

Clinical Significance of SIP1 and E-cadherin in Patients with Esophageal Squamous Cell Carcinoma

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ABSTRACT

Background. Epithelial-mesenchymal transition (EMT), when epithelial cells convert to mesenchymal cells, influences cancer invasion and metastasis. Smad interacting protein 1 (SIP1) is an EMT trigger, which is inversely correlated with E-cadherin in some carcinomas. To elucidate the role of SIP1 in esophageal squamous cell carcinoma (ESCC), the status of EMT and the clinicopathological features were evaluated.

Methods. Immunohistochemical (IHC) analyses of 111 human ESCC tissue specimens for SIP1 and E-cadherin were performed, and the relationships between the expression and clinicopathological features were evaluated.

Results. IHC analyses of esophageal tumors showed the expression of SIP1 and E-cadherin to be significantly inversely correlated. Significant correlations between the SIP1 expression and clinicopathological variables such as differentiation, depth of invasion, vascular invasion, and pathological stage were also seen. Conversely, tumors with a weak expression of E-cadherin tended to exhibit greater histological differentiation. Logistic regression analyses revealed a positive SIP1 expression, lymphatic invasion, and vascular invasion to be factors predicting lymph node (LN) metastasis. Univariate survival analyses revealed a positive SIP1 expression predicted a poorer overall survival than a negative expression.

Conclusion. These results suggest that SIP1 is correlated with LN metastasis and may therefore be an independent marker for metastasis in patients with ESCC.

Esophageal squamous cell carcinoma (ESCC) is still associated with a poor prognosis because of the high frequency of lymph node (LN) metastasis and invasion to neighboring organs.¹ One of the most representative features of a malignant tumor is its invasiveness and tendency to metastasize.

The process by which an epithelial cell loses its epithelial properties and acquires the characteristics of mesenchymal cells (such as fibroblasts)^{2–4} is a phenomenon referred to as epithelial-mesenchymal transition (EMT). The loss of E-cadherin is one of the most important features of EMT, which is considered to contribute to cancer metastasis.⁵

E-cadherin is expressed mainly on the epithelial cell membrane and is required for cell–cell adhesion;⁶ therefore, E-cadherin plays a crucial role in the suppression of tumor invasion.⁷ Because the functional loss of E-cadherin leads to the disruption of adherens junctions, cell migration from the original location, marked phenotypic changes, and a highly motile fibroblastoid, mesenchymal phenotype, allowing the cells to move through the extracellular matrix, a loss of E-cadherin is believed to be one of the hallmarks of EMT.⁸ The transcriptional repression is one of the most important mechanisms in the downregulation of E-cadherin expression.

The expression of E-cadherin is directly or indirectly regulated by various factors, such as snail, slug, and twist, and is repressed via their cell- or tissue-type-dependent binding to the promoter of E-cadherin.^{9–12} In other words,

these factors are considered to trigger EMT. Among these proteins, Smad interacting protein 1 (SIP1), identical to ZEB2, which has been implicated in transforming growth factor- β signaling,^{13,14} binds to the E-cadherin promoter, thus resulting in downregulation of its promoter activities.^{14–16} As such, SIP1 is also one of the key proteins that trigger EMT.¹⁷

The downregulation of E-cadherin expression by snail promotes the invasiveness of human hepatocellular carcinoma.¹⁸ The expression of snail, slug, and twist have been reported to be inversely correlated with that of E-cadherin in ESCC, and all of these contribute to the aggressive clinicopathological features and poor prognosis associated with the disease.^{12,19,20} The expression of SIP1 also contributes to tumor progression and poor prognosis in oral squamous cell carcinoma (SCC).²¹ Nevertheless, the role of SIP1 in ESCC is unknown. We hypothesized that SIP1 expression in ESCC is involved in EMT, and consequently leads to cancer invasion and metastasis.

MATERIALS AND METHODS

Patients

Overall, 111 patients with ESCC were examined in this study. All ESCC tissues were obtained from patients who underwent esophagectomy without any preoperative therapy in the Department of Surgery and Science, Kyushu University Hospital from 1992 to 2002. Adjuvant chemotherapy was not applied routinely. Ethical approval for this study was obtained from the Institutional Review Board (#21-95) of Kyushu University. Histopathological diagnosis was determined according to the TNM classification.²²

Immunohistochemistry

Specimens with the deepest site of the cancerous lesion were selected for immunohistochemical (IHC) staining. IHC staining of SIP1 and E-cadherin was analyzed as previously described.²³ Briefly, sections measuring 3- μ m thickness were deparaffinized and rehydrated.

For SIP1, microwave heat-induced epitope retrieval was performed for 15 min at 95 °C in citrate buffer (pH 6), followed by immersion in 3 % hydrogen peroxide in 100 % ethanol for 30 min to inhibit the endogenous peroxidase activity. After being incubated with normal rabbit serum for 10 min, the sections were then incubated with a goat polyclonal antibody against SIP1 (1:50; sc-18392; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. The streptavidin–biotin method and Histofine SAB-PO (goat) kits (Nichirei Corporation, Tokyo, Japan) were used. The sections were then

incubated with biotinylated rabbit anti-goat immunoglobulins G, A, and M (Nichirei Corporation) for 20 min. The slides were treated with peroxidase-conjugated streptavidin for 20 min. The slides were developed by immersion into 0.01 % H₂O₂ and 0.05 % diaminobenzidine tetrahydrochloride for 4 min, and counterstained with Meyer's hematoxylin.

For E-cadherin, the epitope retrieval was performed with an autoclave at 121 °C for 15 min in citrate buffer (pH 6). The sections were incubated with a mouse anti-human monoclonal E-cadherin antibody (2 μ g/ml; Takara Bio Inc., Shiga, Japan) overnight at 4 °C and labeled with the Envision detection system (Dako Ltd., Glostrup, Denmark). The slides were developed by immersion into 0.01 % H₂O₂ and 0.05 % diaminobenzidine tetrahydrochloride for 5 min, and counterstained with Meyer's hematoxylin.

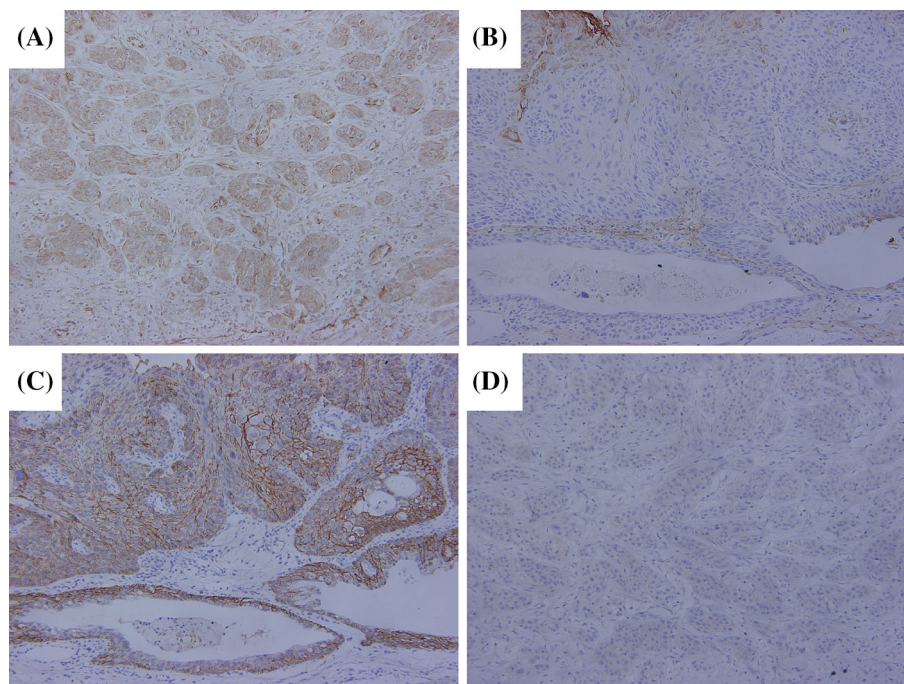
Evaluation of Immunohistochemical Staining

To evaluate the SIP1 and E-cadherin expression, the staining intensity was scored as 0, 1 (weak), 2 (medium), or 3 (strong) (Fig. 1). The extent of staining was scored as 0 (0 %), 1 (1–25 %), 2 (26–50 %), 3 (51–75 %), or 4 (76–100 %) according to the percentage of the positively stained area relative to the total tumor area.²⁴ The value obtained by multiplication of the intensity and extent scores was used as the final staining score (0–12) for SIP1 and E-cadherin. For SIP1 staining, tumors having a final staining score of 0–2 or 3–12 were considered to be negative or positive for expression, respectively. On the other hand, a final staining score of 0–2 or 3–12 was considered to be weak or retained expression for E-cadherin, respectively. The scoring procedure was carried out twice each by three independent observers (RY, MM, YN; each blinded to each others' scores) without any knowledge of the clinical parameters or other prognostic factors.

Statistical Analysis

Student's *t* test, the Chi square test, and Fisher's exact test, when appropriate, were used to compare the clinicopathological data. A *p* value <0.05 was considered to indicate a statistically significant difference. The independent factors associated with LN metastasis were evaluated with a logistic regression analysis. Survival rates were calculated using the Kaplan–Meier method, and differences between survival curves were examined with the log-rank test. All statistical analyses were performing using the statistical package StatView version 5.0 (SAS Institute Inc. Cary, NC, USA).

FIG. 1 The immunohistochemical detection of SIP1 and E-cadherin in esophageal squamous cell carcinoma (a–d). **a** Positive expression of SIP1 was detected in the cytoplasm. **b** Negative expression of SIP1. **c** Retained expression of E-cadherin was detected on the cell membrane. **d** Weak expression of E-cadherin. *SIP1* Smad interacting protein 1



RESULTS

Expression of Smad Interacting Protein 1 (SIP1) and E-cadherin

SIP1 expression was detected in the cytoplasm of the cancer cells, whereas E-cadherin expression was detected at the cell membrane (Fig. 1). Thirty-five tumors (31.5 %) had positive IHC expression of SIP1. On the other hand, membranous expression of E-cadherin was retained in 53 tumors (47.7 %) (Table 1). The E-cadherin expression was weak in 24 (68.6 %) of the 35 cases positive for SIP1 expression, and weak in 34 (44.7 %) of the 76 cases negative for SIP1. Therefore, the expression of SIP1 and E-cadherin was significantly inversely correlated ($p = 0.0195$).

Clinicopathological Analysis

The association between the clinicopathological variables and IHC expression of SIP1 and E-cadherin are shown in Table 1. Significant correlations were observed between SIP1 expression and clinicopathological variables such as histological differentiation, histological depth of invasion, LN metastasis, lymphatic invasion, vascular invasion, and pathological stage ($p = 0.0014$, 0.0003, 0.0007, 0.0322, 0.0046, and 0.0014, respectively), although there were no relationships between SIP1 expression and age, sex, and distant metastasis. On the other hand, only histological differentiation was related to tumors with weak expression of E-cadherin ($p = 0.0395$).

A logistic regression analysis revealed that positive SIP1 expression, lymphatic invasion, and vascular invasion were factors predicting LN metastasis (Table 2).

Univariate Analysis for Survival

Survival analysis according to the Kaplan–Meier method revealed that both the overall survival and disease-free survival rate of patients in the SIP1-positive group were significantly poorer than those of patients in the SIP1-negative group ($p = 0.019$ and 0.001, respectively; Fig. 2). The 5-year survival rate was 31.7 % in the SIP1-positive group compared with 60.3 % in the SIP1-negative group. The disease-free survival rate was 27.4 % in the SIP1-positive group compared with 58.6 % in the SIP1-negative group.

DISCUSSION

The aim of this study was to elucidate the relationship between SIP1 and E-cadherin, and to determine the clinical significance of SIP1 in ESCC. We have immunohistochemically shown that SIP1 expression is inversely correlated with E-cadherin expression, and that its expression is associated with advanced tumor properties and a poor prognosis in ESCC. The IHC inverse correlation between SIP1 and E-cadherin expression has previously been found in gastric cancer, renal cell carcinoma, and oral SCC.^{11,21,25,26} We previously demonstrated that SIP1 is inversely associated with E-cadherin and that SIP1-positive patients have a poorer prognosis in lung cancer. The

TABLE 1 Clinicopathological features of SIP1 and E-cadherin in the 111 ESCCs evaluated in this study

Variable	SIP1		<i>p</i> value	E-cadherin		<i>p</i> value
	Negative (<i>n</i> = 76)	Positive (<i>n</i> = 35)		Weak (<i>n</i> = 58)	Retained (<i>n</i> = 53)	
E-cadherin			0.0195			
Weak	34	24		–		
Retained	42	11				
Age (years)	63.7	65.7	0.2584	64.9	63.8	0.5050
Sex			0.4973			0.0244
Male	67	33		56	44	
Female	9	2		2	9	
Differentiation			0.0014			0.0395
Well+mod	66	21		41	46	
Poorly	10	14		17	7	
T status			0.0003			0.5017
1–2	59	15		37	37	
3–4	17	20		21	16	
N status			0.0007			0.9972
Negative	54	13		35	32	
Positive	22	22		23	21	
M status			0.3153			1.0000
Negative	76	34		59	51	
Positive	0	1		1	0	
Stage			0.0014			0.3483
I–II	66	21		45	42	
III–IV	10	14		15	9	
Lymphatic invasion			0.0322			0.1711
Negative	49	15		37	27	
Positive	27	20		21	26	
Venous invasion			0.0046			0.6118
Negative	61	19		43	37	
Positive	15	16		15	16	

SIP1 Smad interacting protein 1, ESCC esophageal squamous cell carcinoma, SCC squamous cell carcinoma, Well+mod well and moderately differentiated SCC, Poorly poorly differentiated SCC

TABLE 2 Results of the univariate and multivariate analyses of factors associated with lymph node metastasis

Variable	Univariate analysis		Multivariate analysis	
	HR (95 % CI)	<i>p</i> value	HR (95 % CI)	<i>p</i> value
SIP1 (positive vs. negative)	4.158 (1.782–9.681)	0.0010	2.763 (1.004–7.608)	0.0492
E-cadherin (retained vs. weak)	0.999 (0.466–2.139)	0.9972	–	
Tumor depth (T3–4 vs. T1–2)	3.467 (1.520–7.904)	0.0031	0.943 (0.318–2.795)	0.9150
Differentiation (poorly vs. well+mod)	2.659 (1.056–6.711)	0.0380	1.429 (0.475–4.292)	0.5261
Lymphatic invasion (positive vs. negative)	6.303 (2.721–14.597)	0.0001	4.077 (1.567–10.606)	0.0040
Venous invasion (positive vs. negative)	6.444 (2.574–16.136)	0.0001	3.284 (1.092–9.873)	0.0342

HR hazard ratio, CI confidence interval, poorly poorly differentiated SCC, well+mod well and moderately differentiated SCC, SIP1 Smad interacting protein 1, SCC squamous cell carcinoma

relationship was more remarkable in SCC than in adenocarcinoma.²³ As a result, our data showing a reciprocal correlation between SIP1 and E-cadherin in ESCC are

consistent with the findings of the previous immunohistochemical study. In addition, SIP1 messenger RNA (mRNA) expression has previously been reported to be

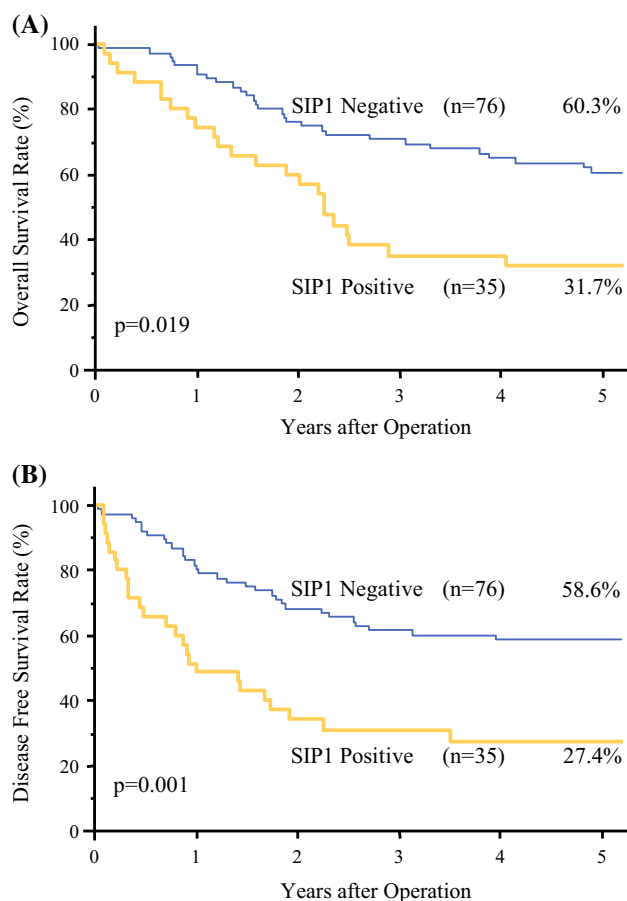


FIG. 2 Comparison of the Kaplan–Meier survival curves in the SIP1-positive group versus the SIP1-negative group. **a** Overall survival rates. **b** Disease-free survival rates. Both survival rates of patients with SIP1-positive tumors (*bold line*) were significantly worse than those of patients with SIP1-negative tumors (*thin line*) ($p = 0.019$ and 0.001 , respectively). *SIP1* Smad interacting protein 1

inversely correlated with E-cadherin mRNA in glioma, pancreatic cancer, and bladder cancer cell lines.^{27–29}

Considering the role of SIP1 as a repressor of E-cadherin, SIP1 expression and concurrent repression of E-cadherin probably result in an advanced tumor profile. Snail, another repressor of E-cadherin, was also reported to promote cancer invasion and metastasis.¹⁵ We herein demonstrated that SIP1 expression shows relationships with clinicopathological factors, including histological differentiation, histological depth of invasion, LN metastasis, pathological stage, lymphatic invasion, and vascular invasion. Histologically, poorly differentiated SCC was significantly predominant in terms of the SIP1-positive cases. Miyoshi et al.³⁰ reported that SIP1 caused the repression of E-cadherin expression and the morphological change from differentiation to dedifferentiation. Vandewalle et al.¹⁷ reported that the exogenous expression of SIP1 in a colon cancer cell line resulted in E-cadherin repression and a morphological change from an epithelial to a

mesenchymal phenotype. Our results might therefore also be consistent with the conversion of epithelial cells to mesenchymal-like cells during the EMT process. EMT plays an important role during the progression of tumor cells to dedifferentiation.²

The process of cancer metastasis requires cell migration and invasion, cell-substrate adhesion, intravasation and extravasation, as well as the cell survival and re-growth of malignant cells at a distant location.^{31,32} EMT that occurs concomitantly with the downregulation of E-cadherin expression^{33,34} is facilitated in terms of the process of the acquisition of migratory properties, the intravasation of tumor cells into the blood or lymph vessels, and the subsequent formation of distant metastasis.³⁵ ESCC cells with SIP1 expression might have a facilitated EMT-like process, and promote cells to have a more infiltrative phenotype, leading to deep invasion and metastasis. Therefore, we analyzed the relationship between SIP1 expression and lymphatic or vascular invasion. In this study, positive SIP1 cases were found to have invaded deeper than negative cases. Furthermore, we demonstrated that tumors positive for SIP1 expression had more lymphatic or vascular invasion than those without SIP1 expression. Our results support that the loss of E-cadherin by SIP1 induces cancers that are more invasive to lymphovascular vessels.

SIP1 expression significantly correlated with the presence of LN metastasis in the univariate analysis. Moreover, the multivariate analysis revealed that the expression of SIP1 is an independent predictive factor for the presence of LN metastasis. The association of LN metastasis with SIP1 is in agreement with the hypothesis that SIP1 could play an important role in tumor progression and metastasis. The snail expression in breast cancer tissue was previously reported to correlate with LN metastasis via the repression of E-cadherin.¹⁰ Taken together, these findings suggest that SIP1 might be responsible for advanced clinicopathological properties, and may also be a predictive marker for the presence of LN metastasis.

The survival rate of patients with positive SIP1 expression was significantly poorer than that of patients with negative expression in the univariate analysis. SIP1 was demonstrated to correlate with aggressive features, as described above. Therefore, the survival rate would be expected to be poorer in the patients with these features. These data are in agreement with another previous report showing that SIP1 is related to a poor prognosis in patients with oral SCC, gastric cancer, breast cancer, and ovarian cancer.^{21,26,36–38} Furthermore, SIP1 is a poor prognostic factor in bladder cancer patients treated with postoperative radiotherapy. SIP1 also has the antiapoptotic activity independent of its effects on cell adhesion, thus suggesting that SIP1 may promote tumor progression.²⁶

CONCLUSIONS

Our data showed that SIP1 expression is associated with aggressive tumor properties and a poor prognosis, and suggest that SIP1 is an independent marker of LN metastasis and is also useful for predicting the malignant properties of ESCC. Therefore, SIP1 might be a candidate molecule that can be targeted to decrease tumor progression. The present study may serve as a stepping stone for improving prognosis via the suppression and prevention of distant metastasis in the future. Further in vitro studies are necessary to clarify the contribution of SIP1 to the EMT process.

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