Bmi-1 expression predicts prognosis in salivary adenoid cystic carcinoma and correlates with epithelial-mesenchymal transition–related factors

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A B S T R A C T

Salivary adenoid cystic carcinoma (AdCC) is known for its high propensity to invade and metastasize. Bmi-1 acts as an oncogene by controlling cell cycle and self-renewal of adult stem cells, and its overexpression correlates with metastasis and poor prognosis in several cancers. Epithelial-mesenchymal transition (EMT) plays a central role in cancer metastasis. A key step in EMT is the down-regulation of E-cadherin that can be repressed by the transcriptional factors, such as Snail and Slug. In the present study, we investigated Bmi-1, Snail, Slug, and E-cadherin expression by immunohistochemistry in 102 patients with AdCC and analyzed statistically whether their expression correlated with clinicopathologic factors and prognosis. Reverse transcription–polymerase chain reaction was also performed in 22 tumor tissues and the adjacent noncancerous tissues to confirm Bmi-1 status in AdCCs. Our data demonstrated significant associations between the tumor metastasis and the expression of Bmi-1, Snail, Slug, and E-cadherin. Furthermore, a high level of Bmi-1 was not only correlated with the overexpression of Snail and Slug but also indicated an unfavorable metastasis-free survival and served as a high-risk marker for AdCC. In addition, Bmi-1 messenger RNA level was found much higher in AdCC tissues than in the adjacent noncancerous salivary gland tissues. Our results suggest that Bmi-1 may play a crucial role in AdCC progression by interaction with EMT-related markers and predict poor survival.

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1. Introduction

Salivary adenoid cystic carcinoma (AdCC) is one of the most common salivary gland malignant tumors that comprises approximately 10% of all epithelial salivary gland neoplasms [1]. It is characterized by slow progression; however, local recurrence and distant metastasis result in a poor long-term survival in that 80% to 90% of patients die of disease in 10 to 15 years [2]. Therefore, extensive efforts are being made to characterize molecular events associated with tumor metastasis and identify prognostic biomarkers in AdCC.

Polycomb group proteins are epigenetic gene-silencing proteins that have been implicated in embryonic development and oncogenesis. B-cell–specific Moloney murine leukemia virus insertion region-1 (Bmi-1), the first functionally identified polycomb group member, is originally identified as an oncogene cooperating with c-myc in a murine lymphomagenesis model [3]. The human Bmi-1 gene is located on chromosome 10p13 [4], containing a conserved RING finger domain at the N-terminus required for tumorigenesis and proliferation regulation [5] and a central helix-turn-helix-turn motif essential for transcriptional repression [6]. As a key epigenetic regulator and a candidate stem cell marker, Bmi-1 controls the cell cycle and self-renewal of tissue stem cells through chromatin and histone modifications. It has been reported that Bmi-1 is dysregulated in several cancer types and correlates with an invasive or metastatic phenotype [7]. In addition, Bmi-1 has been verified as a predictor of prognosis in non–small cell lung cancer, nasopharyngeal carcinoma, gastric carcinoma, metastatic melanoma, and breast carcinoma [8–12]. In spite of the aforementioned link between Bmi-1 and cancer, very few studies have focused on the involvement of Bmi-1 in AdCC.

Epithelial-mesenchymal transition (EMT) is considered to facilitate the conversion of polarized epithelial phenotype to motile fibroblastoid or mesenchymal phenotype and induce stem-like properties in epithelial cells [13]. Several lines of evidence show that cancers are prone to metastasize and establish secondary tumors at distant sites after acquiring the ability to undergo EMT [14]. Loss of E-cadherin and increased N-cadherin expression is a hallmark of EMT and the subsequent metastasis. Snail-related zinc finger transcriptional repressors (Snail/SNAI1 and Slug/SNAI2) are the most prominent suppressors of E-cadherin transcription, which act by binding to specific E-boxes in the E-cadherin promoter [15]. Recently, Bmi-1 has been reported to directly promote EMT and malignancy in nasopharyngeal carcinoma by regulating Snail via modulation of PI3K/Akt/GSK-3β signaling [16].

Based on this information, the aim of this study was to investigate the expression of Bmi-1, E-cadherin, Snail, and Slug in AdCCs and to
analyze the potential association of Bmi-1 and these EMT-related proteins with clinicopathologic parameters and patients’ survival in complete follow-up.

2. Materials and methods

2.1. Patients and tissue samples

Cases diagnosed as AdCC were reviewed from the files of Peking University School and Hospital of Stomatology during 1999 to 2006, and a total of 102 AdCCs were confirmed and selected for this retrospective study. At the same time, the corresponding 102 formalin-fixed, paraffin-embedded tumor samples were derived from the archives of the Department of Oral Pathology, Peking University School of Stomatology, after the approval of the university institutional ethics committee. Clinicopathologic variables, including original tumor site, TNM classification, pathologic subtype, perineural invasion, treatment modality, local regional recurrence, and distant metastasis, were reviewed. Tumor grading was confirmed according to the World Health Organization’s histologic classification of salivary gland tumors [2]. Patients’ follow-up data were obtained by clinical interviews or reviewing the medical records after surgery.

2.2. Immunohistochemistry

Four-micrometer-thick serial sections were cut and then mounted on poly-L-lysine coated slides, deparaffinized in xylene, and sequentially rehydrated through a graded ethanol series. Endogenous peroxidase activity was quenched by incubation with fresh 3% H2O2 in methanol for 20 minutes at room temperature, and then antigen retrieval was accomplished by 0.01 M citrate buffer (pH 6.0) or 10% EDTA buffer (pH 8.0). Immunostaining was performed by adding mouse monoclonal Bmi-1 antibody (1:250; Abcam, Cambridge, UK), rabbit polyclonal Snail antibody (1:400; Abcam), rabbit polyclonal Slug antibody (1:80; Santa Cruz, CA), or monoclonal mouse E-cadherin antibody (1:100; Zymed, San Francisco, CA) and incubated overnight at 4°C, followed by a 30-minute incubation with the secondary antibodies. The immunocomplexes were visualized using liquid DAB + substrate + chromogen system (Zymed). Sections were lightly counterstained with Mayer’s hematoxylin and mounted. Ten cases of noncancerous salivary gland tissue samples served as normal control. Positive controls consisted of tissue previously shown to express the protein of interest, and negative controls were performed by replacing each primary antibody with phosphate-buffered saline.

2.3. Evaluation of immunostainings

Immunohistochemical results were analyzed by 2 independent observers who were blinded for patients’ characteristics and outcome. The staining intensity and the ratio of positive cells were evaluated semiquantitatively within 4 to 6 microscopic fields at magnification ×200. Bmi-1 and E-cadherin stainings were assessed using an intensity reactivity score, in which staining intensity was judged as negative (0), weak (1), moderate (2), or strong (3). Reactivity was also determined according to the percentage of positive cells: up to 10% positive cells were graded as 1 (very low); 11% to 35%, as 2 (low); 36% to 70%, as 3 (moderate); and more than 71% as 4 (high). The final score was calculated by multiplying the staining intensity by the percentage of positive cells. If the score was greater than 4, the expression was considered high; otherwise, it was considered low [17,18]. Moreover, Bmi-1 expression patterns were assessed, and sites of nuclear and cytoplasmic expression were discriminated. The ratio of Snail positive cells was graded as follows: negative (0%-9%), low positive (10%-50%), or high positive (>50%) [19]. For Slug, high expression was defined as detectable immunoreaction in nuclear and cytoplasmic regions of more than 10% of the cancer cells. Stained cells were regarded to be positive regardless of staining intensity [20].

2.4. RNA extraction and reverse transcription–polymerase chain reaction

Total RNA from 22 fresh surgical cancer and adjacent noncancerous tissues was extracted with the RNeasy Mini Kit (Qiagen, Inc, Hilden, Germany), according to the manufacturer’s protocol. The RNA extracted was quantified with a NanoDrop ND-1000 Spectrophotometer (NanoDropTechnologies, Inc, Wilmington, DE). Reverse transcription of RNA was carried out with the cDNA Synthesis kit (Invitrogen, Carlsbad, CA). Bmi-1 was amplified by polymerase chain reaction (PCR) from the complementary DNA samples of non-AdCC and AdCC tissues. The

![Fig. 1](image_url). Immunoreactivity for Bmi-1, E-cadherin, Snail, and Slug in AdCC. High nuclear expression of Bmi-1 in AdCC (A). Intense cytoplasmic staining of Bmi-1 in tumor cells and weak Bmi-1 expression in the adjacent noncancerous salivary gland tissue (B). Strong cytoplasmic and membranous staining of E-cadherin in AdCC (C). Weak membranous staining of E-cadherin in AdCC (D). High positive staining of Snail in AdCC (E). High Slug expression in AdCC (F). All images, original magnifications, ×400.
The following primers were used for amplification of Bmi-1: sense primer, 5'-GTGTATTGTTCGTTACCTGGAGAC-3'; antisense primer, 5'-CATTGC TGCGGAGATCTGTA-3'. GAPDH was amplified as an internal control using sense primer, 5'-CACCATCTTCCAGGACGAG-3', and antisense primer, 5'-TGCGGACACGGCTTGGCAG-3'. The PCR products were analyzed by agarose gel electrophoresis and confirmed by appropriate size and/or sequencing.

2.5. Statistical analysis

All statistical analyses were carried out using the SPSS 17.0 software package. The $\chi^2$ test was used to analyze the correlation between Bmi-1 expression and the expression of EMT-related markers as well as the clinicopathologic characteristics. The overall survival rate, recurrence-free survival (RFS) rate, and metastasis-free survival rate were estimated using the Kaplan-Meier method. Potential prognostic factors were identified by univariate analysis using the log-rank test. Significant variables were further analyzed by the Cox proportional hazards model in the multivariate analysis to test for independent prognosis. Differences at $P < .050$ were considered significant.

3. Results

3.1. Immunostaining of Bmi-1 and EMT-related markers in AdCCs

One hundred two specimens of AdCCs (55 cases with metastasis and 47 without metastasis) and 10 normal salivary gland tissues were examined by immunohistochemistry. Bmi-1 high expression was seen in...
61.8% (63/102) of AdCCs, whereas normal salivary gland tissue indicated weak expression of Bmi-1, and of the 63 cases with Bmi-1 high expression, 34 (54.0%) were stained in the nucleus; and 29 (46.0%), in the cytoplasm (Fig. 1A and B). E-cadherin expression was identified as high in 68.6% (70/102) of AdCCs which was significantly down-regulated in metastatic AdCCs (Fig. 1C and D). Snail reactivity in AdCCs was identified mainly in the nucleus, whereas Slug was expressed in the nucleus and cytoplasm (Fig. 1E and F). Snail and Slug expressions were also detected higher in the AdCCs with metastasis than in those with no metastasis (61.8% positive vs 34.0% positive and 34.5% positive vs 14.9%, respectively). Furthermore, it was found that most patients with high Bmi-1 expression displayed high Snail and Slug expressions. Statistically significant correlations were identified not only between Bmi-1 and Snail expressions (P = .013) but also between Bmi-1 and Slug expressions (P = .005). No statistically significant association was found between E-cadherin and Bmi-1 expressions (P = .737).

To further confirm Bmi-1 status in AdCCs, reverse transcription-PCR was performed in AdCC tissues and the adjacent noncancerous salivary gland tissues. As expected, Bmi-1 messenger RNA (mRNA) level was found much higher in tumor tissues than in noncancerous tissues (Fig. 2), which was in accordance with the protein level examined by immunohistochemistry.

### 3.2. Relationship between Bmi-1 expression and clinical parameters of AdCCs

The relationship of Bmi-1 expression levels with clinicopathologic features of AdCCs was statistically evaluated and presented in Table 1. Bmi-1 high expression was found much more common in AdCCs with metastasis than in those without metastasis (P < .001). However, no evident correlations were observed between Bmi-1 expression profiles and other clinicopathologic features, including age, sex, original tumor site, pathologic subtype, clinical stage, T classification, treatment modality, the presence of lymph node metastasis, perineural invasion, and local regional recurrence (P > .050). The univariate and multivariate analyses of factors related to patient prognosis were performed on 102 patients’ overall survival, RFS, and metastasis-free survival (Tables 2 and 3). The log-rank test indicated that the solid subtype, advanced clinical stage, and negative E-cadherin expression correlated with poor overall survival (Fig. 3A-C). Furthermore, Bmi-1 cytoplasmic staining (in contrast to nuclear expression) had a negative influence on overall survival in the tumor group with Bmi-1 high expression (Fig. 3D). The subsequent multivariate analysis using the Cox proportional hazards model revealed that only histologic subtype, clinical stage, and E-cadherin expression were independent significant prognostic factors (Table 3).

Meantime, the RFS rates in patients more than 50 years old were significantly better than in the younger group (P < .010 Fig. 3E), and the patients who were diagnosed with the cribriform/tubular subtypes also had a better RFS rate compared with solid type (P < .028; Fig. 3F). It seemed that patients who underwent surgery alone appeared to have a lower recurrence rate than patients who received adjuvant radiotherapy or chemoradiotherapy by log-rank test (P = .031; Fig. 3G). However, this significance was no longer present in the multivariate analysis.

Furthermore, the high level of Bmi-1 expression was identified as a high risk of distant metastasis in univariate analyses (P = .001; Fig. 3H). The log-rank test also showed that the solid subtype (P = .000; Fig. 3I), advanced clinical stage (P = .002; Fig. 3J), negative E-cadherin expression (P = .001; Fig. 3K), and high Snall expression (P = .014; Fig. 3L) predicted unfavorable metastasis status. Cox regression proportional hazard analysis showed that Bmi-1 expression, histologic subtype, advanced clinical stage, and negative expression of E-cadherin remained independent covariates that could predict poor metastasis-free survival after ruling out confounding factors (Table 3).

### 4. Discussion

In the present study, we illustrated that high levels of Bmi-1, Snail, and Slug expression as well as reduced E-cadherin expression were significantly linked to distant metastasis in AdCC and subsequently demonstrated the association between Bmi-1 expression, EMT-related markers, and clinicopathologic parameters, which addressed the prog-

### Table 3

<table>
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<th>Variable</th>
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<th>95% CI</th>
<th>Cox P</th>
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</tr>
<tr>
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<tr>
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<td>E-cadherin expression</td>
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<td>2.405-11.026</td>
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P < .05 statistically significant. Abbreviations: C, chemotherapy; MFS, metastasis-free survival; OS, overall survival; R, radiotherapy; T, tumor; Uni, univariate.
nostic role of Bmi-1 in a large series of 102 AdCC cases. Our data showed that 61.8% of AdCCs were observed with high level of Bmi-1 expression, whereas the normal salivary gland tissue indicated weak or negative expression of Bmi-1. Furthermore, Bmi-1 mRNA level was also found much higher in AdCC tissues than in the adjacent noncancerous salivary gland tissues, which was consistent with the protein level examined in AdCC and non-AdCC tissues. These results were in accordance with other studies that reported significantly elevated Bmi-1 expression in AdCCs.

Fig. 3. Kaplan-Meier curves of overall survival, RFS, and metastasis-free survival. Overall survival based on histologic subtypes (A), clinical stages (B), E-cadherin expression (C), and staining sites of Bmi-1 high expression (D) in AdCCs. Recurrence-free survival for patients in different ages (E), histologic subtypes (F), and treatment modalities (G). Metastasis-free survival based on Bmi-1 expression (H), histologic subtypes (I), clinical stages (J), E-cadherin (K), and Snail expression in AdCCs (L). P values were calculated by the log-rank test.
a number of malignancies such as breast cancer, nasopharyngeal carcinoma, and melanoma [9,11,12]. These clues demonstrate that Bmi-1 is involved in neoplastic transformation. Recently, it has been reported that both benign and malignant myoepitheliomas showed elevated levels of Bmi-1 compared with normal tissue [21], and AdCC is identified as a tumor with a biphasic pattern of epithelial and myoepithelial cells. Therefore, to confirm that the elevated Bmi-1 expression is involved in malignant transformation but not due to the increased amount of myoepithelial cells, we investigated the relationship between Bmi-1 expression and the expressions of several myoepithelial specific markers, such as p63, smooth muscle actin, S-100, and calponin. The results showed that there was no significant relation between Bmi-1 expression pattern and myoepithelial markers (data not shown), which suggest that the high level of Bmi-1 in AdCC was not affected by the amount of myoepithelial cells in AdCC.

Distant metastases have been reported to be associated with high expression of Bmi-1 in breast cancer, melanoma, gastric cancer, and nasopharyngeal carcinoma [9–12]. In the present study, a significant relation was found between Bmi-1 overexpression and the distant metastasis of AdCCs, which suggests that a high level of Bmi-1 may play a role in the distant metastasis of AdCC. Meantime, our observations indicated that low membranous E-cadherin expression and high nuclear/cytoplasmic Snail and Slug expression also significantly correlated with distant metastasis in AdCC. Metastatic relapse remains a major challenge in AdCC management. Tumor distant metastases are correlated with distant metastasis in AdCC. Metastatic relapse remains a major challenge in AdCC management. Tumor distant metastases are correlated with distant metastasis in AdCC, we analyzed the associations between individual proteins including Bmi-1, E-cadherin, Snail, and Slug. Interestingly, our data suggested that AdCCs with strong Bmi-1 staining also displayed significantly higher expression of Snail and Slug, but no significant association was found between Bmi-1 and E-cadherin expressions. When we prepared for this article, Chang et al [30] reported that when Bmi-1 was knockdown in AdCC cells in vitro, the expressions of both Snail and Slug were inhibited followed by the reduced clone forming ability and cell proliferation rate, which confirmed that Bmi-1 deregulation plays an important role in the development of AdCC and is involved in EMT and cancer stem cells.

Because current prognostic indicators are not as reliable as desired in AdCC, further efforts should be made to identify a novel molecular predictor of tumor behavior at the time of the diagnosis of AdCC. In the present study, overexpression of Bmi-1 was shown to be strongly associated with metastasis-free survival of AdCC and represented a potential predictor for tumor distant spread. Furthermore, the subcellular localization of Bmi-1 was shown significantly linked to overall survival. Both nuclear and cytoplasmic Bmi-1 expressions have been described in the literatures [17,31]. Cytoplasmic staining was absent in surrounding stromal cells and lymphocytes, and it statistically significantly correlated with overall survival, which suggested that it was more than an unspecific phenomenon. It has been reported that transcription-dependent subcellular trafficking of some transcription factors is a well-known phenomenon and has potential relevance for the biologic behavior of some tumors. In fact, it may also be a sign of the dedifferentiation of tumor cells [31]. In addition, we had shown in univariate and multivariate analyses that negative E-cadherin expression, solid histologic subtype, and advanced clinical stage were prognostic indicators of distant metastasis and poor survival for AdCCs.

In summary, the present study showed the high expression of the stem cell marker Bmi-1 in metastatic phenotype of AdCC and the significant correlation between the expression of Bmi-1 and EMT-related markers, which suggested that Bmi-1 may play a pivotal role in cell invasion and metastasis of AdCC interacting with EMT-related markers. Bmi-1 may have prognostic value and opens the door for further development of biotherapies targeted at specific inhibition of Bmi-1 activities.

Declaration of conflicting interests

The author declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

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