Abstract. The role of promoter methylation in the inactivation of E-cadherin (CDH1) in salivary carcinoma ex pleomorphic adenoma (CXPA) is unknown. The objective of this study was to determine the role and potential clinical implications of CDH1 promoter methylation in salivary CXPA. The CDH1 promoter methylation status was determined by bisulfite sequencing PCR in 37 primary CXPA tissues and 2 CXPA cell lines. E-cadherin expression levels were determined by immunohistochemical analysis of each tumor. E-cadherin protein levels and CDH1 mRNA levels were examined by immunoblotting and quantitative real-time PCR, respectively, in 2 CXPA cell lines. Cells were treated with 5-Aza-dC or TGF-β1 to test the influence of promoter methylation on CDH1 mRNA and protein expression. Associations between CDH1 molecular alterations and patients’ clinicopathologic characteristics and prognosis were statistically evaluated. CDH1 promoter hypermethylation was detected in 21 of 37 tumors (56.76%). Of these 37 tumors, 13 tumors (35.14%) showed low E-cadherin expression. Tumors that had CDH1 promoter methylation had a histological tendency toward luminal differentiation (P=0.004), high tumor grade (P=0.005), high T stage (P=0.024) and high TNM stage (P=0.038) compared with tumors that did not. The two CXPA cell lines exhibited an inverse relationship between CDH1 promoter methylation status and CDH1 mRNA and protein expression. Treatment of the hypermethylated cell line with 5-Aza-dC restored CDH1 mRNA and E-cadherin protein expression. The induction of hypermethylation by TGF-β1 resulted in the repression of CDH1 mRNA and protein expression. CDH1 is commonly silenced in CXPA through promoter methylation. CDH1 methylation is closely related to tumor cell differentiation, histological grade, lymph node metastasis and advanced TNM stage, indicating that CDH1 methylation may play a role in the initiation and progression of CXPA.

Introduction
E-cadherin, a 120-kDa transmembrane glycoprotein encoded by the CDH1 gene located on 16q 22.1, is a prime mediator of calcium dependent cell-cell adhesion and forms the key functional component of adherens junctions between neighboring homozygous cells (1). There is increasing evidence that modulation of this complex by different mechanisms, such as gene mutation (2,3), loss of heterozygosity (LOH) (4,5) and epigenetic and micro-RNA alternations (6-8), is an important step in the initiation and propagation of human cancers. Promoter methylation, a type of epigenetic alteration, is considered to be the predominant mechanism of CDH1 inactivation. This mechanism has been recognized in many solid tumors, including salivary adenoid cystic carcinoma (ACC) (6), eyelid and oral squamous cell carcinoma (SCC) (9), gastric cancer (8), breast cancer (7,10), bladder cancer (11) and colorectal adenocarcinoma (12,13).

The carcinoma ex pleomorphic adenoma (CXPA) is a malignant tumor of the salivary gland that develops in or from a recurrent or long-lasting benign pleomorphic adenoma (PA). This tumor type comprises ~4% of all salivary tumors and 12% of all salivary malignancies (14). Based on the data of our department, CXPA is the second most common (tied with acinic cell carcinoma) malignancy of the salivary gland in the Chinese population, accounting for 8% of all salivary malignancies (15). To date, however, the expression of E-cadherin in human salivary CXPA has been infrequently studied (16-18). Moreover, there are no reports describing the relationship between CDH1 promoter methylation and E-cadherin expression in salivary CXPA. Furthermore, the association between molecular changes to the CDH1 gene and tumor progression remains to be clarified.

In the present study, we evaluated CDH1 promoter methylation status and E-cadherin expression levels in 37 CXPA samples. We also correlated the promoter methylation status in these tumors with clinical and pathological parameters to...
determine the role of CDH1 methylation in the development and progression of salivary CXPA. In addition, we analyzed the promoter methylation status as well as the messenger RNA (mRNA) and protein expression levels of CDH1 in 2 CXPA cell lines: SM-AP1 and SM-AP4. To our knowledge, this is the first report of a comprehensive analysis of CDH1 methylation in salivary CXPA samples.

Materials and methods

Tissue samples and cell lines. Formalin-fixed and paraffin-embedded tissues from 37 cases of salivary CXPA with complete clinical and pathological data were retrieved from the Department of Oral Pathology at Shanghai Ninth People’s Hospital, Shanghai Jiao Tong University in Shanghai, China. Tissue sections (4 µm) were stained with hematoxylin and eosin (H&E) and were reviewed by two investigators. The tumors were histologically examined and classified as high or low grade (19). High-grade tumors exhibited ≥2 of the following features: i, anaplasia with nuclear pleomorphism and prominent nucleoli; ii, frequent mitoses: ≥5 per 10 high-power fields; iii, atypical mitosis; and iv, extensive coagulative tumor necrosis. The clinical stage of each patient’s disease was determined according to criteria of the tumor-lymph node-metastasis (TNM) classification system (2002) International Union Against Cancer (20). CXPAs could be classified into 2 main subtypes according to their morphological and immunohistochemical features. The classification of CXPA-L and CXPA-NL in our study followed the methods detailed by Kim et al (19). This study was approved by the ethics committee of Shanghai Jiao Tong University.

For our in vitro experiments, 2 CXPA cell lines (SM-AP1 and SM-AP4) (21) were cultured in DMEM supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine and 10% fetal bovine serum (FBS) and were incubated at 37°C in a humidified atmosphere containing 5% CO2. Induction of CDH1 promoter hypermethylation in SM-AP1 cancer cells was initiated by the addition of 10 ng/ml TGF-β1 (Peprotech, NJ, USA) to the medium for up to 72 h. Induction of CDH1 promoter demethylation in SM-AP4 cancer cells was initiated by the addition of a demethylation agent, 5-Aza-2’-deoxycytidine (5-Aza-dC; Selleck, TX, USA).

Western blotting and quantitative RT-PCR (qRT-PCR). Western blotting and qRT-PCR were carried out as previously described (23,24). Protein lysates were separated by 10% SDS-PAGE and electrophoretically transferred to a PVDF membrane (Millipore, MA, USA). Subsequently, the membrane was incubated with a primary monoclonal antibody followed by a fluorescent secondary antibody. β-tubulin was used as a protein loading control. Primary antibodies used for western blotting included those against E-cadherin (Abcam), vimentin (Abcam), and β-tubulin (Santa Cruz, CA, USA). Western blot bands were visualized using Imaging system (LI-COR Biosciences, Lincoln, NE, USA), and protein density was quantified using Odyssey version 1.2 software (LI-COR Biosciences). qRT-PCR was performed using SYBR-Green PCR master mix (Applied Biosystems) on an ABI 7300 system. PCR primers were as follows: CDH1 (5’-AGAACAGCAC GTACAGCACCTAA-3’ and 3’-ATCACAGAACTGTTCC TGTTCCA-5’) and β-actin (5’-CTCCATCCTGGCCTCGC TGT-3’ and 3’-GCTGTCACCTTCCAGGTCC-5’).

Immunohistochemistry and evaluation. Immunohistochemistry (IHC) was performed on 4-µm paraffin-embedded sections according to the protocol. An anti-E-cadherin receptor antibody (monoclonal mouse anti-human, dilution 1:200; Life Technologies, USA) was applied as the primary antibody for IHC detection. The IHC procedure was performed by the Envision™ method (Dako, Glostrup, Denmark) according to the manufacturer’s protocol. In the negative control samples, primary antibodies were replaced by PBS. Normal salivary gland tissue slices served as a positive control. In the CXPA samples, E-cadherin was located on the cell membrane and in the cytoplasm. Five random high-power fields were chosen from every slice to assess the E-cadherin score. The score of each slice was based on the percentage and intensity of positively stained cells. The percentage scoring system was as follows: no positive cells (0), <50% positive tumor cells (1), 50-75% positive tumor cells (2), and >75% positive tumor cells (3). The intensity scoring system was as follows: no staining (0), light yellow (1), yellow brown (2), and dark brown (3). The percentage score was multiplied by the intensity score and sections were divided into 2 groups based on the resulting product, as follows: low expression (score ≤6) and high expression (score >6). IHC slides were scored by two pathologists without knowledge of the clinical data in order to eliminate bias. Discrepancies were eliminated by consensus.

Methylation status was defined as low [methylation rate (MR)≤20%], medium (20%<MR≤40%), or high (MR≥40%).
compared using the $\chi^2$ test. Associations between clinico-pathological variables and CDH1 promoter methylation status were evaluated using Pearson's $\chi^2$ test. Patient survival analysis was performed using the Kaplan-Meier method, and differences were evaluated with the log-rank test. Hazard ratios (HR) and their 95% confidence intervals (CI) were estimated using univariate and multivariate Cox proportional hazard models. All statistical analyses were considered significant when the P-value was $\leq$0.05.

**Results**

**Clinical and pathological characteristics.** A total of 37 CXPAs from 26 males (70.27%) and 11 females (29.73%) were investigated in this study. The male to female ratio was 2.36. The age range of the patients was 26-83 years, and the mean age was 61.62 years. Twenty-nine tumors (78.38%) originated from the major salivary glands, and 8 tumors (21.62%) originated from the minor salivary glands. Histologically, 16 (43.24%) tumors were classified as low grade, and 21 (56.76%) were classified as high grade. Perineural invasion was observed in 11 of 37 patients (29.73%). Sixteen patients (43.24%) developed lymph node metastases. The mean follow-up time was 28.86 months. There were 17 deaths, 16 patients died of CXPA and 1 of another disease. Of those that survived, 17 patients survived without tumors and 3 survived with tumors.

**Promoter methylation status of CDH1 and its correlation with E-cadherin expression.** The methylation status of CDH1 was analyzed in 37 salivary CXPA tissues using BSP. As shown in Table 1, low, medium and high methylation of the CDH1 promoter (Fig. 1) was found in 16 (43.24%), 9 (24.32%) and 12 (32.43%) cases, respectively. The correlation between CDH1 methylation status and E-cadherin expression is shown in Table I. The correlation between CDH1 methylation and E-cadherin expression was statistically significant ($P=0.01$).

![Figure 1. Case 1, case 2 and case 3 showed high, medium, and low methylation status on CDH1 promoter, with MR of 42, 17 and 2% respectively.](image-url)
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12 (32.43%) CXPA samples, respectively. IHC analysis was performed to investigate E-cadherin expression. The results showed that 13 (35.14%) of 37 cases had low E-cadherin expression, while 24 (64.86%) cases showed high E-cadherin expression (Table I and Fig. 2). As shown in Table I, we found that CDH1 promoter methylation was significantly lower in the high E-cadherin expression group as compared with the low E-cadherin expression group (P<0.01). This result indicates that the methylation status of CDH1 strongly correlates with E-cadherin expression.

CDH1 promoter methylation and mRNA and protein expression in CXPA cell lines. CDH1 promoter methylation was detected in both SM-AP1 and SM-AP4 cell lines (Fig. 3A). Consistent with the notion that methylation contributes to gene inactivation, CDH1 hypermethylation (MR=48%) status resulted in lower CDH1 mRNA and protein expression in SM-AP4 cell lines than (MR=23%) in SM-AP1 cell lines (Fig. 3B and C). When treated with 5-Aza-dC, a demethylating agent, SM-AP4 cells showed increased CDH1 mRNA and protein expression and decreased CDH1 promoter methylation (Fig. 4A-C). However, SM-AP1 cells showed decreased CDH1 mRNA and E-cadherin expression but an elevated methylation status after TGF-β1 treatment (Fig. 4D-F). Taken together, we suggest that promoter methylation is a predominant factor regulating CDH1 expression in CXPA cell lines.

Associations between CDH1 promoter methylation and clinicopathological parameters. To evaluate the clinical significance of CDH1 promoter methylation, we investigated the association between methylation status and clinicopathological features in CXPA patients. As presented in Table II, CDH1 methylation status was differentially detected according to sex, histological subtype, histological grade, and tumor

Figure 2. E-cadherin immunohistochemistry with high, low and negative expression. E-cadherin was expressed on tumor cell membrane primarily, and cytoplasm partially.

Figure 3. The methylation status and mRNA of CDH1, and E-cadherin and vimentin protein expression in SM-AP1 and SM-AP4 cell lines. (A) CDH1 promoter region methylation rate was 48% in SM-AP1 and 23% in SM-AP4. (B) The mRNA of CDH1 expression in SM-AP4 was significantly lower than that in SM-AP1 cells.
N-stage and TNM-stage. CXPA cases with high histological grade (42.9% versus 7.1%, P=0.005), lymph node metastasis (56.2% versus 14.3%, P=0.024), or advanced TNM-stage (41.7% versus 7.7%, P=0.038) were more likely to display high CDH1 promoter methylation, which indicates that promoter methylation may be a prognostic factor in CXPA. Interestingly, compared with males, females tended to present with higher CDH1 methylation rates (P=0.028). CDH1 methylation status was not significantly correlated with other clinicopathological parameters, such as age, tumor site or neural invasion.

Figure 4. CDH1 promoter methylation status is associated with E-cadherin repression in 2 CXPA cell lines. (A) Schematic diagram showing the position of 10 CpG dinucleotides at the promoter region of CDH1. SM-AP4 cells were treated with 5-Aza-dC (5 µM) for 24 h, and methylation of the CDH1 promoter was analyzed by bisulfite sequencing. (B) The expression of CDH1 mRNA was analyzed by quantitative real-time PCR (mean ± SD from 3 separate experiments) after treated with 5-Aza-dC. (C) The SM-AP4 cells were treated with 5-Aza-dC for the indicated dose and time periods, and E-cadherin expression in these cells was analyzed by western blotting. (D) Schematic diagram showing the position of 10 CpG dinucleotides at the promoter region of CDH1. SM-AP4 cells were treated with TGF-β1 (5 ng/ml) for 3 days, and methylation of the CDH1 promoter was analyzed by bisulfite sequencing. (E) The expression of CDH1 mRNA was analyzed by quantitative real-time PCR (mean ± SD from 3 separate experiments) after treated with TGF-β1. (F) The SM-AP4 cells were treated with TGF-β1 (10 ng/ml) for the indicated time periods, and expression of E-cadherin and vimentin in these cells was analyzed by western blotting.
Survival analysis. Survival curves were generated for all 37 salivary CXPA cases. Methylation of the CDH1 promoter was significantly associated with overall survival (log-rank test, P=0.026) (Fig. 5). In univariate analyses, lymph node metastasis (P=0.004) and CDH1 promoter hypermethylation (P=0.030) were significantly associated with poor overall survival (Table III). To determine whether the association between CDH1 promoter methylation and survival was independent of other parameters, a multivariate analysis was performed including N-stage and CDH1 promoter methylation as co-factors. The multivariate analysis showed that lymph node metastasis (P=0.010) is independently associated with overall survival (Table III) and is an independent prognostic factor in CXPA.
Discussion

Generally, alterations in gene expression are mainly achieved by genetic and epigenetic methods. Genetic alternations primarily change the structure or number of a certain gene, whereas epigenetic alternations occur at the transcriptional level (9). CpG island methylation in the promoter region is a common epigenetic method of modifying gene expression. CpG methylation has been shown to modulate tumor progression in various cancer types, including esophageal squamous cell carcinoma (25,26), oral squamous cell carcinoma (27), salivary CXPA (28) and ACC (29). This modulation occurs mainly via the inactivation of tumor suppressor genes such as p16, MGMT, DAPK and RASSF1A. Altered CDH1 promoter methylation status has been shown to be the key factor in E-cadherin silencing in many tumors (7,9,11). CDH1 silencing is directly related to advanced tumor stage and an aggressive phenotype (7). This is the first study to evaluate CDH1 promoter methylation status in salivary CXPA. In this study, we have also demonstrated the relationship between E-cadherin expression and CDH1 promoter methylation.

In our study, an absence of E-cadherin expression was found in 35.14% (13/37) of CXPA cases. This is similar to a study by Zhang et al (6), which reported a negative E-cadherin detection rate of 38.33% across 60 ACC cases. However, negative E-cadherin expression was found in 68.42% (26/38) of eyelid SCC cases and 87.26% (18/23) of oral SCC cases. A study (7) in breast cancer showed a 42.33% (58/137) rate of reduced E-cadherin expression. It seems that E-cadherin reduction occurs with varying frequencies in different tumor types and at a relatively low frequency in salivary gland tumors specifically. In the meantime, we detected CDH1 promoter methylation using the BSP method, which is considered the ‘gold standard’ for determining DNA methylation and has the advantage of detecting methylation at each CpG site individually. Our study indicated that the CDH1 methylation rate in CXPA was 67.57% (25/37). This rate is similar to that of many other tumors, including primary lung cancer (88%) (30), breast carcinoma (65-95%) (7,10,31,32) and colorectal carcinoma (52%) (33). We found that DNA methylation preferentially occurred in the first four CpG islands compared with the other CpG islands.

We then analyzed the association between CDH1 methylation status and E-cadherin expression in CXPA patients. This analysis demonstrated that CDH1 methylation was significantly correlated with decreased E-cadherin expression (P<0.001) in clinical specimens. In addition, we evaluated the CDH1 methylation status and the corresponding CDH1 mRNA and protein levels in SM-AP1 and SM-AP4 cell lines. Consistent with the above results, cells with higher CDH1 methylation levels showed lower E-cadherin expression. Furthermore, to demonstrate that methylation is the critical factor in the silencing of E-cadherin expression, a dynamic experiment was performed in vitro. The demethylating agent 5-Aza-dC restored CDH1 mRNA and protein expression levels by reversing the high methylation status of SM-AP4 cell lines. Conversely, upregulation of CDH1 methylation levels via TGF-β1 treatment resulted in a repression of CDH1 mRNA and protein levels in SM-AP1 cells. TGF-β1-induced CDH1 promoter methylation was achieved by inducing the expression of the Snail protein, a transcriptional factor that binds the CDH1 promoter region and recruits DNA methyltransferases (DNMT), which

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CXPA, carcinoma ex pleomorphic adenoma; N-, negative lymph node; N+, positive lymph node; CI, confidence interval. *Statistically significant (P<0.05).
subsequently methyle the DNA fragment (34). TGF-β1 is a signaling molecule that mediates the epithelial-mesenchymal transition (EMT) (34). The hallmark of EMT is the loss of E-cadherin expression. In in vitro experiments, TGF-β1 treatment of SM-API cells resulted in the downregulation of the epithelial marker E-cadherin and upregulation of the mesenchymal marker vimentin. This indicated that the EMT process might play a role in the repression of E-cadherin in salivary CXPA.

Despite these results, however, CDH1 promoter methylation was not associated with the downregulation of E-cadherin expression levels in each case. As shown in Table I, E-cadherin expression was absent in one sample in the low-methylation group. Various studies have demonstrated that CDH1 expression levels in each case. As shown in Table I, E-cadherin expression levels are primarily, but not solely, regulated by DNA methylation in CXPA both in vivo and in vitro. Other regulatory mechanisms affecting CDH1 in CXPA may be investigated in further studies.

Consistent with similar studies in eyelid SCC (9), colorectal cancer (13) and breast cancer (32), the association of CDH1 methylation with cervical lymph node metastasis, histological grade and advanced tumor stage suggests that the CDH1 gene may be particularly important in salivary CXPA tumor progression. Consequently, CDH1 methylation, as well as N stage, is a strong predictor of overall survival in patients with CXPA in univariate survival analyses. However, in multivariate survival analyses, lymph node metastasis was shown to be an independent prognostic factor of overall survival for CXPA patients. Our findings provide evidence of the potential usefulness of CDH1 methylation status as an informative prognostic biomarker in patients with CXPA.

Reduction in E-cadherin expression is reportedly correlated with invasion, metastasis and recurrence of tumors in patients with oral squamous cell (36), bladder (37), and breast carcinomas (38). However, we observed no association between E-cadherin expression and any of the clinicopathological parameters that were investigated in the present study (data not shown). This discrepancy may be due to the smaller sample size of our study.

In conclusion, the present study indicates that DNA promoter methylation is the most common molecular abnormality of the CDH1 gene in salivary CXPA. Moreover, CDH1 promoter methylation is associated with histological differentiation, histological grade, tumor N stage and TNM stage. Methylation status may play a significant role in CXPA carcinogenesis and tumor progression and may be a reliable prognostic biomarker of poor patient survival. Further study of the correlation between abnormalities in the CDH1 gene and protein expression as well as interactions with other genes in salivary CXPA is therefore warranted.

Acknowledgements

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References