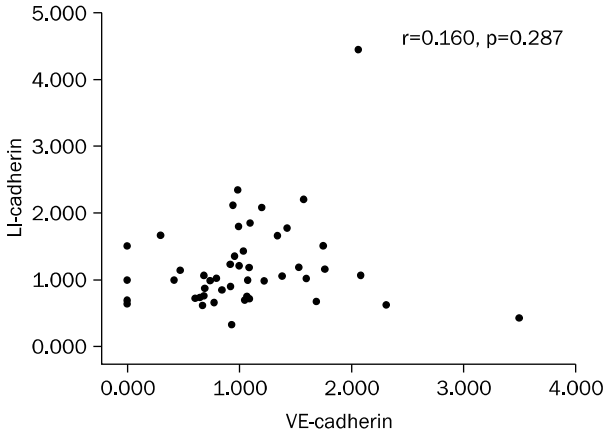

Fig. 11. Correlation between vascular-endothelial cadherin (VE-cadherin) and liver-intestine cadherin (LI-cadherin) mRNA expression. There was no significant relationship between VE-cadherin and LI-cadherin.

between the T/N ratios of VE-cadherin and LI-cadherin (r=0.160, p=0.287) (Fig. 11) and between the T/N ratios of LI-cadherin and VEGF (r=0.067, p=0.655) (Fig. 12).

DISCUSSION

Cadherins play an important role in establishing adherens-type junctions by mediating calcium-dependent cell to cell adhesion.[12] Cadherins were first shown to support cell-cell contact and maintain tissue cohesion, and have been shown to be essential for development and post-embryonic life. In addition, cadherin can also act as cell-signaling receptors by regulating the location of β-catenin. β-cat-

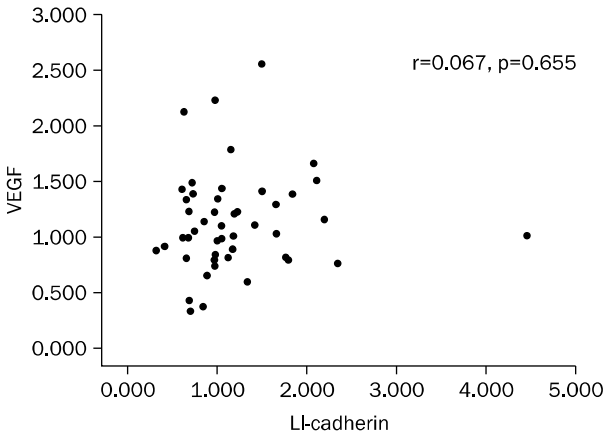

Fig. 12. Correlation between LI-cadherin and vascular endothelial growth factor (VEGF) mRNA expression. There was no significant relationship between LI-cadherin and VEGF.

enin is a distal component of the highly conserved Wnt pathway that governs cell survival, proliferation, and migration.[13] The finding that cadherin cell-cell adhesion receptors are key regulators of tissue architecture during development and tissue homeostasis provides a good molecular evidence to link cell-cell adhesion, morphogenesis and cancer.[14] They are divided into more than 10 subclasses that are distinct in their immunologic specificities and tissue distribution.[15,16]

The formation of new blood vessels (angiogenesis) and lymph vessels (lymphangiogenesis) significantly contribute to malignant growth and metastasis of solid tumors. The assessment of angiogenesis and lymphangiogenesis has emerged as a potentially useful prognostic and predictive factor in human malignancies.[17] A recently characterized member of the cadherin family, VE-cadherin, also known as cadherin 5, is an endothelial cell-specific cadherin located at intercellular adherens junctions, where it is thought to play a role in the cohesion and organization of adherens junctions and in the control of permeability properties of vascular endothelium.[7,18] VE-cadherin is important for tumor angiogenesis, and its expression is up-regulated in the vasculature of breast cancers.[19] Blocking VE-cadherin function with monoclonal antibodies in mouse tumor models leads to the inhibition of tumor angiogenesis and growth. VE-cadherin is crucial for vessel assembly and integrity during angiogenesis, and blocking its function with antibodies has been previously proposed as a promising therapeutic approach against tumor angiogenesis.[5] VE-cadherin is considered to be an important agent that is indirectly responsible for tumor invasiveness due to its involvement in angiogenesis. Highly aggressive melanomas were found to express VE-cadherin on the surface of malignant cells and such expression is indispensable for melanoma to create embryonic-like vasculogenic networks.[20] The intensity or intracapillary extent of positive immune staining for VE-cadherin in the capillary endothelium of hepatocellular carcinoma tissues was significantly associated with tumor size, capsular invasion and tumor cell differentiation.[7] Elevated levels of VE-cadherin protein have been detected in patients with colon cancer, however no correlation was found in comparison between VE-cadherin in a levels and the clinicopathological features of colorectal cancer.[20] This study showed for the first time that mRNA expression, protein expression and immune reactivity of VE-cadherin were noted 46.8%, 50.0% and 66.7% in gastric cancer, respectively and that in AGC, there was a tendency for the T/N ratio of VE-cadherin mRNA to correlate with the lymphatic invasion (p=0.070) and lymph node metastasis (p=0.099) (Table 6). In esophageal cancer, VE-cadherin gene expression showed significant correlations with other angiogenic and lymphangiogenic markers.[17] Up to now there have been no published reports on VE-cadherin expression in gastric cancer. The report of esophageal cancer may support our data and VE-cadherin may be a marker for lymphatic invasion and lymph node metastasis in AGC.

Gastric intestinal metaplasia, an intermediate step in Correa's cascade of gastric carcinogenesis, is generally regarded as a premalignant lesion.[21] Gastric cancer is generally believed to develop from a multistep progression of chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia and subsequently to cancer. Epidemiological studies suggest that patients with intestinal metaplasia have more than a 10-fold increased risk of developing gastric cancer.[22] Intestinal metaplasia of the stomach represents an alteration of the gastric mucosa to epithelium with morphological and biological characteristics of the intestine. Intestinal metaplasia is found in approximately 20% of gastric biopsies and is more frequent in older patients.[23] Intestine specific markers such as villin, sucrase-isomaltase, and aminopeptidase N are known to be expressed in intestinal metaplastic cells and gastric adenocarcinomas while not observed in the normal gastric mucosa.[24] LI-cadherin, which was studied in gastric cancer expression, is another candidate for evaluating whether the gastric epithelium is undergoing neoplastic transformation. LI-cadherin is a structurally unique member of the cadherin superfamily.[25] Whereas the so-called classic cadherins, such as E-, N- and P-cadherin, have five cadherin repeats within their extracellular domain, LI-cadherin consists of seven cadherin repeats. LI-cadherin has only 21 amino acids in the cytoplasm domain, although the classic cadherins have highly conserved cytoplasm domains with 150 to 160 amino acids.[15,26] The expression of LI-cadherin differs by species. In the rat, LI-cadherin is expressed in the liver and intestinal epithelial cells, while in humans and the mouse, LI-cadherin is expressed in the intestinal epithelial cells, but not in the liver. It has been reported that LI-cadherin is expressed in colorectal and pancreatic cancers.[27,28] In colorectal cancer, a reduced LI-cadherin expression level was associated with a

high tumor grade, lymphatic invasion, lymph node metastasis and an advanced TNM stage.[29] Recent studies have reported that LI-cadherin is overexpressed in gastric cancer and in gastric intestinal metaplasia.[9,27] Dong et al.[27] reported that immunohistochemistry showed LI-cadherin was mainly present on the mucosal cell membrane and absent in normal gastric tissues and the positive rate for LI-cadherin was 78.4%. Also in this study, immune reactivity of LI-cadherin was shown in the mucosal cells of gastric cancer tissues and positive rate of immunohistochemical stain was 72.2% and LI-cadherin mRNA was overexpressed in 53.2% gastric cancer cases.

The evidence for the expression of LI-cadherin in gastric cancer continues to be debated. It was reported that there is a close association between the reduced expression of LI-cadherin and lymph node metastasis in gastric cancer. LI-cadherin was expressed in the well-differentiated adenocarinoma cells but this expression was reduced in the dedifferentiated adenocarcinoma cells. Therefore, the adhesion molecules associated with the invasion and metastasis of cancer tissues appear to depend on how little they are expressed.[2] It was also reported that lymph node metastasis was associated with the overexpression of LI-cadherin.[9,27] LI-cadherin is found to be complementary to the co-expressed classical cadherins such as E-cadherin. LI-cadherin may be up-regulated in some situations where the classical cadherin-mediated adhesion is disrupted. It may be involved in the intermediate steps of gastric carcinogenesis. More advanced disease may result in a greater change in the up-regulation of LI-cadherin. This may explain the significant association with lymph node metastasis.[9,27] In this study, the T/N ratio of LI-cadherin was significantly correlated with distant metastasis and lymphatic invasion specially in AGC patients and there was a tendency for the T/N ratio of LI-cadherin mRNA to correlate with the nodal status. When nodal status was divided into two subgroups, local and distant nodal metastasis, (N0/1 *vs.* N2/3), mRNA expression of the distant nodal metastasis group was significantly higher than that of the local nodal metastasis group which is favored to the second opinion. As the exact adhesive function of LI-cadherin is still unknown, more studies are required to elucidate this further.

VEGF was first described as a potent vascular permeability factor secreted by tumor cells that stimulates a rapid and reversible increase in microvascular permeability without mast cell degranulation or endothelial cell damage. This tumor secreted vascular permeability factor was later shown to be a highly selective and remarkably potent growth factor of endothelial cells. VEGF has been shown to promote the migration, growth and survival of endothelial cells and is essential for vasculogenesis and angiogenesis.[30] In adults, deregulated expression of VEGF is involved in a variety of disease states, ranging from inflammation to cancer and metastasis.[31] VEGF expression is rapidly increased in hypoxic tissues, providing a mechanism that can assist tissue reoxygenation by stimulating angiogenesis and induces the proliferation of endothelium, contributes to tubulogenesis and increases vascular permeability.[10,30] VEGF has been shown to be a marker for a poor prognosis. In addition, VEGF expression correlates with the degree of gastric wall involvement and production of ascites and the peritoneal dissemination of gastric cancer.[31,32] In this study, overexpression of VEGF mRNA showed a tendency to correlate with distant metastasis.

VEGF and VE-cadherin are required for efficient angiogenesis. A possible dependency of VEGF and VE-cadherin was suggested in an experimental model of nude mice with implanted human renal cell carcinoma in which antibodies against VEGF were shown to restrict tumor growth by decreasing both VEGF and murine VE-cadherin mRNA expression.[33] The mRNA level of VE-cadherin increases in the conditions of enhanced angiogenesis like pregnancy or cancer before treatment. In such conditions, VEGF also increased. Apoptosis of endothelial cells causes gradual expulsion of VE-cadherin for the cell surface. So VE-cadherin and VEGF seem to be independent markers of angiogenesis and separately reflect the dynamics of neovascularization.[34] In colon cancer, VE-cadherin and VEGF were elevated in cancer patients than in controls but there were no correlations between VE-cadherin and VEGF. These finding also suggest that VE-cadherin and VEGF seem to be independent markers of angiogenesis in colon cancer.[20] Similarily in our study, there was no significant correlation between VE-cadherin and VEGF mRNA ratio in gastric cancer. And there were also no significant correlation between VE-cadherin and LI-cadherin mRNA ratio and between LI-cadherin and VEGF mRNA ratio. These finding may suggest that VE-cadherin, LI-cadherin, and VEGF seem to be independent markers for gastric cancer.

There were limitations in this study. First, the number of cancer specimens was relatively small. Only 47 gastric cancer cases were available for RT-PCR, 24 cases for Western blot analysis, and 18 cases were available for immunohistochemistry. Because of the small number of cancer specimens and enrolling only the patient who underwent the surgical procedure, the portion of histologically poorly differentiated adenocarcinoma and signet ring cell carcinoma group was relatively big in our study. Also, we have to consider the possible discrepancy between endoscopic forcep biopsies and surgically resected specimen.

Secondly, the normal (noncancerous) gastric specimen, which were used in this study, were not confirmed normal by microscopically but suspected to be normal by macroscopically and the specimens were from the gastric cancer patients, not from normal healthy control. It is known that the non-cancerous mucosa surrounding gastric cancer is often affected by gastritis, atrophy and intestinal metaplasia, so the normal gastric specimen may not be really normal gastric mucosa.

In this study, LI-cadherin had significant relationships with distant metastasis and lymphatic invasion in advanced gastric cancer. VE-cadherin transcription trends to increase with lymphatic invasion and lymph node metastasis in advanced gastric cancer. VEGF transcription tend to increase when distant metastasis was presented. As an RT-PCR assay can be performed easily with small amount of specimens, examination of LI-cadherin in biopsy specimens, obtained before surgery, may provide useful preoperative informations on tumor aggressiveness.

REFERENCES

1. Ministry for Health Welfare and Family Affairs. Yearbook of health and welfare statistics (1993-2007). Seoul: Ministry of Health and Welfare (Korea), 2009.
2. Park SS, Kang SH, Park JM, et al. Expression of liver-intestine cadherin and its correlation with lymph node metastasis in gastric cancer: can it predict N stage preoperatively? Ann Surg Oncol 2007;14:94-99.
3. Grötzinger C, Kneifel J, Patschan D, et al. LI-cadherin: a marker of gastric metaplasia and neoplasia. Gut 2001;49:73-81.
4. Konturek PC, Konturek SJ, Brzozowski T. Gastric cancer and *Helicobacter pylori* infection. J Physiol Pharmacol 2006;57 (Suppl 3):51-65.
5. Cavallaro U, Liebner S, Dejana E. Endothelial cadherins and tumor angiogenesis. Exp Cell Res 2006;312:659-667.
6. Blaschuk OW, Devemy E. Cadherins as novel targets for anti-cancer therapy. Eur J Pharmacol 2009;625:195-198.
7. Kato K, Takada T, Fukusato T. Expression of vascular endothelial-cadherin in human hepatocellular carcinoma tissues. Hepatol Res 2007;37:444-453.
8. Dong WG, Yu QF, Xu Y, Fan LF. Li-cadherin is inversely correlated with galectin-3 expression in gastric cancer. Dig Dis Sci 2008;53:1811-1817.
9. Ko S, Chu KM, Luk JM, et al. Overexpression of LI-cadherin in gastric cancer is associated with lymph node metastasis. Biochem Biophys Res Commun 2004;319:562-568.
10. Carmeliet P, Collen D. Molecular basis of angiogenesis. Role of VEGF and VE-cadherin. Ann N Y Acad Sci 2000;902:249-262.
11. Lampugnani MG, Dejana E. Adherens junctions in endothelial cells regulate vessel maintenance and angiogenesis. Thromb Res 2007;120(Suppl 2):S1-S6.
12. George SJ, Dwivedi A. MMPs, cadherins, and cell proliferation. Trends Cardiovasc Med 2004;14:100-105.
13. Brantjes H, Barker N, van Es J, Clevers H. TCF: Lady Justice casting the final verdict on the outcome of Wnt signalling. Biol Chem 2002;383:255-261.
14. Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. Science 1991;251:1451-1455.
15. Gessner R, Tauber R. Intestinal cell adhesion molecules. Liver-intestine cadherin. Ann N Y Acad Sci 2000;915:136-143.
16. Yagi T, Takeichi M. Cadherin superfamily genes: functions, genomic organization, and neurologic diversity. Genes Dev 2000; 14:1169-1180.
17. Loges S, Clausen H, Reichelt U, et al. Determination of microvessel density by quantitative real-time PCR in esophageal cancer: correlation with histologic methods, angiogenic growth factor expression, and lymph node metastasis. Clin Cancer Res 2007;13:76-80.
18. Wheelock MJ, Johnson KR. Cadherin-mediated cellular signaling. Curr Opin Cell Biol 2003;15:509-514.
19. Labelle M, Schnittler HJ, Aust DE, et al. Vascular endothelial cadherin promotes breast cancer progression via transforming growth factor beta signaling. Cancer Res 2008;68:1388-1397.
20. Sulkowska M, Famulski W, Wincewicz A, et al. Levels of VE-cadherin increase independently of VEGF in preoperative sera of patients with colorectal cancer. Tumori 2006;92:67-71.
21. Ko S, Chu KM, Luk JM, et al. CDX2 co-localizes with liver-intestine cadherin in intestinal metaplasia and adenocarcinoma of the stomach. J Pathol 2005;205:615-622.
22. Leung WK, Sung JJ. Review article: intestinal metaplasia and gastric carcinogenesis. Aliment Pharmacol Ther 2002;16: 1209-1216.
23. Filipe MI, Potet F, Bogomoletz WV, et al. Incomplete sulphomucin-secreting intestinal metaplasia for gastric cancer. Preliminary data from a prospective study from three centres. Gut 1985;26:1319-1326.
24. Osborn M, Mazzoleni G, Santini D, Marrano D, Martinelli G, Weber K. Villin, intestinal brush border hydrolases and keratin polypeptides in intestinal metaplasia and gastric cancer; an immunohistologic study emphasizing the different degrees of intestinal and gastric differentiation in signet ring cell carcinomas.

Virchows Arch A Pathol Anat Histopathol 1988;413:303-312.
25. Ito R, Oue N, Yoshida K, et al. Clinicopathological significant and prognostic influence of cadherin-17 expression in gastric cancer. Virchows Arch 2005;447:717-722.
26. Takamura M, Sakamoto M, Ino Y, et al. Expression of liver-intestine cadherin and its possible interaction with galectin-3 in ductal adenocarcinoma of the pancreas. Cancer Sci 2003;94:425-430.
27. Dong W, Yu Q, Xu Y. Altered expression of a Li-cadherin in gastric cancer and intestinal metaplasia. Dig Dis Sci 2007;52:536-542.
28. Su MC, Yuan RH, Lin CY, Jeng YM. Cadherin-17 is a useful diagnostic marker for adenocarcinomas of the digestive system. Mod Pathol 2008;21:1379-1386.
29. Takamura M, Ichida T, Matsuda Y, et al. Reduced expression of liver-intestine cadherin is associated with progression and lymph node metastasis of human colorectal carcinoma. Cancer Lett 2004;212:253-259.
30. Gavard J, Gutkind JS. VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. Nat Cell Biol 2006;8:1223-1234.
31. Bazas VM, Lukyanova NY, Demash DV, Galakhin KO, Myasoedov DV. Relation between cell-to-cell adhesion and angiogenesis and clinico-morphological prognostic factors in patients with gastric cancer. Exp Oncol 2008;30:235-239.
32. Aoyagi K, Kouhuji K, Yano S, et al. VEGF significance in peritoneal recurrence from gastric cancer. Gastric Cancer 2005;8:155-163.
33. Dagnaes-Hansen F, Rasmussen LM, Tilton R, Denner L, Flyvbjerg A. A murine vascular endothelial growth factor antibody inhibits in vivo growth of human Caki-I renal adenocarcinoma. Anticancer Res 2003;23:1625-1630.
34. Rabascio C, Muratori E, Mancuso P, et al. Assessing tumor angiogenesis: increased circulating VE-cadherin RNA in patients with cancer indicates viability of circulating endothelial cells. Cancer Res 2004;64:4373-4377.