E-Cadherin Expression in Patients With Esophageal Squamous Cell Carcinoma

Promoter Hypermethylation, Snail Overexpression, and Clinicopathologic Implications

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Key Words: E-cadherin; Snail; Methylation; Esophageal cancer; Metastasis; Prognosis

Abstract

Hypermethylation in the E-cadherin promoter region and expression of the transcription factor Snail were analyzed in 41 cases of esophageal squamous cell carcinoma (ESCC) and paired normal squamous epithelium by methylation-specific polymerase chain reaction (PCR) and reverse transcription–polymerase chain reaction (RT-PCR) to clarify the mechanism regulating E-cadherin deletion; 93 cases of ESCC were analyzed immunohistochemically to determine the clinicopathologic impact of E-cadherin deletion.

Hypermethylation of the E-cadherin promoter and Snail overexpression were detected in 25 cases (61%) by methylation-specific PCR and 34 cases (83%) by RT-PCR, respectively. Reduced E-cadherin expression, observed immunohistochemically in 55 cases (59%), correlated with hypermethylation \( (P = .0011) \) but not Snail overexpression \( (P = .685) \). Hypermethylation and Snail overexpression correlated significantly with E-cadherin deletion \( (P = .0018) \). Snail overexpression was unrelated to clinicopathologic factors. Reduced E-cadherin expression correlated with tumor invasion \( (P = .019) \) and vascular invasion \( (P = .052) \) but not other factors. E-cadherin deletion had prognostic impact in univariate \( (P = .023) \) and multivariate \( (P = .034) \) analyses.

E-cadherin deletion was regulated by hypermethylation and Snail expression. Examination of reduced E-cadherin expression is important for assessing biologic behavior, including clinical outcome, in patients with ESCC.

Esophageal cancer is a commonly occurring cancer that generally has an unfavorable prognosis despite the availability of multimodal therapies. In addition to the TNM classification, many genes have been evaluated as possible molecular markers for clarifying the biologic behavior of esophageal cancer or its clinical outcome, and several possible molecular markers have been reported. One of the potential molecular markers, E-cadherin, has been identified as a cell-adhesion molecule and is located at the 16q22 locus. To date, several reports have demonstrated the prognostic impact of reduced E-cadherin expression in various cancers of the digestive tract. In the esophagus, the impact of E-cadherin expression on cancer metastasis or progression has also been studied. Recently, the prognostic impact of reduced E-cadherin expression was reported in a multi-institutional study by the Japanese Society of Esophageal Disease. However, the mechanism regulating E-cadherin expression is still unclear.

Cytosine methylation of the CpG island in the 5' promoter region is involved in the transcriptional inactivation of various genes and is a significant alternative to mutational inactivation in the development of cancer. Because the importance of cytosine methylation has become increasingly appreciated, many studies of methylation status have been conducted since Herman et al described a methylation-specific polymerase chain reaction (PCR) assay. Hypermethylation of the CpG island has been reported to be a major gene silencing mechanism for the E-cadherin gene in stomach and breast cancer and malignant melanoma.

In contrast, the transcription factor Snail, located at 20q13.2, is a repressor of E-cadherin gene expression in epithelial tumor cells. The E-cadherin gene has an E-pal element containing 2 E-boxes including the sequence 5'-CACCTG in
the promoter. *Snail* has been reported to repress E-cadherin expression by binding to the E-boxes directly, although this function has been demonstrated in vitro but not in vivo. Therefore, study of *Snail* expression in surgically obtained tissue might clarify the regulatory mechanism controlling E-cadherin expression in the cancer tissue and reveal potential applicability to gene therapy.

Although the incidence of adenocarcinoma of the esophagus is increasing in western countries, esophageal squamous cell carcinoma (ESCC) is the predominant histologic type globally, particularly in Japan and Asia in general. To our knowledge, the mechanism regulating E-cadherin expression in ESCC tissue has not been reported, and few studies have focused on adenocarcinoma of the esophagus. The present study is the first to clarify the regulation of E-cadherin expression in ESCC, and the results potentially might reveal a target for metastasis inhibition and provide treatment benefits to patients. In the present study, methylation-specific PCR assays of the E-cadherin promoter region, reverse transcription (RT)-PCR of *Snail* gene expression, and immunohistochemical staining for E-cadherin were conducted to clarify the regulation and role of E-cadherin expression in ESCC.

**Materials and Methods**

**Patients and Tissue Samples**

We included 93 cases of esophageal squamous cell carcinoma (79 men and 14 women; mean age, 64.0 years; range, 36-84 years) in the present study. The patients had undergone esophagectomy and lymphadenectomy without preoperative supplemental therapy at our institute between January 1990 and December 2000. Resected specimens were classified in accordance with the TNM classification system of the International Union Against Cancer. In addition, invasion of the cancer into lymphatic and blood vessels was assessed microscopically.

**Genomic DNA and Total RNA Extraction**

For analysis of methylation status, fresh frozen tissue samples were obtained from a recent series that included 41 of the 93 ESCC cases. A section of the viable tumor was dissected macroscopically and stored at -80°C until use. Genomic DNA and total RNA were isolated from fresh frozen tissue samples using the QIAamp DNA Mini Kit and RNeasy Mini Kit (QIAGEN, Hilden, Germany), respectively, following the manufacturer’s protocol.

**Bisulfite Modification and Methylation-Specific PCR**

Aberrant methylation of the CpG island in the E-cadherin promoter region was studied via methylation-specific PCR. Bisulfite modification of genomic DNA, which converts unmethylated cytosines to uracil, was performed using the CpGenome DNA Modification Kit (Intergen, Purchase, NY) following the manufacturer’s protocol. A 1-µg sample of genomic DNA was used for bisulfite modification. CpGenome Universal Methylated DNA (Intergen) was used as a positive control for the methylated E-cadherin promoter region.

PCR was carried out using 2 µL of bisulfite-modified genomic DNA under the conditions described in a previous report. Primer sequences for the methylated E-cadherin gene were (forward) 5’-TTAGGGTTAGAGGTATCCGAT-3’ and (reverse) 5’-TAATCTAAATTACCTACCGAG-3’, and those for the unmethylated E-cadherin gene were (forward) 5’-TAATTTTAGGTTAGGGTTATGAT-3’ and (reverse) 5’-CACAACCAATCCAACACACA-3’. The annealing temperature was set at 57°C for methylated DNA and 53°C for unmethylated DNA.

**RT-PCR for *Snail* Expression**

Total RNA (1 µg), random primer (Roche Diagnostics, Indianapolis, IN), and a 10-µmol/L concentration of deoxynucleoside triphosphate and Super Script II (Invitrogen, Paisley, Scotland) were used for first-strand complementary DNA synthesis. Detailed conditions of reverse transcription were described in a previous report.

Nested PCR was performed for *Snail* gene amplification as described in the literature. Primer sequences for the *Snail* gene were (forward) 5’-GCGCGGAATCGGCGACG-3’, (reverse) 5’-CCAAGAACCCCTTTTCCGAC-3’, (nested forward) 5’-ACTACGGGAGCTCGAG-3’, and (nested reverse) 5’-GTGGCTCGATGGTGC-3’. The annealing temperature was set at 60°C for the first and nested PCRs. The gene expression status of *Snail* was determined as overexpression when the PCR product was more amplified in tumor tissue compared with matched normal esophageal epithelium after visualization by electrophoresis.

**Visualization of PCR Products**

PCR products (10 µL) were analyzed by electrophoresis through a 2% agarose gel (High Strength Analytical Grade Agarose, BIO-RAD, Hercules, CA) containing ethidium bromide and visualized on a Mini-Transilluminator (Funakoshi, Tokyo, Japan).

**Immunohistochemical Analysis**

Immunohistochemical staining for E-cadherin was carried out using representative paraffin-embedded specimens from 93 patients. Sections (4-µm) were cut from resected specimens fixed in 10% buffered formalin and embedded in paraffin. After deparaffinization and rehydration, tissue sections were autoclaved at 121°C in citrate.
buffer (10-mmol/L concentration, pH 6.0) for 10 minutes for antigen retrieval. The specimens then were incubated with anti-E-cadherin monoclonal antibody (dilution 1:30; Novocastra, Newcastle upon Tyne, England) for 12 hours at 4°C. Immunohistochemical staining, following a standard avidin-biotin-peroxidase complex technique, was carried out using the Histofine SAB-PO (M) kit (Nichirei, Tokyo, Japan) and 3,3’-diaminobenzidine as the chromogen. Nuclei were counterstained with hematoxylin.

Noncancerous epithelium was evaluated as a positive control sample, and a specimen analyzed without E-cadherin monoclonal antibody was used as a negative control sample. Immunohistochemical staining was evaluated by a combination of distribution and intensity in the cancer area. Concerning intensity, a positive reaction was determined as equal or strong intensity, and an unclear or weak reaction was determined as reduced intensity compared with matched normal squamous epithelium. Then, adding to intensity, specimens staining for E-cadherin were considered as positive when a preserved positive reaction could be observed in more than 50% of the cancer cells, whereas those with negative or unclear or weak reactions in more than 50% were characterized as reduced expression. The specimens were evaluated by 2 independent observers unaware of the clinical information (S.T. and Y.K.).

**Statistical Methods**

Correlations between Snail or E-cadherin expression and clinicopathologic factors were analyzed by using the Mann-Whitney U test, the Fisher exact probability test, or the χ² test. The Kaplan-Meier method was used to assess the clinical outcome after surgery. A Cox proportional hazards regression model was used for multivariate analysis. Differences were evaluated by using the log-rank test. A P value of less than .05 was considered significant.

**Results**

**Regulation of E-Cadherin Gene Expression**

Hypermethylation of the E-cadherin gene was detected in 24 (59%) of 41 cases. In contrast, the unmethylated E-cadherin promoter region was amplified in all cases. **Image 11**. Eighteen of 24 cases exhibiting E-cadherin hypermethylation also demonstrated reduced protein expression immunohistochemically. This interaction between methylation status and the protein expression pattern was highly significant (P = .0011) **Table 1**.

The Snail gene was amplified by RT-PCR of normal squamous epithelium in all cases. In tumor tissue, Snail gene overexpression, compared with matched normal epithelium, was detected in 34 (83%) of 41 cases. **Image 21**. In the present series, Snail overexpression did not seem to be correlated with any clinicopathologic factors of ESCC **Table 2**.

Of 34 samples overexpressing Snail, 19 exhibited reduced immunohistochemical expression of E-cadherin, although a significant correlation between these factors was not observed **Table 3**. Likewise, no statistically significant correlation was identified between hypermethylation of the E-cadherin gene and Snail overexpression **Table 4**. However, interestingly, E-cadherin expression was preserved in all cases that did not exhibit promoter hypermethylation or Snail overexpression. In contrast, reduced E-cadherin

<table>
<thead>
<tr>
<th>CpG in the Promoter Region</th>
<th>Hypermethylation</th>
<th>Unmethylated</th>
<th>P</th>
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<td>Reduced</td>
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* Fisher exact probability test.

**Table 1**

E-Cadherin Expression and Hypermethylation in the Promoter Region in 41 Cases

**Image 11** Hypermethylation could be detected in 24 (59%) of 41 cases by methylation-specific polymerase chain reaction (PCR) analysis of E-cadherin promoter region. M, methylated PCR product; U, unmethylated PCR product; (–), negative control; (+), positive control.

**Image 21** Snail overexpression in cancer tissue was observed in 34 (83%) of 41 cases compared with matched normal esophageal epithelium by reverse transcription–polymerase chain reaction. G3PDH, glyceraldehyde-3-phosphate dehydrogenase; N, matched normal squamous epithelium of the esophagus; T, tumor tissue; (–), negative control; (+), positive control.
expression was observed in most cases that exhibited both hypermethylation and Snail overexpression ($P = .0018$).

**Clinicopathologic Implications of E-Cadherin Protein Expression**

Of the 93 cases in this series, E-cadherin expression was preserved in cancer specimens from 38 patients (41%). In the other 55 cases, E-cadherin expression was completely absent or deleted in part of the carcinoma (59%). Only depth of tumor invasion (pT) was correlated significantly with the deletion of E-cadherin expression ($P < .05$). Esophageal cancers exhibiting deleted E-cadherin expression tended to invade more aggressively. Although a statistically significant relationship was not observed, vascular invasion was uncommon in patients with preserved E-cadherin expression ($P = .052$). In contrast, E-cadherin expression status was not associated with any of the other clinicopathologic parameters, including lymph node (pN), distant (pM) metastasis status, or lymphatic vessel invasion.

With respect to clinical outcome after surgery, univariate analysis indicated that patients with deleted E-cadherin expression had an unfavorable prognosis ($P = .0234$). Multivariate analysis of prognostic parameters including pT, pN, pM, TNM stage, lymphatic invasion, vascular invasion, and E-cadherin suggested that E-cadherin expression status ($P = .0285$), pM ($P = .0059$), and lymphatic vessel invasion ($P = .0319$) were independent prognostic factors.

**Discussion**

E-cadherin is a cell-adhesion molecule located on 16q22, and its loss or reduced expression has been used as a molecular biomarker to predict the clinical outcome for patients with ESCC and for patients with other solid malignant tumors. Therefore, in the present study, the mechanism down-regulating E-cadherin expression was studied in ESCC tissues. A previous study using the comparative genomic hybridization technique demonstrated that loss of 16q was
infrequent (observed in <5% of ESCC cases). However, immunohistochemical analysis demonstrated reduced E-cadherin expression in more than half of the ESCC cases (59%) and in a previous multi-institutional study (57.7%). These results suggest that chromosomal imbalance (16q loss) might not be critical in the elimination or reduction of E-cadherin expression in ESCC.

Herman et al described the methylation-specific PCR method, which has attracted interest for its potential role in the study of methylation status, especially for tumor suppressor genes. Activated E-cadherin has been reported to cause growth retardation and function as a tumor suppressor gene and as a cell-adhesion molecule. However, the methylation status of the E-cadherin gene in surgically resected ESCC tissue series has not been evaluated, despite the high frequency of hypermethylation (83.9%) in adenocarcinoma of the esophagus. Therefore, we studied the role of E-cadherin methylation status in ESCC. Indeed, hypermethylation of the promoter region was frequent (59%) in ESCC, although it was relatively low compared with that in adenocarcinoma. In the present study, hypermethylation was associated significantly with the loss or reduction in E-cadherin expression (P = .0011). This result suggested that hypermethylation might be an important mechanism in E-cadherin silencing in ESCC.

The human Snail gene, located on human chromosome 20q13, is a reported repressor of E-cadherin. The Snail gene was overexpressed at high frequency (83%) in ESCC in the present study, although no significant correlation could be demonstrated between Snail overexpression and reduced E-cadherin expression in our series. An inverse correlation between these parameters was reported in vitro in melanoma, oral squamous cell carcinoma, and hepatocellular carcinoma. However, expression in hepatocellular carcinoma also was studied by the implantation of cancer cells, but not in human tissue. One previous report focused on resected human breast cancer tissue, although to our knowledge, the present study is the first report on ESCC tissue. In breast cancer tissue, Blanco et al also reported a negative correlation between Snail overexpression and reduced E-cadherin expression.

In contrast with established cell lines, tumor tissue usually exhibits much more heterogeneity. The lack of correlation between Snail overexpression and reduced E-cadherin expression in our study might be a result of tumor heterogeneity, which supports the observations reported by Blanco et al. Interestingly, E-cadherin expression was preserved in

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**Table 6**

Clinicopathologic Implications of E-Cadherin Expression in 93 Cases

<table>
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<th>Reduced</th>
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<tr>
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*pM, distant metastasis; pN, lymph node metastasis; pT, tumor invasion.
† Fisher exact probability test.
‡ χ² test.
§ Mann-Whitney U test.
the cases that did not exhibit either E-cadherin hypermethylation or Snail overexpression but was reduced in most cases in which both were observed (P = .0018). Although Snail overexpression was not correlated significantly with promoter hypermethylation, our results suggest that these 2 pathways work cooperatively, but not independently, to down-regulate E-cadherin expression. Snail binds to E-boxes in the CpG island of the E-cadherin promoter region and inhibits the promoter activity.16,17 Taken together, the epigenetic events, hypermethylation or inactivation of the promoter, might have critical roles in silencing E-cadherin expression.

Snail also has been reported to control epithelial-mesenchymal transitions by down-regulation of E-cadherin expression.31,32 Blanco et al30 reported a significant correlation between Snail overexpression and cell differentiation and between Snail overexpression and lymph node metastasis in breast cancer tissue. However, Snail overexpression revealed no significant interactions with clinicopathologic factors in our study. This apparent contradiction might be attributable to differences in the methods used to examine Snail expression. In fact, more frequent Snail overexpression was demonstrated by RT-PCR (83%) in the present study compared with that by in situ hybridization (42.9%) in the study by Blanco et al.30 However, the frequency variations also might be affected by histologic differences between ESCC and adenocarcinoma of the breast. Further studies of various cancers are required to clarify these observations.

The use of E-cadherin expression as a prognostic indicator in ESCC has been reported in a multi-institutional immunohistochemical study.8 Univariate and multivariate analyses identified reduced E-cadherin expression as a significant indicator of poor prognosis in our study and in the previous studies. Furthermore, our study and the multi-institutional study found that reduced E-cadherin expression correlated with pT but not pN or distant metastasis pM. E-cadherin is a cell adhesion molecule that is reported to have an important role in cancer metastasis. However, our results suggest that reduced E-cadherin induces cancer metastasis via tumor invasion by suppressing cell-to-cell attachment, and the literature suggests that it might activate cancer growth and invasion by loss of function as a tumor suppressor gene.23-26

Our study suggests that further investigations to clarify the mechanisms regulating E-cadherin expression in ESCC are very important, and the control of epigenetic silencing of E-cadherin might be critical for suppressing cancer invasion or metastasis and might be a potential target of gene therapy.

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References


