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## Snail plays a key role in E-cadherin-preserved esophageal squamous cell carcinoma

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**Abstract.** Snail is a zinc-finger transcription factor that triggers the epithelial-mesenchymal transition (EMT) by directly repressing E-cadherin expression. However, the relationship between E-cadherin and Snail expression remains unclear in esophageal squamous cell carcinoma (ESCC). The purpose of the present study was to evaluate the clinical significance of E-cadherin and Snail expression in ESCC. Immunohistochemistry was used to investigate the expression of E-cadherin and Snail proteins in 194 patients with ESCC. The relationship between expression of these proteins and clinicopathological factors was analyzed, and the usefulness of Snail in disease prognosis was evaluated in relation to E-cadherin expression. E-cadherin expression was preserved in 41.2% of tumors, and Snail expression was confirmed in 61.7%. Tumors with reduced E-cadherin expression invaded deeper ( $P < 0.0001$ ), had more lymph node metastasis ( $P < 0.0001$ ) and had more lymphatic invasion ( $P = 0.0011$ ) than tumors with preserved expression. Tumors that were positive for Snail expression invaded deeper ( $P = 0.0385$ ), had more distant lymph node metastasis (pM) ( $P = 0.0051$ ) and had a more advanced stage ( $P = 0.0044$ ) than those that were negative for Snail expression. Snail expression was not significantly correlated with reduced E-cadherin expression. Patients with reduced E-cadherin expression or positive Snail expression had poor clinical outcomes. In the preserved E-cadherin group, overall survival rate was better in patients with negative Snail expression than in those with positive Snail expression ( $P = 0.035$ ). Snail appears to play a key role in preserved E-cadherin expression. Further studies on other molecules in the pathways related to reduced E-cadherin expression in ESCC from the view-point of EMT are necessary.

### Introduction

E-cadherin is a member of the cadherin family, which is known to play an important role in the regulation of intercellular adhesion in epithelial tissues (1). Because one of the first changes in the metastatic process is a decrease in this adhesion (2,3), it has been postulated that abnormal or reduced E-cadherin expression acts to facilitate tumor invasion and the subsequent formation of metastases. Indeed, E-cadherin expression has been found to be closely associated with tumor invasiveness, dedifferentiation, formation of metastases and prognosis in various human carcinomas (4-8). In esophageal squamous cell carcinoma (ESCC), loss of E-cadherin expression is associated with tumor invasiveness, metastasis and prognosis (9,10).

The change in cancer cell phenotype to increased mortality and invasiveness is reminiscent of the epithelial-mesenchymal transition (EMT) that occurs during embryonic development. Snail is a zinc-finger transcription factor that triggers EMT by directly repressing E-cadherin expression (11). Snail overexpression in different epithelial cells leads to dramatic conversion towards a fibroblastic phenotype at the same time that E-cadherin expression is lost, and invasive and migratory properties are acquired (12). At present, the role of Snail in ESCC has not been elucidated. The purpose of the present study was to examine the clinical significance of Snail expression in ESCC and the correlation between Snail expression and E-cadherin expression in ESCC.

### Materials and methods

*Patients and specimens.* Subjects were 194 patients with ESCC who underwent esophagectomy with lymph node dissection between 1988 and 1998 at Kagoshima University Hospital, Kagoshima, Japan. The patients, 176 males and 18 females, ranged in age from 36 to 85 years (mean, 63.6 years). None of these patients underwent endoscopic mucosal resection, palliative resection, preoperative chemotherapy or radiotherapy, and none of them had synchronous or metachronous multiple cancer in other organs. Specimens of cancer tissues and adjacent non-cancerous tissue were collected from the patients after informed consent was obtained, in accordance with institutional guidelines.

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*Key words:* cell adhesion, transcriptional factor, immunohistochemistry, prognosis

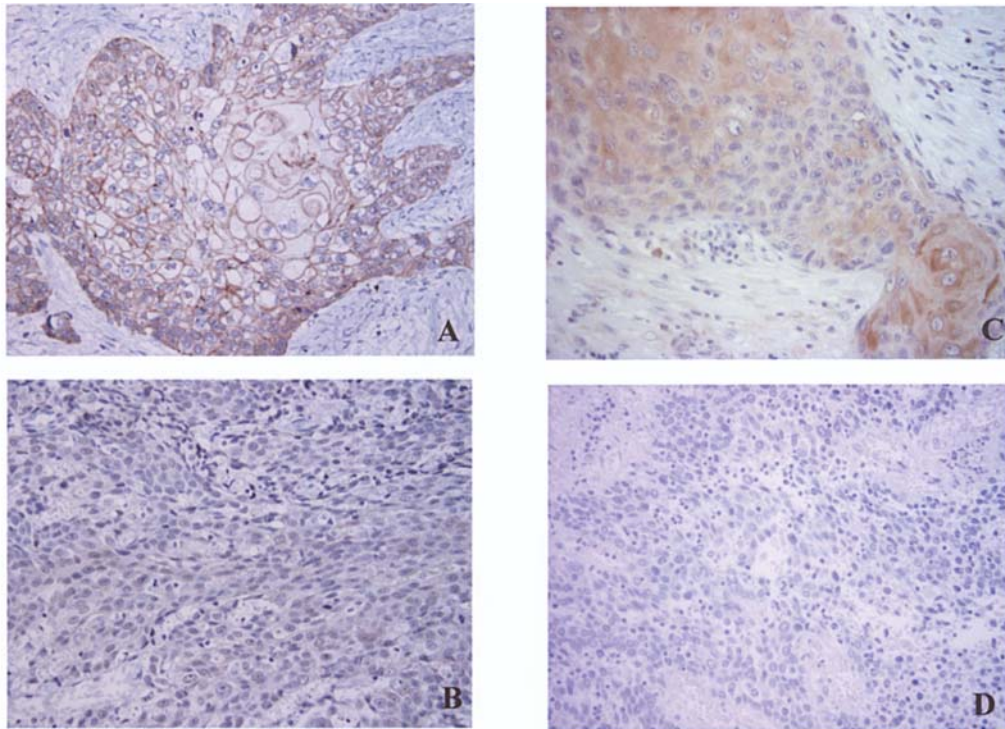


Figure 1. Expression of E-cadherin and Snail in esophageal squamous cell carcinomas. (A) Preserved expression of E-cadherin is detected in the cell membrane (x400). (B) Reduced expression of E-cadherin (x400). (C) Expression of Snail is detected in the cytoplasm (x400). (D) No expression of Snail (x400).

Using the tumor node metastasis (TNM) classification of the International Union against Cancer (13), all of the M1 tumors were attributable to distant lymph node metastases (Table I). All patients were followed-up after discharge, with X-ray examination and tumor marker assays (squamous cell carcinoma antigen and carcinoembryonic antigen) performed every 1-3 months, computed tomography performed every 3-6 months, and ultrasonography performed every 6 months. Bronchoscopic and endoscopic examination was performed when necessary. Postoperative follow-up data were obtained from all patients, with a median follow-up period of 30 months (range, 1-173 months).

**Immunohistochemical staining and evaluation.** Specimens were cut into 3- $\mu$ m sections, which were mounted on glass slides. Immunohistochemical staining was performed using the streptavidin-biotin peroxidase method as described previously (14). Briefly, after sections were deparaffinized in xylene and dehydrated in ethanol, endogenous peroxidase activity was blocked by incubating sections for 15 min in 0.3% H<sub>2</sub>O<sub>2</sub> in methanol. Sections were then heated in citrate buffer (0.01 M, pH 6.5) at 120°C for 10 min (microwave oven, 450 W) to reveal antigens. After cooling, sections were pre-incubated in 3% skim milk/PBS for 30 min. Sections were then incubated with either anti-E-cadherin monoclonal antibody (1:100; E-cadherin; Takara Biotechnology, Inc., Takara, CA) or anti-Snail polyclonal antibody (1:100; SNAI 1:E-18; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. Sections were then incubated with biotinylated anti-goat IgG and streptavidin-biotin peroxidase [Histofine SAB-PO (G) kit, Nichirei Corp.], and reactions were visualized using diaminobenzidine tetrahydrochloride.

Evaluation of immunohistochemistry was performed by 2 independent investigators (S.N. and Y.U.). Expression of E-cadherin was compared between malignant cells and normal epithelial cells located distant from the tumor. Tumor cells that stained as strongly as normal epithelial cells were considered to have preserved expression, whereas those that exhibited weaker staining patterns than normal epithelial cells or did not stain at all were considered to have reduced expression (15). For Snail, positive expression was defined as detectable immunoreaction in the perinuclear and other cytoplasmic regions in >10% of cancer cells. To evaluate expression of Snail and E-cadherin, 10 fields (within the tumor and at the invasive front) were selected, and expression in 1000 tumor cells (100 cells/field) was evaluated at high magnification (x200).

**Statistical analysis.** Statistical analysis of group differences was performed using the  $\chi^2$  test and t-test. The Kaplan-Meier method was used for survival analysis, and differences in survival were estimated using the log-rank test. Prognostic factors were examined by univariate and multivariate analyses (Cox proportional hazards regression model). A P-value of <0.05 was considered to indicate statistical significance. All statistical analyses were performed using JMP 5 for Windows software (SAS Institute Inc. Cary, NC, USA).

## Results

**Expression of E-cadherin and Snail in ESCC.** E-cadherin expression was observed on the cell membranes of cancer cells, indicating preserved expression, in 41.2% of all patients (80 of 194) (Fig. 1A and B). Expression of Snail was observed

Table I. Relationship between E-cadherin expression and clinicopathological findings.

	E-cad(+) n=80 (41.2%)	E-cad(-) n=114 (58.8%)	P-value
Age	63.5±8.2	63.6±7.8	0.9379
Gender (male/female)	72/8	104/10	0.7716
Tumor location			
Upper	12 (15.0)	14 (12.3)	0.7648
Middle	42 (52.5)	58 (50.9)	
Lower	26 (32.5)	42 (36.8)	
Histology			
Well	28 (35.0)	45 (39.5)	0.0342
Moderate	45 (56.3)	46 (40.3)	
Poor	7 (8.7)	23 (20.2)	
pT			
T1	33 (41.3)	11 (9.6)	0.0001
T2	9 (11.2)	13 (11.4)	
T3	22 (27.5)	64 (56.1)	
T4	16 (20.0)	26 (22.9)	
pN			
pN0	56 (70.0)	28 (24.6)	0.0001
pN1	24 (30.0)	86 (75.4)	
pM			
pM0	69 (86.3)	69 (60.5)	<0.0001
pM1	11 (13.8)	45 (39.5)	
Stage			
1	28 (12.4)	7 (38.8)	0.0001
2A	22 (22.0)	17 (9.4)	
2B	6 (9.5)	7 (16.5)	
3	13 (24.8)	38 (20.0)	
4	11 (31.4)	45 (15.3)	
Lymphatic invasion			
Negative	44 (55.0)	36 (31.6)	0.0011
Positive	36 (45.0)	78 (68.4)	
Venous invasion			
Negative	58 (72.5)	73 (64.0)	0.2152
Positive	22 (27.5)	41 (36.0)	

in the cytoplasm of cancer cells in 61.7% of all patients (84 of 194) (Fig. 1C and D).

*Relationship between E-cadherin and Snail expression and clinicopathological features.* E-cadherin expression was significantly associated with the following clinicopathological parameters: depth of tumor invasion, lymph node metastasis, stage and lymphatic invasion (Table I). E-cadherin expression

Table II. Relationship between Snail expression and clinicopathological findings.

	Snail(+) n=84 (61.7%)	Snail(-) n=110 (38.3%)	P-value
Age	64.3±7.8	63.1±8.0	0.2871
Gender (male/female)	79/5	97/13	0.1540
Tumor location			
Upper	8 (9.5)	18 (16.4)	0.6747
Middle	41 (48.8)	59 (53.6)	
Lower	35 (41.7)	33 (30.0)	
Tumor depth			
T1	11 (13.1)	33 (30.0)	0.0385
T2	9 (10.7)	13 (11.8)	
T3	43 (51.2)	43 (39.1)	
T4	21 (25.0)	21 (19.1)	
Histology			
Well	41 (48.8)	32 (29.1)	0.0186
Moderate	33 (39.3)	58 (52.7)	
Poor	10 (11.9)	20 (18.2)	
pN			
pN0	31 (36.9)	53 (48.2)	0.1162
pN1	53 (63.1)	57 (51.8)	
pM			
pM0	51 (60.7)	87 (79.1)	0.0051
pM1	33 (39.3)	23 (20.9)	
Stage			
1	9 (10.7)	26 (23.6)	0.0044
2A	15 (17.9)	24 (21.8)	
2B	2 (2.4)	11 (10.0)	
3	25 (29.8)	26 (23.6)	
4	33 (39.2)	23 (21.0)	
Lymphatic invasion			
Negative	30 (35.7)	50 (45.5)	0.1721
Positive	54 (64.3)	60 (54.5)	
Venous invasion			
Negative	53 (63.1)	78 (70.9)	0.2495
Positive	31 (36.9)	32 (29.1)	
E-cad expression			
Positive	29 (34.5)	51 (46.4)	0.0969
Negative	55 (65.5)	59 (53.6)	

was related to histological differentiation (P=0.0342). Tumors with reduced E-cadherin expression invaded deeper (P<0.0001), had more lymph node metastasis (P<0.0001), had more distant

Table III. Relationship between Snail expression and clinicopathological findings according to E-cadherin expression.

	E-cad(+)		P-value	E-cad(-)		P-value
	Snail(+) n=29, (%)	Snail(-) n=51, (%)		Snail(+) n=55, (%)	Snail(-) n=59, (%)	
<b>Tumor location</b>						
Upper	1 (3.4)	11 (21.6)	0.0214	7 (12.7)	7 (11.9)	0.9344
Middle	14 (48.3)	28 (54.9)		27 (49.1)	31 (52.5)	
Lower	14 (48.3)	12 (23.5)		21 (38.2)	21 (35.6)	
<b>Tumor depth</b>						
T1	7 (24.1)	26 (51.0)	0.0364	4 (7.3)	7 (11.9)	0.8451
T2	2 (6.9)	7 (13.7)		7 (12.7)	6 (10.2)	
T3	12 (41.4)	10 (19.6)		31 (56.4)	33 (55.9)	
T4	8 (27.6)	8 (15.7)		13 (23.6)	13 (22.0)	
<b>Histology</b>						
Well	14 (48.3)	14 (27.5)	0.1218	27 (49.1)	18 (30.5)	0.0628
Moderate	12 (41.1)	33 (64.7)		21 (38.2)	25 (42.4)	
Poor	3 (10.3)	4 (7.8)		7 (12.7)	16 (27.1)	
<b>pN</b>						
pN0	18 (62.1)	38 (74.5)	0.2431	13 (23.6)	15 (25.4)	0.8247
pN1	11 (37.9)	13 (25.5)		42 (76.4)	44 (74.6)	
<b>pM</b>						
pM0	22 (75.9)	47 (92.2)	0.0419	29 (52.7)	40 (67.8)	0.1000
pM1	7 (24.1)	4 (7.8)		26 (47.3)	19 (32.2)	
<b>Stage</b>						
1	7 (24.1)	21 (41.3)	0.0037	2 (3.6)	5 (8.5)	0.4246
2A	8 (27.7)	14 (27.5)		7 (12.7)	10 (16.9)	
2B	0 (0)	6 (11.7)		2 (3.6)	5 (8.5)	
3	7 (24.1)	6 (11.7)		18 (32.8)	20 (33.9)	
4	7 (24.1)	4 (7.8)		26 (47.3)	19 (32.2)	
<b>Lymphatic invasion</b>						
Negative	15 (51.7)	29 (56.9)	0.6570	15 (27.3)	21 (35.6)	0.3396
Positive	14 (48.3)	22 (43.1)		40 (72.7)	38 (64.4)	
<b>Venous invasion</b>						
Negative	19 (65.5)	39 (76.5)	0.2915	34 (61.8)	39 (66.1)	0.6339
Positive	10 (34.5)	12 (23.5)		21 (38.2)	20 (33.9)	

nodal metastasis (pM) ( $P < 0.0001$ ) and had more lymphatic invasion ( $P = 0.0011$ ) than tumors with preserved expression.

Snail expression was related to histological differentiation ( $P = 0.0186$ ). Tumors that were positive for Snail expression invaded deeper ( $P = 0.0385$ ), had more distant lymph node metastasis (pM) ( $P = 0.0051$ ) and had a more advanced stage ( $P = 0.0044$ ) than those that were negative for Snail expression ( $P = 0.0385$ ,  $0.0051$  and  $0.0044$ , respectively) (Table II).

Of the 80 tumors with preserved E-cadherin expression, 29 (36.3%) were positive for Snail expression. On the other hand, 55 tumors (46.0%) were positive for Snail expression among the 114 tumors with reduced E-cadherin expression. Significant differences were not found between E-cadherin and Snail expression ( $P = 0.0969$ ).

*Relationship between Snail expression and clinicopathological variables according to E-cadherin expression.* Tumors were divided into the preserved E-cadherin group and the reduced E-cadherin group. In the preserved E-cadherin group, expression of Snail was related to depth of tumor invasion, distant lymph node metastasis (pM1), and stage. In the reduced E-cadherin group, expression of Snail was not related to any clinicopathologic factors (Table III).

*Relationship between prognosis and expression of E-cadherin and Snail.* Seven of the patients died of postoperative complications within 30 days of the beginning of the study period, leaving 187 patients for survival analysis. The 5-year survival rate of patients with tumors with preserved E-cadherin

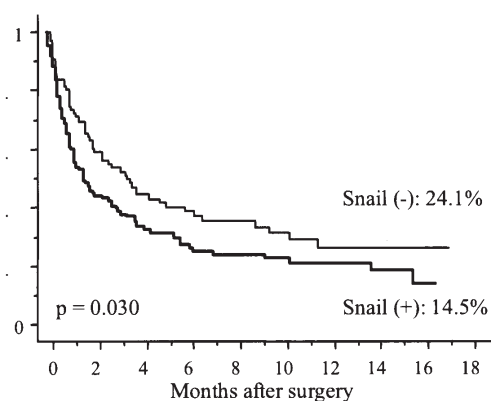
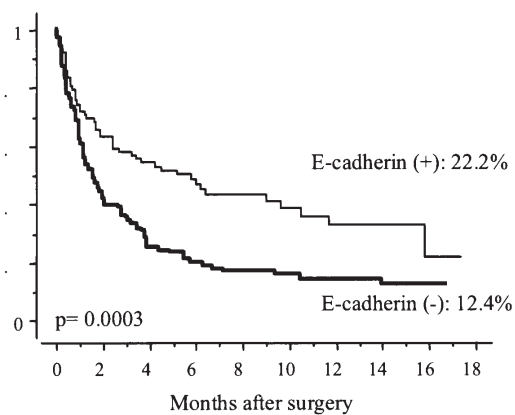


Figure 2. The postoperative overall survival curve of patients according to the expression of E-cadherin (A) or Snail (B). There was a significant difference in survival between the patients with preserved and reduced expression of E-cadherin ( $P=0.003$ ). There also was a significant difference in overall survival between the patients with (+) and without (-) expression of Snail ( $P=0.030$ ).

expression was 22.2%, whereas the rate for reduced E-cadherin expression was 12.4%. There was a significant difference in 5-year survival between preserved and reduced expression of E-cadherin ( $P=0.003$ ; Fig. 2A). The 5-year survival rate of patients negative and positive for expression of Snail was 24.1% and 14.5%, respectively, and the difference was significant ( $P=0.030$ ) (Fig. 2B).

*Relationship between prognosis and Snail expression in preserved and reduced E-cadherin groups.* In the preserved E-cadherin group, the overall survival rate was significantly higher for patients negative for Snail expression than for those positive for Snail expression ( $P=0.029$ ; Fig. 3A). However, in the E-cadherin reduced group, there were no significant differences between patients positive and negative for Snail expression (Fig. 3B).

*Univariate and multivariate analyses of survival.* Table IV shows the univariate and multivariate analyses of factors related to patient prognosis. Univariate analysis revealed that the following factors were significantly related to postoperative survival: depth of tumor invasion, lymph node metastasis, stage, lymphatic invasion, venous invasion, E-cadherin expression, Snail expression and combination of E-cadherin and Snail expression. Multivariate regression analysis indicated that depth of invasion and lymph node metastasis,

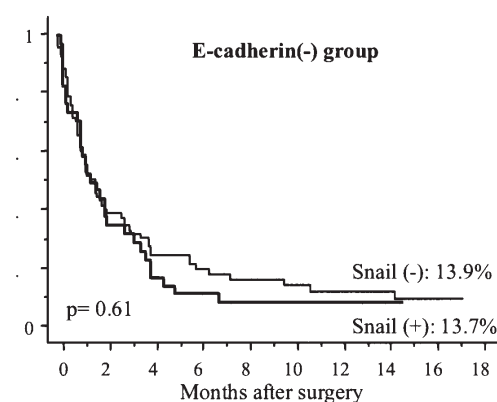
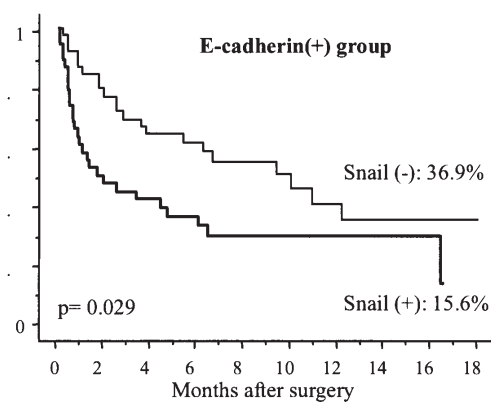


Figure 3. The postoperative survival curves between the patients with (+) and without (-) Snail expression according to E-cadherin expression. (A) In the preserved E-cadherin expression group, the patients without Snail expression (-) had a better outcome than those with Snail expression (+) ( $P=0.029$ ). (B) In the reduced E-cadherin expression group, there was no difference between the patients with Snail expression (+) and without Snail expression (-).

stage and venous invasion were independent prognostic factors, but that E-cadherin expression, Snail expression, and combination of E-cadherin and Snail expression were not independent prognostic factors.

## Discussion

Cell-cell adhesion molecules, which are present in tight junctions, intermediate junctions, desmosomes and gap junctions, play an important role both in normal cell structures, and in cancer invasion and metastasis (16). E-cadherin is one such molecule. As E-cadherin expression decreases, the incidence of lymph node metastasis increases, resulting in poor prognosis in various types of cancer, including ESCC (17). The concept of EMT, which is a determinant of carcinoma progression, was recently proposed. Loss of E-cadherin expression is emerging as one of the most common indicators of EMT onset (18). EMT induction by Snail in epithelial cells is mediated by direct transcriptional repression of E-cadherin. We hypothesized that an inverse correlation between E-cadherin and Snail expression is present in ESCC.

In the present study, E-cadherin expression was related to histological differentiation, tumor depth, lymph node metastasis, lymphatic invasion and stage. Positive Snail expression was associated with histology, tumor depth, distant nodal metastasis (pM1) and stage. As Snail expression is

Table IV. Univariate and multivariate analyses of prognostic factors.

Prognostic factors	Univariate P	Multivariate P	Hazard ratio	95% confidence interval
pT (pT1-2/pT3-4)	<0.0001	0.0025	1.923	1.251-2.953
pN (pN0/pN1)	<0.0001	0.0003	1.849	1.184-2.888
Stage (I,II/III,IV)	<0.0001	0.0004	2.933	1.882-4.571
Venous invasion	<0.0001	0.0044	1.776	1.210-2.607
Lymphatic invasion	<0.0001	0.1858	1.346	0.870-2.083
E-cadherin (preserved/reduced)	0.0003	0.9191	1.029	0.681-1.553
Snail (negative/positive)	0.03	0.8103	1.047	0.662-1.326
Combination of E-cadherin and Snail (E-cadherin preserved + Snail negative/ other groups)	0.0008	0.6228	1.133	0.713-1.800

related to tumor depth in human breast cancer, it is possible that Snail might repress E-cadherin expression during development and tumor progression through interaction with the proximal E-boxes of E-cadherin promoters (19). Inverse correlations between E-cadherin and Snail expression have been found in liver, bladder, pancreas, colon and breast cancer cells (12,18,20,21). In the present study, however, significant correlations were not found between E-cadherin and Snail expression. Molecules other than Snail may affect the loss of E-cadherin expression. Slug is also a zinc-finger transcription factor which is a member of the Snail family. When we previously examined the relationship between E-cadherin and Slug expression in ESCC, a close relationship was found (22). In breast cancer cell lines, expression of Slug, rather than Snail, was strongly correlated with the absence of E-cadherin transcripts (23). Tan *et al* (24) reported that inhibition of the integrin-linked kinase pathway in colon cancer cells, which suppresses  $\beta$ -catenin signaling, also repressed Snail promoter activity. Thus, the expression of Snail may be regulated by integrin-linked kinase, and Snail expression is at least one molecular event in the acquisition of more invasive characteristics in ESCC.

In the present study, prognosis was poorer in patients with reduced E-cadherin than in those with preserved expression, which agrees with previous reports. Similarly, the prognosis was worse in patients with Snail expression than in those without Snail expression. However, some patients with preserved E-cadherin expression had a poor prognosis. Thus, we analyzed the data by dividing Snail expression into two groups based on E-cadherin expression. In the preserved E-cadherin group, Snail expression was correlated with tumor depth and stage. Thus, overall survival was poorer in patients with Snail expression than in those without Snail expression. On the other hand, in the reduced E-cadherin group, no significant correlations were seen between Snail expression and clinicopathologic factors, including prognosis. Although the patients with preserved E-cadherin and negative Snail expression had the best clinical outcome according to univariate analysis, it was not an independent prognostic factor on multivariate analysis.

These results, together with our previous reports on Slug expression (22), suggested that the function of Snail varies

with E-cadherin expression in ESCC. In tumors with preserved E-cadherin expression, Snail and Slug may play a key role in EMT regulation. In tumors with reduced E-cadherin expression, E-cadherin expression may be regulated not only by Snail and Slug, but also by other transcription factors, such as Twist or Sip1, or genetic/epigenetic alternation (25). In gastric cancer, Rosivatz *et al* (26) suggested that, in the context of EMT regulation with respect to E-cadherin expression, two different pathways are present in the diffuse or intestinal type. In the former, Twist, mutant E-cadherin and N-cadherin were related to tumor progression, while in the latter, Sip1 was related to tumor progression. Twist, Goosecoid and Snail repress E-cadherin and induce FOXC2; they also enhance cell migration *in vitro* and metastatic potential *in vivo*. Further studies are necessary to determine whether these genes regulate individual or overlapping pathways related to EMT and metastasis in ESCC (27).

In conclusion, Snail may play a key role in E-cadherin-preserved ESCC. Because other molecules may be related to tumor progression and metastasis in ESCC, further examination is necessary, particularly in E-cadherin-reduced ESCC.

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