Promoter Methylation as a Common Mechanism for Inactivating *E-cadherin* in Human Salivary Gland Adenoid Cystic Carcinoma

Chun-Ye Zhang, MD1
Li Mao, MD2,3
Lei Li, AD4
Zhen Tian, MD, PhD1
Xiao-Jian Zhou, AD4
Zhi-Yuan Zhang, MD, PhD4
Jiang Li, MD, PhD1

1 Department of Oral Pathology, College of Stomatology, Shanghai Ninth People’s Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China.
2 Department of Thoracic/Head and Neck Medical Oncology, University of Texas M. D. Anderson Cancer Center, Houston, Texas.
3 Cancer Biology Program, University of Texas Graduate School of Biomedical Sciences at Houston, Houston, Texas.
4 Department of Oral and Maxillofacial Surgery, College of Stomatology, Shanghai Ninth People’s Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China.

**BACKGROUND.** The role of promoter methylation in the inactivation of *E-cadherin* (*E-cad*) in salivary gland adenoid cystic carcinoma (ACC) is unknown. The objective of this study was to determine the role and potential clinical implications of promoter methylation of *E-cad* in salivary gland ACC.

**METHODS.** The promoter methylation status of *E-cad* was determined by using methylation-specific polymerase chain reaction (PCR) analysis in 60 primary salivary gland ACC tissues and 3 ACC cell lines. The level of E-cad protein expression was determined by immunohistochemical analysis of each tumor. E-cad protein and messenger RNA (mRNA) expression levels were examined by immunohistochemical analysis and reverse transcriptase-PCR in 3 ACC cell lines. Associations between molecular alterations and patients’ clinicopathologic characteristics were analyzed statistically. E-cad mRNA expression was examined in a 5-azacytidine-treated ACC-2 cell line.

**RESULTS.** Promoter methylation of *E-cad* was detected in 34 of 60 tumors (57%). Of those 34 tumors, 18 tumors (53%) showed no E-cad protein expression, whereas only 5 of the remaining 26 tumors (19%) without *E-cad* promoter methylation showed no E-cad protein expression (*P* = .01). Tumors that had *E-cad* promoter methylation had a significantly higher histologic grade (*P* = .01) and more perineural invasion (*P* = .02) compared with tumors that did not have methylation. All 3 ACC cell lines exhibited *E-cad* promoter methylation and a lack of E-cad mRNA and protein expression, whereas 5-azacytidine restored E-cad mRNA expression in the ACC-2 cell line.

**CONCLUSIONS.** *E-cad* frequently is inactivated in salivary gland ACC through promoter methylation, and *E-cad* promoter methylation may play a role in tumor cell differentiation and perineural invasion. *Cancer* 2007;110:87–95. © 2007 American Cancer Society.

**KEYWORDS:** *E-cadherin*, promoter methylation, salivary gland adenoid cystic carcinoma, gene inactivation, carcinogenesis.

Salivary gland tumors are among the most commonly occurring head and neck tumors, and approximately 40% of these tumors are malignant.1,2 In our database, adenoid cystic carcinoma (ACC) is the most common malignancy in the salivary gland (32%)3 and is characterized by slow but aggressive growth, nerve and blood vessel invasion, multiple recurrences, and distant metastases.4-6 Although the 5-year survival rate is high for patients with ACC, probably because of the slow growth of the tumor, the 10- and 15-year prognoses are poor because of the frequent local recurrences and distant metastases.5,9,10
The molecular mechanism involved in the development and progression of salivary gland ACC remains uncertain. However, it has been reported that epigenetic mechanisms, such as CpG-enriched promoter methylation and histone deacetylation, play an important role in the transcriptional inactivation of tumor suppressor genes, including p16\(\text{INK4a}\), E-cadherin (E-cad) (also known as CDH1), Ras association domain family 1A (RASSF1A), death-associated protein kinase homolog (DAPK), 6-O-methylguanine-DNA methyltransferase (MGMT), and human mutLI homolog 1 (hMLH1), in many types of tumors.\(^{11-18}\) In a previous study, we demonstrated the frequently occurring promoter methylation of p16\(\text{INK4a}\), RASSF1A, and DAPK in salivary gland ACC, suggesting that promoter methylation is a common mechanism in the inactivation of tumor suppressor genes in ACC.\(^{19}\)

E-cad is a transmembrane glycoprotein that is expressed on epithelial cells and is responsible for calcium-dependent cell-cell adhesion.\(^{20-22}\) Through catenin, E-cad plays a critical role in transducing signals to influence several important biologic processes.\(^{23-25}\) Reduced expression of E-cad, caused by genetic and epigenetic events,\(^{20}\) has been observed in aggressive carcinomas of the bladder, breast, and colorectum.\(^{26-29}\) Although E-cad may be inactivated by gene mutations or deletions in human carcinomas,\(^{28,30}\) promoter methylation of E-cad is a more common mechanism for its inactivation.\(^{31,32}\)

In the current study, we evaluated the promoter methylation status of E-cad and the expression levels of E-cad protein in samples from 60 unrelated patients with salivary gland ACC. We also correlated the E-cad status in these tumors with clinical and pathologic parameters to determine the role of E-cad in the development and progression of salivary gland ACC. In addition, we analyzed the promoter methylation status and the messenger RNA (mRNA) and protein expression of E-cad in 3 salivary gland ACC cell lines (ACC-2, ACC-3, and ACC-M) that were developed in our laboratory.\(^{33,34}\) To our knowledge, this is the first report of a comprehensive analysis of E-cad in a large cohort of patients with salivary gland ACC.

**MATERIALS AND METHODS**

**Tissue Samples and Cell Lines**

Samples of salivary gland ACC were obtained from 60 unrelated patients in whom this disease was diagnosed between 1999 and 2002 in the Department of Oral Pathology at Shanghai Ninth People's Hospital, Shanghai Jiao Tong University in Shanghai, China. Tumor samples were fixed in formalin and embedded in paraffin. Tissue sections (4 μm) were stained with hematoxylin and eosin and were reviewed by 2 pathologists (J.L. and C.-Y.Z.).

In each tumor, the histologic grade was determined according to the following criteria: grade 1, a tubular and cribriform pattern but no solid component; grade 2, tubular, cribriform, and solid pattern but solid component <30%; and grade 3, tubular, cribriform, and solid pattern but solid component ≥30%.\(^{2}\) The clinical stage of each patient’s disease was determined according to criteria from the Tumor, Lymph Node, Metastases (TNM) classification system of the 2002 International Union Against Cancer.\(^{35}\) For our in vitro experiment, we cultured 3 ACC cell lines (ACC-2, ACC-3, and ACC-M) in RPMI-1640; until their use, we incubated them at 37°C in a humidified atmosphere containing 5% CO\(_2\).

**Microdissection and DNA Extraction**

Tissue sections (10 μm) were stained with hematoxylin and eosin. Areas that contained tumor cells were dissected under a stereomicroscope and then digested in 300 μL of digestion buffer (500 mM Tris; 20 mM ethylenediamine tetraacetic acid [EDTA]; 10 mM NaCl, pH 9.0; 1% sodium dodecyl sulfate; and 0.5 mg/mL of proteinase K) at 42°C for 36 hours. The digested products were purified twice using phenol and chloroform. DNA was precipitated with ethanol and resuspended in double-distilled water. The genomic DNA of the ACC-2, ACC-3, and ACC-M cells was extracted similarly, except microdissection was used in tissue sections.

**Methylation-specific Polymerase Chain Reaction**

The methylation status in the E-cad promoter was determined by methylation-specific polymerase chain reaction (MSP) analysis. Genomic DNA (2 μg), with 1 μg salmon sperm DNA as a carrier and in a total volume of 50 μL, was denatured using NaOH (final concentration, 0.2 M) for 10 minutes at 37°C; 30 μL of 10-mM hydroquinone (Sigma Chemical, St. Louis, Mo) and 520 μL 3-M sodium bisulfite (Sigma Chemical, St. Louis, Mo), pH 5.0, both freshly prepared, were added and mixed. The samples were then incubated at 50°C for 16 hours.

Modified DNA was purified using the Wizard DNA purification resin (Promega, Madison, Wis) according to the manufacturer’s instructions and then eluted into 50 μL of water. The reaction was stopped with NaOH (final concentration, 0.3 M) for 10 minutes at room temperature, followed by ethanol precipitation. The DNA was resuspended in double-distilled water and was either used immediately or was stored at −20°C.
Bisulfite-modified DNA was then amplified by MSP using methylation-specific primers for E-cad (forward 5'-TTAGGTTAGGGTATCGGC-3', reverse 5'-TAACTAAATTACCTACCGAC-3') and annealing at 57°C, which produced a 116-base pair (bp) fragment, and using unmethylated-specific primers for E-cad (forward 5'-AGCCATGGCCCTTGGAG-3', reverse 5'-CCAGGGCTTGCACCTTC-3') and annealing at 53°C, which produced a 97-bp fragment. The polymerase chain reaction (PCR) mixture contained 10 × PCR buffer (0.166 M NH₄SO₄; 0.67 M Tris, pH 8.8; 67 mM MgCl₂; 100 mM β-mercaptoethanol; 67 μM EDTA; and 9% dimethyl sulfoxide), HotStar Taq DNA polymerase (0.625 units) (Qiagen, Valencia, Calif), dimethyl sulfoxide (0.5 μL), deoxyribonucleoside triphosphates (dNTPs) (1.25 mM of each), primers (75 ng per reaction), and bisulfite-modified DNA (50–100 ng) in a final volume of 12.5 μL. Water was substituted for DNA as a negative control. The PCR products were subjected to electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized directly under ultraviolet illumination. The PCR products from ACC-2, ACC-3, and ACC-M cells were sequenced directly.

Immunohistochemistry
Formalin-fixed, paraffin-embedded tissues were cut into 4-μm sections and then deparaffinized in graded alcohol. Antigen retrieval was accomplished by microwave irradiation (in citrate buffer, pH 6.0) for 20 minutes. Endogenous peroxidase activity was blocked with 3% H₂O₂ for 20 minutes. The slides were incubated overnight at 4°C with monoclonal mouse antihuman E-cad antibody (Zymed Laboratories Inc., South San Francisco, Calif), followed by incubation with secondary peroxidase-labeled polymer conjugated to goat-antimouse immunoglobulin antibody (Dako, Carpinteria, Calif) at room temperature for 30 minutes. E-cad expression was detected with the diaminobenzidine chromogen system (Dako). The nuclei were counterstained with hematoxylin. Negative controls, with omission of the first antibody, were run simultaneously. Sections of oral mucosa were used as positive controls for E-cad. Results were scored according to the percentage of positively stained cells: no positive tumor cells = 0; <30% positive tumor cells = 1; 30% to 50% positive tumors cells = 2; and >50% positive tumor cells = 3.

Immunostain was applied to the cultured ACC-2, ACC-3, and ACC-M cells cultured on coverslips and fixed with acetone. The staining procedures were the same as those applied to the tissue sections.

Reverse Transcriptase-PCR
Total RNA was extracted from cultured ACC cells (ACC-2, ACC-3, ACC-M, and 5-azacytidine-treated ACC-2) with TRIzol reagent (Invitrogen, Carlsbad, Calif) according to the manufacturer's protocol. Combinational DNA was prepared by reverse transcription of 1 μg of total RNA using the first-strand synthesis system. PCR was performed in a total volume of 25 μL containing 0.25 μL of Taq, 4 μL of PCR buffer, 4 μL of MgCl₂, 2 μL of dNTP, 1 μL of primers, 0.5 μL of DNA, and 13.25 μL of double-distilled water. The primers used for E-cad were forward 5'-GGGTCTTGCTATGTTGCC-3' and reverse 5'-GTTCCGCTCTGCTTGGG-3' (420 bp), and the primers used for glyceraldehyde 3-phosphate dehydrogenase were forward 5'-GGATTTGTCGTATTGGG-3' and reverse 5'-GGAAGATGGTGATGGGATT-3' (207 bp). The amplification conditions were at 94°C for 5 minutes followed by 35 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, with a final 10-minute extension at 72°C. The PCR products were subjected to electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized directly under ultraviolet illumination.

Statistical Analyses
All data were analyzed by using SAS statistical software, version 6.2 (SAS Institute Inc., Cary, NC). P values ≤0.05 were considered statistically significant.

RESULTS
Clinical and Pathologic Characteristics
Of all 60 patients with salivary gland ACC, 28 were women and 32 were men, and the median age was 53 years (range, 16-71 years). Twenty-seven tumors (45%) originated from the major salivary glands, and 33 tumors (55%) originated from the minor salivary glands. Histologically, 25 tumors were classified as grade 1, 19 were classified as grade 2, and 16 were classified as grade 3. Fifteen patients (25%) had clinical stage I disease, 28 patients (47%) had stage II disease, 9 patients (15%) had stage III disease, and 8 patients (13%) had stage IV disease. Perineural invasion was observed in 33 of 60 patients (55%). Six patients (10%) developed recurrent tumors.
Promoter Methylation of the *E-cad* Gene in ACC

*E-cad* promoter methylation was observed in 34 tumors (57%) (Table 1, Fig. 1). These findings, along with those from our previous study, indicated that 55 of the 60 tumors (92%) exhibited methylation in at least 1 of the *E-cad, RASSF1A, p16INK4a, DAPK,* or *MGMT* gene promoters; 32 tumors (53%) exhibited methylation in at least 2 of the promoters; 14 tumors

### TABLE 1

Associations Between the Promoter Methylation Status of E-cadherin and Clinicopathologic Characteristics

<table>
<thead>
<tr>
<th>Clinicopathologic characteristic</th>
<th>Methylation status</th>
<th>Positive</th>
<th>Negative</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td></td>
<td>17</td>
<td>15</td>
<td>.55</td>
</tr>
<tr>
<td>Women</td>
<td></td>
<td>17</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td></td>
<td>17</td>
<td>10</td>
<td>.37</td>
</tr>
<tr>
<td>≥50</td>
<td></td>
<td>17</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>9</td>
<td>16</td>
<td>.01</td>
</tr>
<tr>
<td>2 and 3</td>
<td></td>
<td>25</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I and II</td>
<td></td>
<td>23</td>
<td>20</td>
<td>.43</td>
</tr>
<tr>
<td>III and IV</td>
<td></td>
<td>11</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Perineural invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td>23</td>
<td>10</td>
<td>.02</td>
</tr>
<tr>
<td>No</td>
<td></td>
<td>11</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major</td>
<td></td>
<td>12</td>
<td>13</td>
<td>.25</td>
</tr>
<tr>
<td>Minor</td>
<td></td>
<td>22</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

TNM indicates the International Union Against Cancer tumor, lymph node, metastases classification system.

**FIGURE 1.** Methylation analysis of *E-cadherin* promoter in adenoid cystic carcinoma (ACC) tissues. M indicates tumor DNA amplified with methylation-specific primer; U, tumor DNA amplified with unmethylation-specific primer; +, positive control; CM, negative control for methylation; CU, negative control for unmethylation.

**FIGURE 2.** Immunohistochemical analysis of E-cadherin in adenoid cystic carcinoma (ACC) tissues. (a,c) A positive reaction occurred in tubular and cribriform patterns. (b) Higher power view of a. E-cadherin expression occurred primarily in tumor cell membranes (arrow). (c) Positive reactions were stronger in the glandular epithelia than in the myoepithelia. (d) No positive reaction was observed in solid patterns (original magnification, ×200 in a, c, and d; ×400 in b).
(23%) exhibited methylation in at least 3 of the promoters; 6 tumors (10%) exhibited methylation in at least 4 of the promoters; and no tumor exhibited methylation in all 5 promoters.

E-cad Protein Expression in Salivary Gland ACC and Its Correlation With Promoter Methylation Status

E-cad expression was observed primarily on the cell membranes in the tumors (Fig. 2). The levels of E-cad expression in salivary gland ACC were variable in different tumors and in different regions within individual tumors. Positive staining was scored in 37 tumors (62%). In the E-cad-positive tumors, positively stained cells were observed mainly in tubular and cribriform patterns, whereas no staining or only weakly stained cells were observed in solid patterns. The positive staining in the glandular epithelia was stronger than that observed in the myoepithelia in the tubular pattern (Fig. 2). Of the 34 tumors with E-cad promoter methylation, 18 tumors (53%) showed no E-cad expression, whereas only 5 of the remaining 26 tumors (19%) without promoter methylation exhibited no E-cad protein expression ($P = .01$) (Table 2).

Promoter Methylation, mRNA, and Protein Expression of E-cad in ACC Cell Lines

E-cad promoter methylation was detected in all 3 cell lines (ACC-2, ACC-3, and ACC-M) (Fig. 3). Consistent with the notion that methylation contributes to the inactivation of the gene, no E-cad mRNA or protein expression was detected in any of the 3 salivary gland ACC cell lines (Figs. 4, 5). The bisulfite sequencing of the MSP products confirmed the promoter methylation by showing that all cytosine residues remained unchanged, a hallmark of cytosine methylation (Fig. 6). However, E-cad mRNA expression was detected in 5-azacytidine-treated ACC-2 cells (Fig. 7).

Correlation between E-cad Promoter Methylation or Lack of Protein Expression and Clinicopathologic Parameters

A higher histologic grade was observed in tumors that had E-cad promoter methylation than in tumors without it. Of the 34 tumors that exhibited E-cad promoter methylation, 25 tumors (74%) were grade 2 or 3, compared with 10 of 26 tumors (38%) grade 2 or 3 tumors that exhibited no E-cad promoter methylation ($P = .01$) (Table 1). E-cad promoter methylation also was correlated with perineural invasion. Of the 34 tumors that exhibited E-cad promoter methylation, 23 tumors (68%) had perineural invasion, compared with 11 of 26 tumors (42%) without E-cad promoter methylation ($P = .02$) (Table 1). No association was observed between E-cad promoter methylation and the patients’ sex, age, TNM stage, or tumor location (Table 1) or between E-cad protein expression and the patients’ sex, age, tumor location, tumor grade, TNM stage, or perineural invasion.

DISCUSSION

Promoter methylation is a common mechanism underlyng the inactivation of a number of tumor suppressor genes, such as E-cad, p16$^{INK4a}$, RASSF1A, DAPK, hMLH1, and MGMT, in various types of head and neck carcinomas, including nasopharyngeal, thyroid, laryngeal, and oral squamous cell $^{13,36-39}$ We previously reported that promoter methylation of
**p16^{INK4a}, RASSF1A, and DAPK** commonly occurred in salivary gland ACC.\(^{19}\)

In the current study, *E-cad* promoter methylation was observed in 57% of patients with salivary gland ACCs, with the highest frequency among the 5 gene promoters that we analyzed in the same tumors.\(^{19}\) These results are consistent with earlier reports in which 16 of 23 patients (70%) with salivary gland ACC showed *E-cad* promoter methylation.\(^{40}\) Furthermore, *E-cad* methylation also was observed in all 3 salivary gland ACC cell lines that we tested. Because *E-cad* promoter methylation is rare in normal salivary gland tissues (data not shown), these data strongly suggest that the methylation of *E-cad* promoter is involved in the tumorigenesis of salivary gland ACC. Our findings from this study, along with our previous data,\(^{19}\) suggest that abnormal promoter methylation is a major contributor to salivary gland ACC, because >90% of the tumors exhibited methylation in at least 1 of the 5 promoters studied. Whether environmental factors, such as radiation and tobacco exposure, contribute to the epigenetic abnormalities in ACC remains to be determined. Nevertheless, evidence has indicated an association between chewing tobacco and the hypermethylation of several tumor suppressor genes in oral squamous cell carcinoma.\(^{41}\)

Maruya et al.\(^{40}\) described an association between promoter methylation status and protein expression of *E-cad* in 23 salivary gland ACCs: In their report, areas with high expression of *E-cad* were negative for promoter methylation, whereas areas with no expression of *E-cad* were positive. In the current study, we also observed that promoter methylation of *E-cad* was associated with a loss of *E-cad* protein expression in patients with ACC, suggesting that *E-cad* methylation results in the down-regulation of *E-cad* protein expression. This notion is supported by the perfect correlation between *E-cad* promoter methylation and the lack of *E-cad* mRNA and protein expression as well as the re-expression of *E-cad* after treatment with a demethylation agent in the salivary gland ACC cell lines. However, some other mechanisms, such as mutations, genomic deletion, or increased protein degradation, also may contribute to inactivation of the gene.

Methylation of the *E-cad* gene reportedly is correlated with microvascular invasion, metastasis, and the recurrence of tumors in patients with hepatocellular, oral squamous cell, endometrial, breast, and prostate carcinomas.\(^{11,42-44}\) In the current study, *E-cad* promoter methylation status was correlated strongly with the histologic grade and with the perineural invasion of salivary gland ACCs. Maruya et al.\(^{40}\) however, observed no correlation between promoter methylation and the histologic grade of salivary gland ACCs. This discrepancy may have been caused by the smaller sample size in the study by

**FIGURE 5.** Immunohistochemical analysis of *E-cadherin* showed that no positive reaction occurred in the 3 adenoid cystic carcinoma (ACC) cell lines ACC-2 (a), ACC-3 (b), and ACC-M (c) (original magnification, ×200 in a-c).
Maruya et al., the different etiologic profiles of the patient populations, or the differences in the sensitivity of the detection assays used.

A reduced E-cad protein level has been associated with the invasion and metastasis of tumors in patients with oral squamous cell,43 bladder,27 and breast carcinomas.28 However, we observed no association between E-cad protein expression and any of the clinicopathologic parameters that were investigated in the current study. Our immunohistochemical assay may not have been sufficient to distinguish moderately reduced protein expression from the normal expression level. It is also possible that the antibody we used recognized closely related cadherins in some cases of salivary ACC in our experimental conditions. Further experiments are encouraged to address these possibilities.

REFERENCES


38. Elisei R, Shiohara M, Koeffler HP, Fagin JA. Genetic and epigenetic alterations of the cyclin-dependent kinase inhibitors p15INK4b and p16INK4a in human thyroid carci-


