Vimentin expression is associated with decreased survival in gastric cancer

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Received September 8, 2010; Accepted November 4, 2010

DOI: 10.3892/or.2011.1185

Abstract. Gastric cancer rich in stromal tissue, such as diffuse-type disease, carries a poor prognosis. In some cancers, expression of vimentin, a mesenchymal maker, is associated with poor survival. The expression of mesenchymal markers such as vimentin is observed after epithelial-mesenchymal transition (EMT), an important initial behavioral change related to the adhesion and migration properties of tumor cells that is required for local tumor invasion. A hallmark of EMT is the loss of E-cadherin. EMT-inducing regulators, including SIP1, Slug, and Twist, repress E-cadherin transcription by interacting with E-cadherin promoter. We investigated the expression of vimentin and EMT-related genes, including SIP1, Slug, and Twist, in frozen cancer tissues and normal tissues by real-time quantitative reverse-transcriptase polymerase chain reaction. Tumor samples were obtained from 106 patients with gastric adenocarcinomas who underwent a gastrectomy. The relation of the expression of these genes to clinicopathological factors and outcomes was studied. Vimentin mRNA was significantly higher in diffuse type compared to intestinal type according to Lauren's classification (p=0.048) and was significantly elevated in patients with recurrent or distant metastatic disease (p=0.049). Immunohistochemically, however, vimentin was detected only in cancer stroma. Twist mRNA expression significantly correlated with tumor depth (p=0.042) and advanced tumor stage (I-II vs. III-IV, p=0.030). E-cadherin immunohistochemical expression was significantly associated with Lauren's histopathological type (p<0.001). Univariate analysis of relapse-free survival showed that tumor depth, lymph node metastasis, Lauren's histopathological type, and vimentin mRNA expression were significant prognostic factors (p<0.001, p=0.013, p=0.011, and p=0.019). On multivariate analysis, vimentin mRNA expression was an independent prognostic factor [hazard ratio (HR)=2.1; 95% confidence interval (CI), 1.0-4.4; p=0.036], coming after tumor depth (HR=9.7; 95%CI, 3.7-24; p<0.001). Vimentin mRNA expression is associated with recurrence or distant metastasis and decreased survival in gastric cancer.

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Key words: vimentin, gastric cancer

Introduction

Gastric cancer is the second leading cause of oncologic death worldwide (1). Clinical outcomes remain poor in patients with advanced disease. Scirrhous-type gastric cancer is characterized by extensive fibrosis with infiltration of sparse tumor cells and clinically has poorer outcomes than any other type of gastric cancer. Interference between tumor cells and host cells modulates tumor-cell phenotype and tumor progression in the stromal microenvironment (2). Some molecular markers expressed in cancer stroma may influence tumor progression or the survival of patients. Vimentin is a type III intermediate filament protein that is involved in cell attachment, migration, and signaling (3,4). It is present in stromal cells, including fibroblasts, endothelial cells, macrophages, neutrophils, and lymphocytes (5). One study found that vimentin was an independent prognostic factor, detected immunohistochemically only in the stroma of colon cancer (6). In other studies, vimentin was detected in both the parenchyma and mesenchyma of various types of cancers, including breast cancer, non-small cell lung cancer, and oral squamous cell carcinoma (7-12). Vimentin expression in cancer epithelial cells has been associated with metastasis and poor survival (10,12). In gastric cancer, the prognostic value of vimentin remains unclear.

At the molecular level, the expression of vimentin in cancer cells is regulated by epithelial-mesenchymal transition (EMT), considered an important change in the adhesion and migration properties of cancer cells (13). EMT is required for local tumor invasion, an initial step toward distant metastasis. The hallmark of EMT is the loss of E-cadherin, a central component of cell-to-cell adhesion junctions that contributes to the maintenance of cell polarity and environment. Loss of this protein leads to destabilization of the epithelial architecture (14). E-cadherin plays a critical role in the suppression of tumor progression. Down-regulation or inactivation of E-cadherin was shown to be caused by mutation or hypermethylation in gastric cancer (15,16). Low expression

of E-cadherin has been associated with better survival in gastric cancer (17). Several EMT-inducing regulators, including SIP1 (also known as ZEB2), Slug (also known as SNAI2), and Twist, repress E-cadherin transcription by interacting with specific E-boxes of the proximal E-cadherin promoter (18). Expression of these transcription factors correlate with the histopathological type of gastric cancer (19). Poorer survival has been demonstrated in cancers with high expression of SIP1 (20), Slug (21-24), and Twist (25-27).

The present study examined correlations of the expression of EMT-related modulators, including E-cadherin, Twist, SIP1, Slug, and vimentin, with clinicopathological features and outcomes in gastric cancer.

Patients and methods

Patients. The study group comprised 106 patients with primary gastric adenocarcinomas who underwent gastrectomy from January 2004 through December 2007 at the Department of Esophagogastric Surgery, Tokyo Medical and Dental University. Each tumor was classified according to the tumor-node-metastasis classification recommended by the International Union against Cancer. All patients were evaluated for recurrent disease by diagnostic imaging, including computed tomography, ultrasonography, and endoscopy, every 3-6 months. The median follow-up time was 48 months (range: 29-72). Recurrent disease was diagnosed in 35 (35%) of 99 patients who had undergone R0 operations. Thirty-two patients (30%) died of metastatic gastric cancer, and 11 (10%) died of other diseases without recurrence. This study was approved by the Institutional Review Board of Tokyo Medical and Dental University. Written informed consent was obtained from all patients.

RNA extraction and cDNA synthesis. Immediately after surgery, a small piece of gastric cancer tissue and one of matched adjacent normal mucosa (taken from the borders of the surgical specimen) were separately placed directly in RNA stabilization reagent (RNAlater®, Qiagen, Valencia, CA) and stored at -80˚C until further analysis. Total RNA for each sample was extracted using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The concentration of total RNA was determined by measuring absorption at 260 and 280 nm with an ultraviolet spectrophotometer (Beckman Coulter, Fullerton, CA). For cDNA synthesis, 10 μg of total RNA was reverse-transcribed into cDNA samples using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Real-time quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). Expression levels of vimentin, SIP1, Slug, and Twist, as well as of ß-actin, used as an endogenous control, were determined by real-time quantitative PCR using a 7300 Real-Time PCR system (Applied Biosystems). TaqMan gene expression assays were purchased from Applied Biosystems (vimentin, Hs00185584_m1; SIP1, Hs00207691_m1; Slug, Hs00950344_m1; Twist, Hs01675818_s1; ß-actin, Hs99999903_m1). The PCR reaction was carried out using TaqMan Universal PCR Master mix (Applied Biosystems) with 1 μl of cDNA in a 24-μl final reaction volume. Thermal cycling conditions were: 50˚C for 2 min, 95˚C for 10 min, 40 cycles of 15-sec denaturation at 95˚C, and 1 min of annealing at 60˚C. cDNA synthesized by HCT15 was used as the calibrator. Each sample was run in duplicate for both the target and endogenous genes. The amounts of vimentin, SIP1, Slug, and Twist normalized to the endogenous control and relative to the calibrator were determined by the comparative Ct method for relative quantification ($\Delta\Delta$Ct method) using Relative Quantification Study software (7300 Sequence Detection system, version 1.4, Applied Biosystems).

Immunohistochemical analysis and evaluation. Immunohistochemical analyses of E-cadherin and vimentin were performed by the streptavidin-biotin method using a Histofine SAB-PO kit (Nichirei Co., Tokyo, Japan). Monoclonal mouse anti-human antibodies against E-cadherin and vimentin were purchased from Takara Bio, Inc. (Shiga, Japan) and Dako (Glostrup, Denmark). All available hematoxylin and eosin-stained slides of the surgical specimens were reviewed. For each case, representative paraffin blocks were selected for immunohistochemical studies. Sections (4 μm) were cut from formalin-fixed, paraffin-embedded tissue blocks. After deparaffinization and rehydration, antigen retrieval treatment was performed at 121˚C (autoclave) for 5 min in 10 nmol/l sodium citrate buffer (pH 9.0), followed by treatment with 3% hydrogen peroxide for 15 min to quench endogenous peroxidase activity. Nonspecific binding was blocked by treating the slides with 5% EzBlock (including 5% normal goat serum and 0.1% Tween-20) for 60 min at room temperature. The slides were incubated with primary antibody (dilution 1:50) overnight at 4˚C. Immunodetection was performed by the conventional streptavidin-biotin method with a Nichirei SAB-PO kit (Nichirei Co.). The slides were counterstained with 1% Mayer's hematoxylin.

The membranous expression of E-cadherin was evaluated and classified semi-quantitatively into 4 categories as described by Gabbert *et al* (17). The extent of membranous staining was scored as 3+ (>60%), 2+ (20-60%), 1+ (<20%), or 0 (0%). Staining intensity was not relevant in this evaluation. Tumors with a score of 3+ were classified as having preserved expression, and all others were classified as having decreased expression. We also attempted to evaluate vimentin expression, but no cancer cells were stained.

Statistical analysis. Differences between groups in mRNA expression were evaluated with the Mann-Whitney U-test, and those in E-cadherin expression were assessed with the χ^2 test. Kaplan-Meier curves were plotted to assess the relations of the expression of each gene to relapse-free survival (RFS) and disease-specific survival (DSS). Survival curves were compared using the log-rank test. Only recurrent disease was considered an event in the calculation of RFS. The mRNA expression of tumors was classified as being higher or lower than the median value, and the relations of the expression levels to RFS and DSS were examined. Multivariate proportional Cox models were used to assess prognostic significance. $p<0.05$ were considered to indicate statistical significance. All analyses were performed with the statistical software package SPSS 17 (SPSS Japan Inc., Tokyo, Japan).

Table I. Correlation coefficient among mRNA expression in cancer tissue and non-cancer tissue.

A, Cancer tissue

	Correlation	Slug	Twist	Vimentin
SIP1	Correlation coefficient	0.58	0.30	0.37
	p-value	<0.001	0.002	<0.001
Slug	Correlation coefficient		0.40	0.074
	p-value		<0.001	0.45
Twist	Correlation coefficient			0.22
	p-value			0.026

B, Non-cancer tissue

	Correlation	Slug	Twist	Vimentin
SIP1	Correlation coefficient	0.62	0.33	0.42
	p-value	<0.001	0.001	<0.001
Slug	Correlation coefficient		0.27	0.30
	p-value		0.005	0.002
Twist	Correlation coefficient			0.26
	p-value			0.008

Results

Gene expression in cancer tissue and corresponding non-cancer tissue. The mRNA expression of vimentin and Twist were significantly higher in cancer tissue than in non-cancer tissue (median value of vimentin, 0.20 vs. 0.11, p<0.001; Twist; 0.99 vs. 0.32, p=0.001). In contrast, Sip1 mRNA expression was significantly lower in cancer tissue than in non-cancer tissue (0.076 vs. 0.096, p<0.001), and Slug expression did not differ significantly between the two types of tissue (0.43 vs. 0.45, p=0.18). Significant positive correlations were observed among SIP1, Slug, and Twist expression in both cancer tissue and non-cancer tissue. Vimentin expression was significantly associated with SIP1 expression and Twist expression in both types of tissue, but with Slug expression only in cancer tissue (Table I).

Immunoreactivity of E-cadherin and vimentin (Fig. 1). Expression of membranous E-cadherin was decreased in 46 tumors (43%). The staining score was 2+ in 17 tumors (16%), 1+ in 19 (18%), and 0 in 10 (9%). Vimentin was strongly expressed in stromal cells of both cancer and normal tissue, but was not expressed at all in cancer cells.

Relation between immunohistochemical E-cadherin expression and the expression of other genes (Table II). Vimentin mRNA expression was significantly higher in tumors with decreased immunohistochemical expression of E-cadherin than in tumors with preserved expression (p=0.023). The mRNA expression of other genes was not significantly associated with E-cadherin expression.

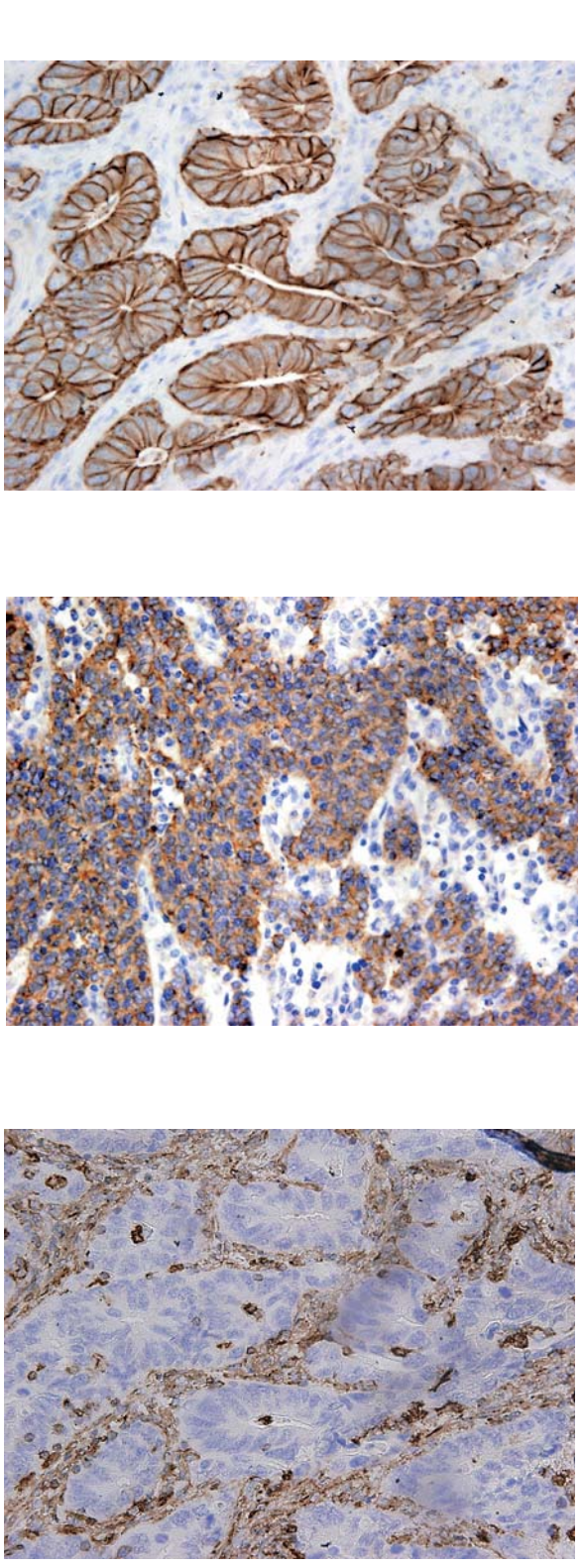

Figure 1. Representative gastric carcinomas showing immunostaining for E-cadherin predominantly in the membrane (A) and predominantly in the cytoplasm (B); immunostaining for vimentin predominantly in cancer stroma (C), magnification, x400.

Relation between expression of each gene and clinicopathological variables (Table III). Vimentin mRNA expression was significantly higher in diffuse-type tumors (including 5 unclassified-type tumors) than in intestinal-type tumors

Table II. Correlations between the mRNA expression levels of EMT-related genes and immunohistochemistry of E-cadherin.

	E-cadherin		
	Decreased (n=46)	Preserved (n=60)	p
SIP1	0.072 (0.013-0.74)	0.084 (0.009-0.89)	0.79
Slug	0.28 (0.051-14)	0.43 (0.040-4.5)	0.092
Twist	1.1 (0.018-33)	0.87 (0.024-48)	0.6
Vimentin	0.26 (0.016-1.7)	0.17 (0.016-1.6)	0.023

(p=0.048) and was significantly higher in patients with recurrent or distant metastatic disease (p=0.049). High Twist mRNA expression significantly correlated with tumor depth (p=0.042) and advanced tumor stage (I-II vs. III-IV, p=0.030). SIP1 and Slug mRNA expression was not significantly associated with any clinicopathological factor. Preserved immunoreactivity of E-cadherin was significantly associated with Lauren's intestinal type (p<0.001), and tended to correlate negatively with tumor depth (p=0.063).

Relation between expression of each gene and RFS (Table IV). On univariate analysis, patients with high vimentin mRNA expression had significantly poorer RFS than those with low expression (p=0.019). Tumor depth, lymph node metastasis, and Lauren's histopathological type were associated significantly with RFS (p<0.001, p=0.013, and p=0.011). On multivariate analysis, vimentin mRNA expression was an independent prognostic factor of RFS [hazard ratio (HR)=2.1; 95% confidence interval (CI), 1.0-4.4; p=0.036], coming after tumor depth (HR=9.7; 95%CI, 3.7-24; p<0.001).

Relation between expression of each gene and DSS (Table V). On univariate analysis, patients with high vimentin mRNA expression had significantly poorer DSS than those with low expression (p=0.046). Tumor depth, lymph node metastasis, and Lauren's histopathological type significantly correlated with DDS (p<0.001, p=0.007, and p=0.007). On multivariate analysis, vimentin mRNA expression was not an independent prognostic factor of DSS (HR=1.8; 95% CI, 0.81-3.8; p=0.16).

Table III. Correlations between the expression levels of each gene and clinicopathological factors.

		SIP1 mRNA		Slug mRNA		Twist mRNA		Vimentin mRNA		E-cadherin immunohistochemistry		
	n (%)	Median	p	Median	p	Median	p	Median	p	Decreased	Preserved	p
Age (years)												
<71	57 (54)	0.072	0.79	0.38	0.85	0.71	0.10	0.22	0.38	24	33	0.77
≥71	49 (46)	0.082		0.34		1.20		0.19		22	27	
Gender												
Male	84 (79)	0.080	0.35	0.36	0.46	1.1	0.081	0.22	0.29	35	49	0.48
Female	22 (21)	0.063		0.31		0.75		0.17		11	11	
Histopathology (Lauren)												
Intestinal	47 (44)	0.076	0.77	0.38	0.47	1.0	0.54	0.17	0.048	10	37	<0.001
Diffuse/Unclassified	59 (56)	0.072		0.33		0.88		0.25		36	23	
Tumor depth												
T1/2	58 (55)	0.074	0.94	0.35	0.59	0.82	0.042	0.17	0.20	20	37	0.063
T3/4	48 (45)	0.077		0.37		1.29		0.24		26	23	
Lymph node metastasis												
Negative (N0)	31 (29)	0.071	0.69	0.53	0.08	0.69	0.60	0.17	0.28	12	19	0.53
Positive (N1/2/3)	75 (71)	0.077		0.34		1.1		0.22		34	41	
Stage												
I/II	53 (50)	0.076	0.76	0.36	0.65	0.69	0.030	0.17	0.11	20	33	0.24
III/IV	53 (50)	0.077		0.35		1.2		0.24		26	27	
Recurrence or distant metastasis												
No	62 (58)	0.079	0.72	0.37	0.23	0.95	0.30	0.17	0.049	23	39	0.12
Yes	44 (42)	0.075		0.34		1.1		0.25		23	21	

Table IV. Prognostic factors in univariate and multivariate Cox proportional-hazards regression models for RFS.

	Univariate			Multivariate		
	HR	95% CI	p	HR	95% CI	p
Age (years)						
<71	1.0					
≥71	0.63	0.32-1.2	0.18			
Gender						
Male	1.0					
Female	0.60	0.23-1.5	0.28			
Histopathology (Lauren)						
Intestinal	1.0			1.0		
Diffuse/Unclassified	2.6	1.2-5.3	0.011	1.0	0.46-2.2	0.99
Tumor depth						
T1/2	1.0			1.0		
T3/4	9.7	4.2-22	<0.001	9.0	3.7-24	<0.001
Lymph node metastasis						
Negative (N0)	1.0			1.0		
Positive (N1/2/3)	3.0	1.3-7.2	0.013	1.0	0.45-2.2	0.93
Vimentin mRNA						
Low	1.0			1.0		
High	2.3	1.1-4.6	0.019	2.1	1.0-4.4	0.036
Sip1 mRNA						
Low	1.0					
High	1.1	0.56-2.1	0.82			
Slug mRNA						
Low	1.0					
High	0.76	0.40-1.5	0.42			
Twist mRNA						
Low	1.0					
High	1.1	0.59-2.1	0.73			
E-cadherin staining						
High	1.0					
Low	1.5	0.77-2.8	0.25			

Tumor depth was the only significant independent predictor of DDS (HR=24; 95% CI, 5.1-110; p<0.001).

Discussion

Our results showed that high vimentin mRNA expression significantly correlated with poor survival in gastric cancer. Vimentin is a major intermediate filament protein of mesenchymal cells and serves as an organizer of a number of critical proteins involved in cell attachment, migration, and signaling. Vimentin promotes the formation and turnover of adhesive structures by regulating integrins, which are heterodimer transmembrane cell adhesion receptors, especially in endothelial cells (28,29). Vimentin can affect the operation of protein complexes on the cell membrane and signaling pathways, such as MAP kinase (30,31). Therefore, vimentin expression in parenchymal or mesenchymal cells of cancer tissue may influence tumor progression and the survival of patients. We suspected that vimentin expression in stromal cells is associated with poor survival in gastric cancer, because strong immunoreactivity of vimentin was detected only in stromal cells of cancer tissue. Our results are consistent with the findings of Ngan *et al*, who demonstrated that vimentin was detected only in stromal cells of colon cancer and not in cancer cells (6). They revealed that vimentin expression assessed by computer imaging in cancer stroma correlated significantly with worse survival. However, their evaluation technique was complex and is generally not used.

Table V. Prognostic factors in univariate and multivariate Cox proportional-hazards regression models for DSS.

	Univariate			Multivariate		
	HR	95% CI	p	HR	95% CI	p
Age (years)						
<71	1.0					
≥71	0.72	0.35-1.5	0.36			
Gender						
Male	1.0					
Female	0.99	0.41-2.4	0.99			
Histopathology (Lauren)						
Intestinal	1.0			1.0		
Diffuse/Unclassified	3.1	1.3-7.3	0.007	1.1	0.46-2.7	0.81
Tumor depth						
T1/2	1.0			1.0		
T3/4	26.0	6.3-110	<0.001	24.0	5.1-110	<0.001
Lymph node metastasis						
Negative (N0)	1.0			1.0		
Positive (N1/2/3)	5.2	1.6-17	0.007	1.1	0.31-4.1	0.86
Vimentin mRNA						
Low	1.0			1.0		
High	2.1	1.0-4.5	0.041	1.8	0.81-3.8	0.16
Sip1 mRNA						
Low	1.0					
High	1.2	0.63-2.6	0.48			
Slug mRNA						
Low	1.0					
High	0.98	0.49-2.0	0.97			
Twist mRNA						
Low	1.0					
High	1.3	0.63-2.6	0.51			
E-cadherin staining						
High	1.0					
Low	1.7	0.83-3.4	0.15			

Vimentin has been observed in both stromal cells and cancer cells in breast, lung, and oral cancer (3,10-12). Liu *et al* reported that immunoreactivity of vimentin in cancer cells was associated with tumor progression and worse survival in patients with oral squamous cell carcinomas (12). In that study, vimentin was expressed at the invasive front of cancer cells. We found no vimentin expression in sparse cells of cancer stroma. Future studies should separate stromal cells from cancer tissue by laser-capture microdissection techniques, but our method for the analysis of whole samples of cancer tissue is more convenient and may be better suited for routine use.

Immunoreactivity of E-cadherin was significantly decreased in diffuse-type gastric cancer in the present study, consistent with the results of previous studies (17,32). Inactivation of E-cadherin by mutations or hypermethylation has been demonstrated in gastric cancer (15,33-35). In addition, E-cadherin mutations have been detected only in diffuse-type gastric cancer (15). Decreased expression of E-cadherin in the membrane of gastric cancer cells has been significantly associated with poor survival, and cytoplasmic expression of E-cadherin was observed in diffuse-type disease (17). We evaluated the expression of E-cadherin by immunohistochemical staining, because decreased membranous expression of E-cadherin appears to be essential for the initiation of EMT. Vimentin mRNA expression was significantly elevated in tumors with decreased E-cadherin expression in the present study, although it remains unclear whether EMT acted on cancer stroma to increase vimentin expression.

Loss of E-cadherin function during tumor progression can be caused by transcriptional repression binding to CDH1-E box elements, including Slug, Sip1, and Twist. Therefore, tumors with higher expression of these elements might have lower expression of E-cadherin. Alves *et al* showed that Slug was up-regulated in gastric cancers in which E-cadherin was down-regulated (35). In contrast, we found a slight but insignificant trend toward a positive association between Slug mRNA expression and E-cadherin immunoreactivity. This discrepancy might have been caused by the different methods used to evaluate mRNA expression (cancer to non-cancer ratio vs. quantitative value of cancer) or to analyze E-cadherin (gene expression vs. immunohistochemistry).

A negative correlation between the immunohistochemical expression of Slug and E-cadherin was reported in esophageal squamous cell carcinoma (21), whereas no correlation between Slug and E-cadherin was found in colorectal cancer (23) or ovarian cancer (24). There was no correlation of Slug or SIP1 expression with survival in the present study. However, Slug expression has been associated with metastasis or recurrence in breast cancer (25), as well as with worse survival in ovarian carcinoma (22), colon cancer (23), and esophageal squamous cell carcinoma (21). The SIP1/E-cadherin ratio correlated significantly with survival in ovarian cancer (22).

Rosivatz *et al* showed that up-regulated SIP1 was associated with intestinal-type gastric cancer and therefore suggested that SIP1 might be a main component of the E-cadherin dependent pathway in that type of cancer (19). They also suggested that Twist might have a key role in diffuse-type gastric cancer because it was up-regulated in that type. However, neither SIP1 nor Twist was associated with histological type in the present study. Positive correlations among Slug, SIP1, and Twist in our study were in agreement with the results of Yoshida *et al* in ovarian cancer. Twist overexpression has been associated with advanced stage or poor survival in various cancers (24,27,36). In the present study, Twist was not associated with survival, but was significantly related to advanced stage. This finding might be explained by the lack of a relation between Twist and recurrent disease.

In conclusion, vimentin mRNA expression was associated with recurrence or distant metastasis and poor survival in gastric cancer; however, immunohistochemical expression of vimentin was detected only in cancer stroma.

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