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Original Article

B-cell specific Moloney murine leukemia virus insertion site 1 contributes to invasion, metastasis, and poor prognosis in salivary adenoid cystic carcinoma

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KEYWORDS
BMI-1;
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Prognostic predictor

Abstract *Background/purpose:* Upregulation of B-cell specific Moloney murine leukemia virus insertion site 1 (BMI-1) has been involved in the invasion, metastasis, and poor prognosis of many cancers. The aim of this study was to evaluate the levels and clinical significance of BMI-1 in saliva of patients with salivary adenoid cystic carcinoma (SACC), and to analyze biological function and mechanism of BMI-1 in the invasion and metastasis of SACC.
Materials and methods: The levels of BMI-1 in saliva and tumor tissues of SACC patients were determined. The correlation of salivary BMI-1 levels with clinicopathological parameters and clinical outcomes in patients with SACC was analyzed. Additionally, the effects of BMI-1 on wound-healing, transwell invasion, and epithelial-mesenchymal transition (EMT)-related protein expression *in vitro* as well as on tumorigenicity and experimental lung metastasis *in vivo* were investigated through exogenous overexpression and silencing of BMI-1 in SACC cells.
Results: BMI-1 levels increased in saliva and tumor tissues in SACC patients with invasion or metastasis. High salivary BMI-1 levels were correlated with poor TNM stage, poor overall survival, and disease-free survival. Exogenous expression of BMI-1 in SACC-83 promoted its

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migration and invasion, while silencing BMI-1 in SACC-LM inhibited its migration and invasion *in vitro* and suppressed tumorigenesis and lung metastasis *in vivo*. Furthermore, BMI-1 regulated the expression of EMT-related proteins in SACC.
Conclusion: Our study shows that BMI-1 can serve as a valuable biomarker to identify tumor invasion and metastasis in SACC, predict its prognosis, and act as a promising therapeutic target for SACC.

Introduction

Salivary adenoid cystic carcinoma (SACC) is one of the most common salivary gland tumors, accounting for approximately 22%—25% of salivary gland malignancies.[1] It is characterized by a strong local invasion and a high rate of distant metastasis, resulting in poor survival. Therefore, characterizing the molecular mechanisms involved in the invasion and metastasis of SACC and identifying effective biomarkers for diagnosing and predicting SACC is crucial and urgent.

B-cell specific Moloney murine leukemia virus insertion site 1 (BMI-1), a member of the polycomb group of transcriptional repressors, was originally identified as an oncogene contributing to murine lymphoma. Subsequent studies revealed the involvement of BMI-1 in multiple biological functions, including cell proliferation, apoptosis, immortalization, and initiation and progression of various cancers.[2—9] In addition, increased BMI-1 expression has been reported to promote the invasion and metastasis of several human cancers, including breast, gastric and esophageal cancers.[10—12] BMI-1 is also involved in the poor prognosis of colorectal cancer, non-small cell lung cancer, head and neck squamous cell carcinoma, pediatric acute lymphoblastic leukemia, and renal cell carcinoma.[13—17] However, the levels, biological function, and clinical significance of BMI-1 in salivary adenoid cystic carcinoma (SACC) remain unclear.

Materials and methods

Patients and specimens

Saliva and tumor tissue specimens were obtained from a cohort of 94 patients who were histologically diagnosed with SACC (38 patients with local invasion or distant metastasis and 56 patients without local invasion or distant metastasis) and underwent initial surgical treatment in the Department of Stomatology, The Affiliated Wuxi People's Hospital of Nanjing Medical University Wuxi, China, Center of Stomatology, The Central Hospital of Enshi Tujia and Miao Autonomous Prefecture, Enshi, China, between December 2006 and January 2010. Patients' saliva was collected between 9 a.m. and 11 a.m. before the surgery. Saliva was also obtained from 20 healthy donors, matched by age and sex using the same conditions and methods to be used as normal controls. The clinicopathological characteristics of 94 patients are summarized in Supplementary Table S1. The study was approved by the Institutional Ethics Committee of the two hospitals mentioned above. Written informed consent was provided by all patients and healthy donors following the institutional guidelines. The study adhered to the tenets of the Declaration of Helsinki.

Cell culture

The human SACC cell lines, SACC-83 and SACC-LM, were purchased from Peking University (Beijing, China). ACC-2 and ACC-M were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). SACC-LM and ACC-M are highly metastatic cells derived from lung metastases of SACC-83 and ACC-2 xenografts, respectively.[18] All cells were cultivated in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco BRL), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified 5% CO_2 atmosphere.

Enzyme-linked immunoassay (ELISA) analysis

The levels of BMI-1 in saliva were assessed using a human BMI-1 ELISA Kit (JL22649, Jianglaibio, Shanghai, China) according to the manufacturer's protocols. The median was set as the cut-off value to determine high or low levels of BMI-1 in saliva. Regarding the patients with SACC, the cut-off value was 148.16 (range 40.92—346.75); a value $>$ 148.16 was considered a high salivary BMI-1 level, and a value $<$ 148.16 was considered a low salivary BMI-1 level.

Immunohistochemical analysis

Paraffin-embedded specimens were cut into 4 μm sections, deparaffinized, rehydrated, and treated with citrate buffer for heat-induced epitope retrieval. The sections were then incubated with an anti-BMI-1 antibody (6964, Cell Signaling Technology, Danvers, MA, USA, 1:200), followed by incubation with a peroxidase-conjugated secondary antibody. Image-pro plus 6.0 (IPP 6.0) software was used to quantitatively determine the integrated optical density (IOD) of BMI-1 staining. Five lesion areas of each section were randomly selected under the same conditions for further analysis.

Quantitative real-time polymerase chain reaction (real-time PCR)

Real-time PCR reactions were performed using an ABI StepOne real-time PCR system and the One Step TB Green PrimeScript RT-PCR Kit (RR066A, Takara, Shiga, Japan). The real-time PCR primer sequences used are as follows: BMI-1, forward: 5′-CAAGACCAGACCACTACTGAAT-3′, reverse: 5′-TATCTTCA TCTGCAACCTCTCC-3′; β-actin, forward: 5′-CCTGGCACCCAGCACAAT-3′, reverse: 5′-GGGCCGG ACTCGTCATACT-3′.

Western blot analysis

Cell lysates were electrophoresed through 10%–15% polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was incubated with anti-BMI-1 (6964, 1:1000), anti-E-cadherin (14,472, 1:1000), anti-Vimentin (5741, 1:1000), anti-N-cadherin (13,116, 1:1000), or anti-Snail antibodies (3879, 1:1000) (all from Cell Signaling Technology). Secondary antibodies were labeled with IRDyes. Signals were observed using an Odyssey Infrared Imaging System.

Transwell invasion assay

The cell invasion assay was performed with Matrigel coated on the upper surface of the Transwell chamber (pores 0.8 μm, Merck Millipore, Burlington, MA, USA). Cells invading the membrane were fixed and stained with 0.1% Coomassie Brilliant Blue. Images of three randomly selected fields of the fixed cells were captured, and the cells were counted using Image-pro plus 6.0 software.

Tumorigenicity assay *in vivo*

Ten 4-week-old BALB/C nude male mice were used for the study. 1×10^6 SACC-LM cells stably transfected with shRNA BMI-1 or shRNA NC lentivirus in 100 μL serum-free RPMI-1640 were subcutaneously injected into the left buttocks of the animals. Each treatment group consisted of five mice. The tumor volume was measured every 3 days from the third day after injection and calculated using the following formula: tumor volume = length × width × width/2. The animals were sacrificed 18 days after injection, the tumor nodules were removed, and tumor weights were measured.

Experimental metastasis assay *in vivo*

Ten 4-week-old athymic female mice were used for *in vivo* experimental metastasis assay. 1×10^6 SACC-LM cells stably transfected with shRNA BMI-1 or shRNA NC lentivirus in 200 μL serum-free RPMI-1640 were intravenously injected into the lateral tail vein of the mice. Each treatment group consisted of five mice. The weights of the mice were measured every 5 days. The animals were sacrificed 25 days after injection, and their lungs were removed. Surface metastatic nodules per lung were then determined.

Statistical analysis

The statistical significance was determined using Statistical Package for Social Sciences Version 19.0 (SPSS19.0). Student's *t* test and one-way ANOVA were used to compare the means of two groups or more. The log-rank test analyzed univariate associations between BMI-1 protein levels in saliva and overall survival and disease-free survival. The Cox proportional-hazards model was used for multivariate analyses. All tests were two-sided, and *P*-values < 0.05 were considered statistically significant.

Results

BMI-1 levels increased in SACC patients with invasion or metastasis

To determine BMI-1 levels, we first performed ELISA on samples from 20 healthy volunteers and 94 patients with SACC. We found that the saliva of SACC patients with local invasion or distant metastasis had a higher level of BMI-1 than patients without local invasion or distant metastasis (Fig. 1A). Correspondingly, the salivary BMI-1 levels in both groups were notably higher than that in healthy volunteers (Fig. 1A). We then performed immunohistochemistry to detect the BMI-1 level in tumor tissues of the same cohort of patients with SACC to validate the accuracy of saliva testing by ELISA. As expected, the level of BMI-1 in tumor tissues of SACC patients with local invasion or distant metastasis was significantly higher than patients without local invasion or distant metastasis (Fig. 1B). Representative images showed weak staining of BMI-1 in tumor tissue of SACC patient without lung metastasis (Fig. 1D and F), whereas strong staining of BMI-1 was observed in tumor tissue of SACC patient with lung metastasis (Fig. 1E and F). Furthermore, Pearson's correlation analyses were performed to estimate the consistency of BMI-1 levels detected by these two methods in the same cohort of SACC patients. A significantly positive correlation between the results of immunochemistry and ELISAs was observed ($R = 0.8804$, $P < 0.0001$, (Fig. 1C).

High salivary BMI-1 levels correlated with poor TNM stage and poor overall and disease-free survival

The correlations between the salivary BMI levels and clinicopathological parameters in patients with SACC were analyzed to evaluate the clinical significance of salivary BMI levels. We found that high salivary BMI-1 levels correlated with poor TNM stage (Fig. 2G). In contrast, no significant associations were found between the salivary BMI levels and age ($P = 0.8526$), gender ($P = 0.4034$), smoking history ($P = 0.1683$), alcohol history ($P = 0.3398$), pathology subtype ($P = 0.3496$), or disease site ($P = 0.8778$, Fig. 2A–F). Next, the overall and disease-free survival probabilities were estimated using Kaplan–Meier survival analysis to determine the correlation between the salivary BMI levels and clinical outcomes in patients with SACC. We observed that patients whose saliva contained high levels of BMI-1 had significantly poorer overall ($P = 0.0036$, Fig. 2H)

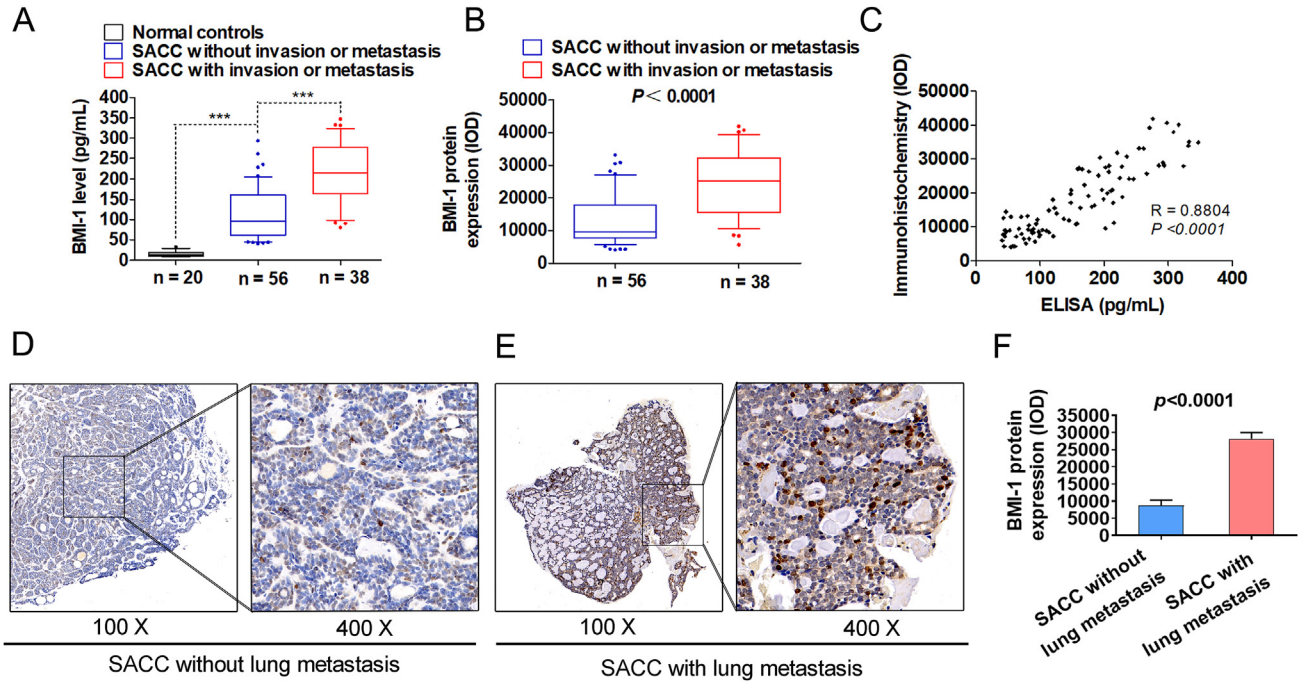

Fig. 1 BMI-1 level increases in saliva and tumor tissues in SACC patients with invasion or metastasis. (A) Enzyme-linked immunoassays show salivary BMI-1 levels of 38 SACC patients with invasion or metastasis, 56 SACC patients without invasion or metastasis, and 20 healthy donors (Normal controls). (B) Immunohistochemical staining shows BMI-1 levels in tumor tissues of 38 SACC patients with invasion or metastasis and 56 SACC patients without invasion or metastasis. (C) Pearson's correlation analysis evaluated the conformance of BMI-1 levels in saliva and tumor tissues from 94 patients with SACC. (D, E) Representative images show BMI-1 immunohistochemical staining in tumor tissues from SACC patients with or without lung metastases. (F) The immunohistochemical staining of BMI-1 of D and E were quantitatively analyzed with Image-pro plus 6.0 software (IOD, integrated optical density; ***$P < 0.001$).

and disease-free survival ($P = 0.0001$, Fig. 2I). In addition, multivariate Cox proportional analyses indicated that the salivary BMI levels (HR, 2.482; 95% CI, 1.060—5.813; $P = 0.036$) could be an independent predictor of clinical outcome in patients with SACC (Table 1).

BMI-1 levels increased in SACC cells with high invasion and metastasis potential

We further aimed to confirm the expression of the BMI-1 gene in SACC cells with different invasion and metastasis potentials. We performed real-time PCR and Western blot analyses to investigate the transcriptional and translational status of BMI-1 in SACC-83 and ACC-2 with low invasion and metastasis potential and SACC-LM and ACC-M with high invasion and metastasis potential. We found significantly higher BMI-1 mRNA and protein levels in SACC-LM and ACC-M, as compared to SACC-83 and ACC-2 (Fig. 3A and B). Moreover, the expression levels of the BMI-1 gene in the two groups of SACC cells were remarkably higher than those in normal salivary gland tissues (Normal-1 and Normal-2).

BMI-1 affected migration and invasion of SACC cells *in vitro*

Based on the BMI-1 expression patterns, we transfected an shRNA BMI-1 lentivirus into SACC-LM with high endogenous BMI-1 (Fig. 4A and B) and an Ad BMI-1 adenoviral vector overexpressing BMI-1 into SACC-83 cells with low BMI-1 expression (Fig. 5A and B). Using the wound-healing and transwell invasion assays, we found that downregulating BMI-1 inhibited the migration and invasion of SACC-LM (Fig. 4C—F). As expected, migration and invasion abilities were increased in SACC-83 cells transfected with Ad BMI-1 compared with mock-transfected control cells (Fig. 5C—F).

BMI-1 silencing inhibited tumorigenicity and lung metastasis of SACC cells *in vivo*

We next performed a xenograft assay using SACC-LM cells stably transfected with shRNA BMI-1 or shRNA NC lentivirus to determine whether BMI-1 affected tumorigenicity of SACC cells *in vivo*. We found that the silencing of BMI-1 expression by shRNA BMI-1 lentivirus significantly inhibited the growth rate, size, and weight of subcutaneous tumors of SACC-LM when compared with shRNA NC control cells (Fig. 6A—C). Moreover, we investigated the effect of BMI-1 downregulation on tumor metastasis of SACC cells by injecting SACC-LM stably transfected with shRNA BMI-1 or shRNA NC lentivirus into the lateral tail of athymic mice. The shRNA BMI-1-mediated silencing of BMI-1 in SACC-LM cells ameliorated the weight loss in mice and decreased metastatic nodules of lungs when compared with shRNA NC control cells (Fig. 6D—F).

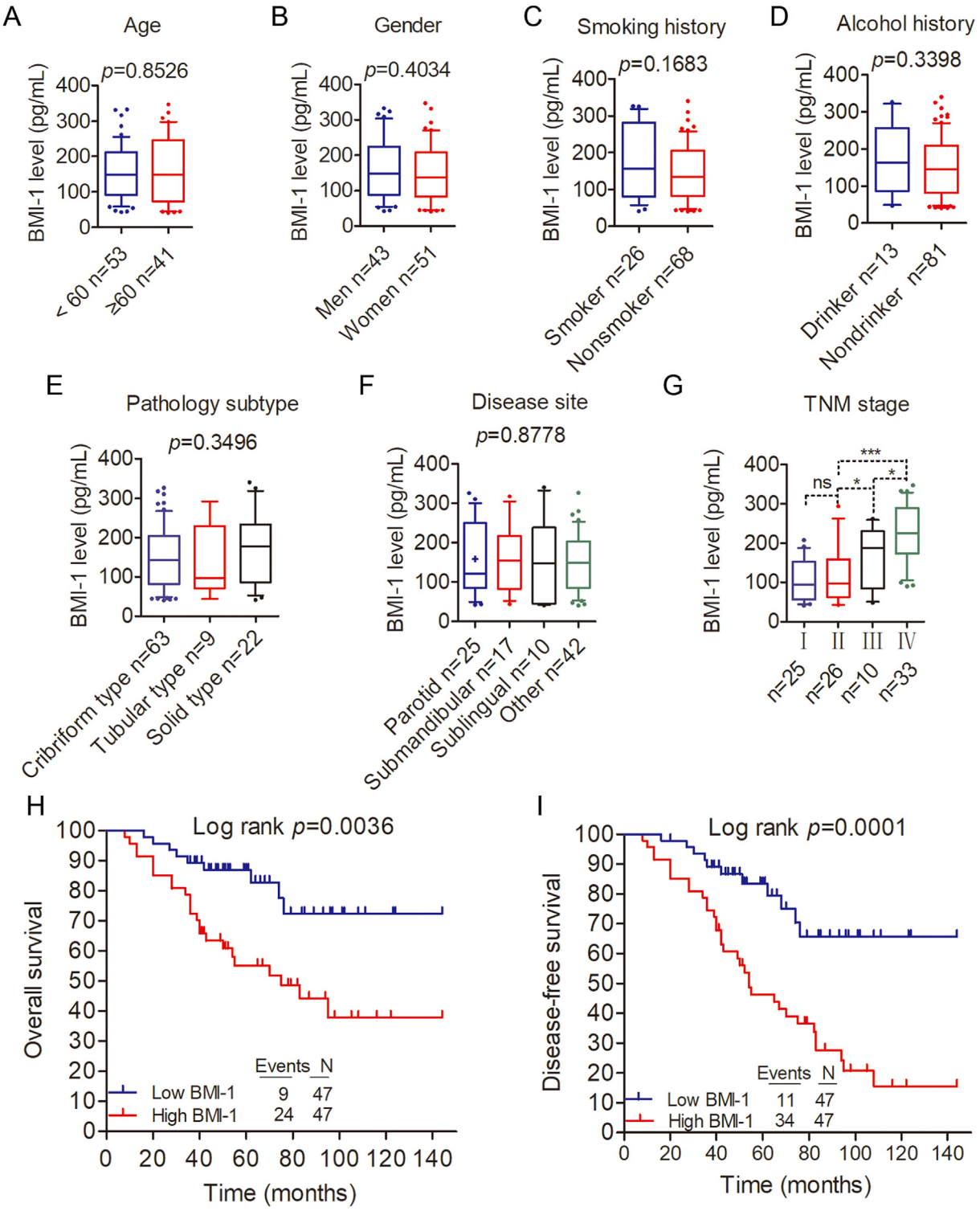

Fig. 2 High salivary BMI-1 levels correlate with poor TNM stage and poor overall and disease-free survival. (A–G) The associations between the salivary BMI-1 levels and clinicopathological parameters (age, gender, smoking history, alcohol history, pathology subtype, disease site, and TNM stage) were analyzed in patients with SACC. (H, I) Kaplan–Meier survival curves illustrate the overall survival (H) and disease-free survival (I) of patients with SACC according to the BMI-1 protein levels in saliva (ns, no significant difference; $*P < 0.05$; $***P < 0.001$).

Table 1 Univariate and multivariate cox proportional hazards regression models for estimating overall survival in patients with salivary adenoid cystic carcinoma (SACC).

Characteristics	HR	95%CI	*P* value
Univariate analysis			
Age (<60 y vs ≥ 60 y)	1.198	0.594–2.413	0.614
Sex (men vs women)	0.888	0.447–1.762	0.733
Smoking history (smoker vs nonsmoker)	1.201	0.571–2.258	0.629
Alcohol history (drinker vs nondrinker)	0.85	0.326–2.212	0.738
Pathology subtype	1.544	1.065–2.238	0.022
TNM stage	1.431	1.066–1.922	0.017
BMI-1 level (high vs low)	2.944	1.368–6.335	0.006
Disease site	0.973	0.742–1.275	0.84
Multivariate analysis			
TNM stage	1.151	0.828–1.600	0.403
Pathology subtype	1.468	1.014–2.123	0.042
BMI-1 level (high vs low)	2.482	1.060–5.813	0.036

Abbreviations: SACC, salivary adenoid cystic carcinoma; HR, hazard ratio; CI, confidence interval.

BMI-1 regulated the expression of EMT-related proteins in SACC

BMI-1 has been shown to contribute to invasion and metastasis by regulating EMT in many cancers. Therefore, we performed western blotting to evaluate whether the EMT-related protein expression was altered in SACC cells downregulating and overexpressing BMI-1. As shown in Fig. 7, BMI-1 downregulation increased the level of E-cadherin and decreased N-cadherin, Vimentin, and Snail in SACC-LM cells transfected with the shRNA BMI-1 lentivirus. On the other hand, the expression of E-cadherin was decreased, and that of N-cadherin, Vimentin, and Snail were increased in SACC-83 cells transfected with Ad BMI-1 compared with mock-transfected control cells (Fig. 7).

Discussion

In our present study, the BMI-1 levels in saliva and tumor tissues of patients with SACC were detected using ELISA and immunohistochemistry respectively. We found that the results of these two methods were in good agreement. As a secretion of the salivary glands, saliva has long been regarded as a valuable biological fluid for analyzing electrolytes, hormones, DNA, antibodies, enzymes, and various other proteins.[19–23] Because of the unique advantages of saliva testing, such as noninvasiveness, pain-free, ease of collection, and affordability, compared to histopathology, immunohistochemistry, and other diagnostic methods, it has been widely utilized to diagnose numerous cancers.[24–28] Our results showed that the salivary BMI-1 levels in SACC patients with local invasion or distant metastasis were significantly higher than in SACC patients without invasion or metastasis. These results were consistent with the findings in tumor tissues of endometrial adenocarcinoma, gastric cancer, and pancreatic cancer.[29–31]

In addition, several recent studies reported that the upregulation of BMI-1 was associated with poor prognosis in many cancers, including cancer of the renal cell, tongue, uterine, and lung.[17,32–34] Therefore, we analyzed the correlations between the salivary BMI-1 levels and clinical

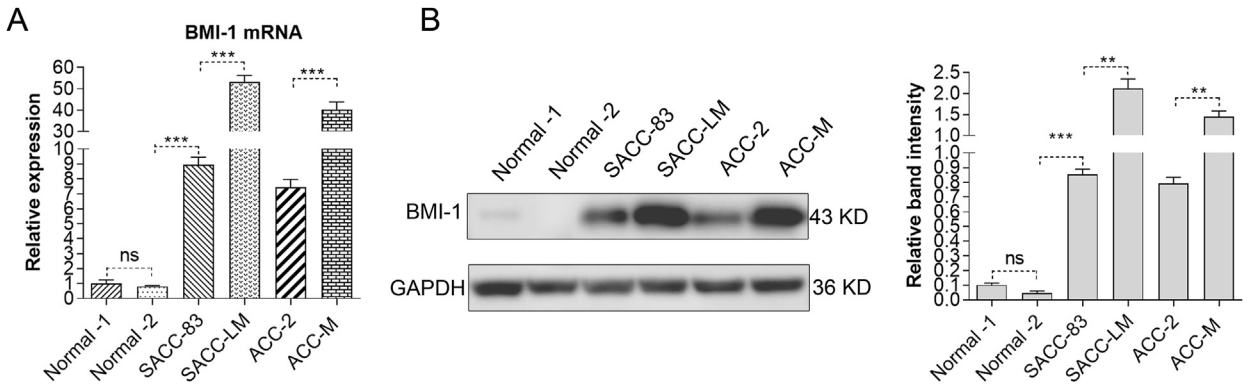

Fig. 3 BMI-1 levels increase in SACC cells with high invasion and metastasis potential. (A) Real-time PCR and (B) Western blotting analyses show the mRNA and protein levels of BMI-1 in SACC cell lines (SACC-LM and ACC-M with high invasion and metastasis potential, and SACC-83 and ACC-2 with low invasion and metastasis potential) and normal salivary gland tissue (Normal-1 and Normal-2) as control (ns, no significant difference; $^{**}P < 0.01$, $^{***}P < 0.001$).

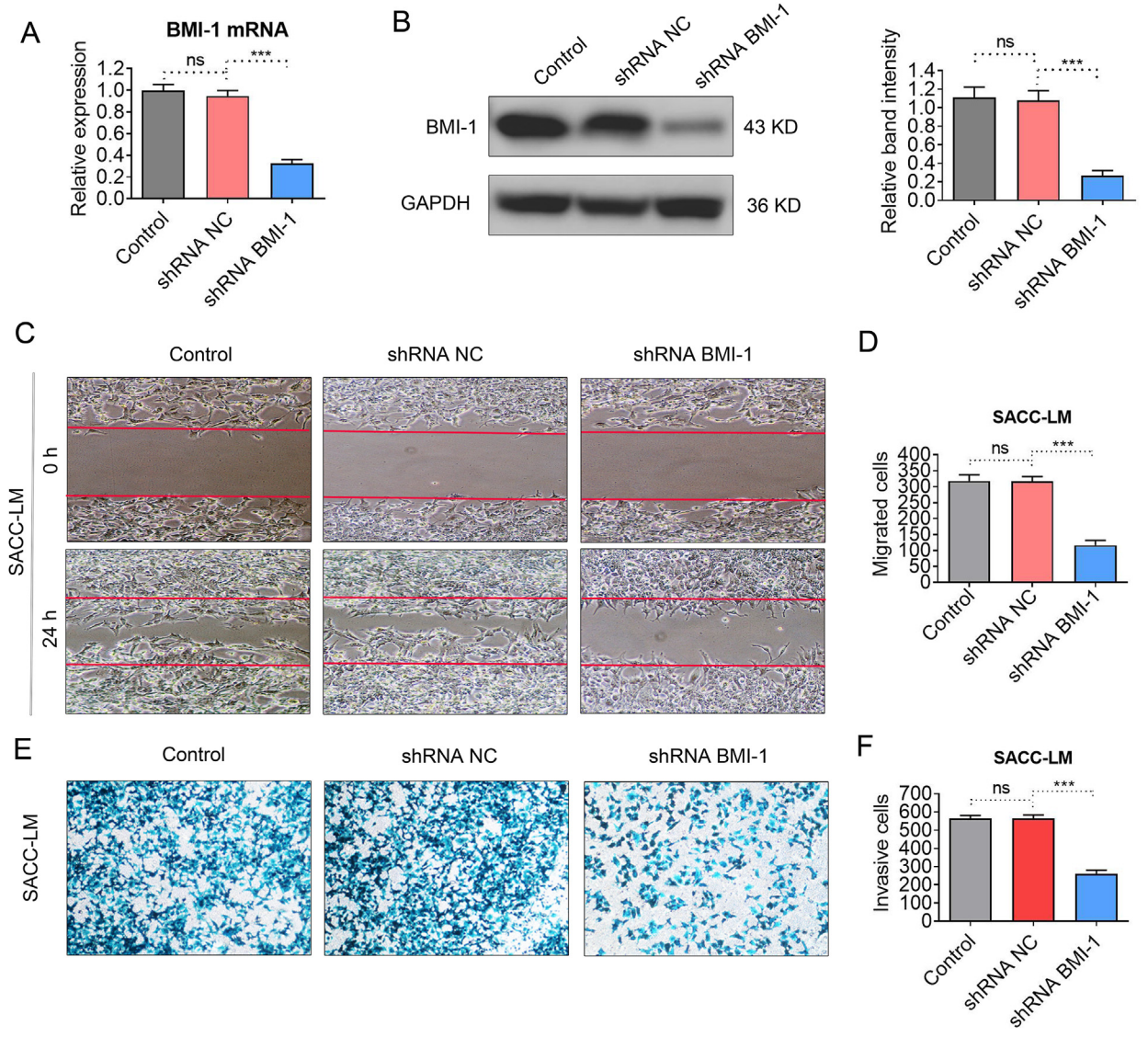

Fig. 4 Silencing of BMI-1 expression inhibits the migration and invasion of SACC cells. (A, B) The downregulation of BMI-1 by shRNA BMI-1 lentivirus was evaluated in SACC-LM cells using real-time PCR (A) and western blotting (B) analyses. shRNA NC was used as a negative control. (C, D) The effect of BMI-1 knockdown on the migration of SACC-LM cells was analyzed using the wound-healing assay. (E, F) The effect of BMI-1 knockdown on the invasion of SACC-LM cells was assessed using the transwell migration assay (ns, no significant difference; ***$P < 0.001$).

outcomes in patients with SACC. Our data showed that patients whose saliva contained high levels of BMI-1 had poor overall and disease-specific survival. Multivariate analyses showed that the salivary BMI-1 level could serve as an independent prognostic factor in patients with SACC. This finding was consistent with the results detected in SACC tumor tissues by immunohistochemistry.[35,36]

Although the biological function of BMI-1 in other cancer cells has been validated, functional studies of BMI-1 on invasion and metastasis of SACC cells remained unclear. In this study, we conducted a series of gain-of-function and loss-of-function assays. Consistent with the findings described in pancreatic cancer stem cells, glioma cells, and colon cancer cells,[31,37,38] our results indicated that BMI-1 downregulation inhibited the migration and invasion abilities of SACC-LM cells. In contrast, exogenous BMI-1 expression promoted the migration and invasion potential of SACC-83 cells *in vitro*. Furthermore, the experimental lung metastasis assay confirmed that BMI-1 silencing significantly inhibited the metastasis of SACC cells *in vivo*.

During the initiation of tumor metastasis, the epithelial-derived tumor cells enhance their potential for migration and invasion, enabling them to detach from the primary tumor site and invade the surrounding tissues, a process called EMT. BMI-1 promotes the invasion and metastasis of endometrial cancer, colon cancer, and hepatocellular carcinoma cells by regulating the EMT of

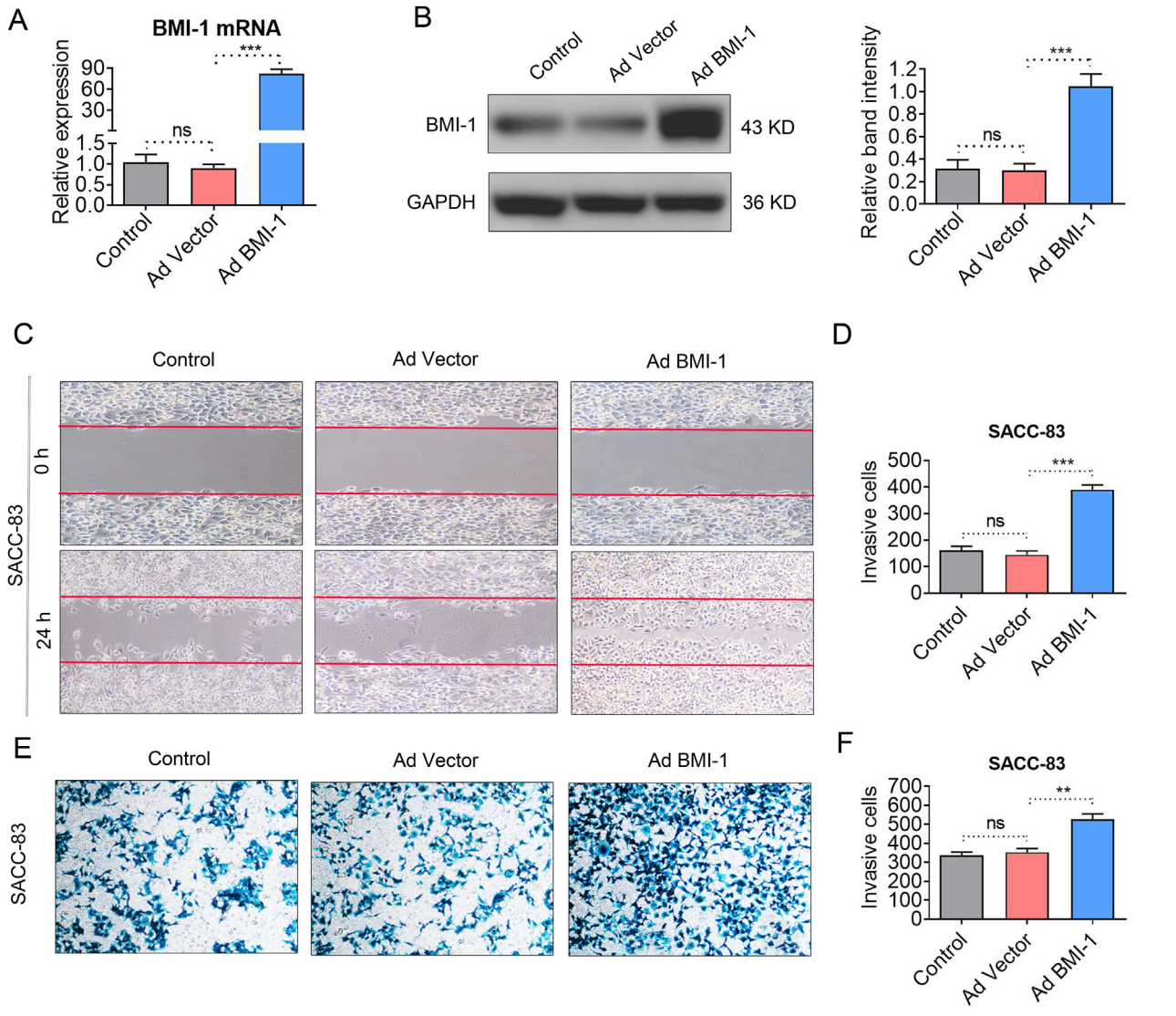

Fig. 5 Exogenous expression of BMI-1 promotes the migration and invasion of SACC cells *in vitro*. (A, B) Real-time PCR (A) and western blotting (B) analyses confirmed the overexpression of BMI-1 by the Ad BMI-1 adenoviral vector in SACC-83 cells. The Ad vector was used as the empty adenoviral vector. (C, D) The effect of BMI-1 overexpression on the migration of SACC-83 cells was analyzed using the wound-healing assay. (E, F) The effect of BMI-1 overexpression on the invasion of SACC-83 cells was assessed using the transwell migration assay (ns, no significant difference; $^{**}P < 0.01$, $^{***}P < 0.001$).

these cancer cells.[29,38,39] A characteristic of EMT is that the metastasizing cells transit from an epithelial to a mesenchymal state. Therefore, the downregulation of epithelial-specific proteins, such as E-cadherin, and upregulation of mesenchymal-specific proteins, such as N-cadherin and Vimentin, can be used as markers to indicate that an epithelial-derived tumor cell has undergone an EMT. The alteration of EMT-related protein expression in SACC cells with overexpressing or downregulating BMI-1 indicated that BMI-1 might mediate the invasion and metastasis of SACC cells by regulating the EMT. As for the mechanism of BMI-1 regulating EMT, previous studies revealed that Snail could transcriptionally repress E-cadherin by binding to the E-box elements of its promoter,[40] and BMI-1 could stabilize Snail through the PI3K/AKT/GSK-3β pathway.[41,42] Our findings that the targeted knockdown or ectopic expression of BMI-1 resulted in downregulation or increased expression of Snail protein level in SACC cells demonstrated that BMI-1 mediates EMT, at least partly, by regulating Snail expression.

In conclusion, our results demonstrated that BMI-1 might be a candidate biomarker to identify tumor invasion and metastasis in SACC, predict the prognosis of SACC and act as a promising candidate therapeutic target for SACC.

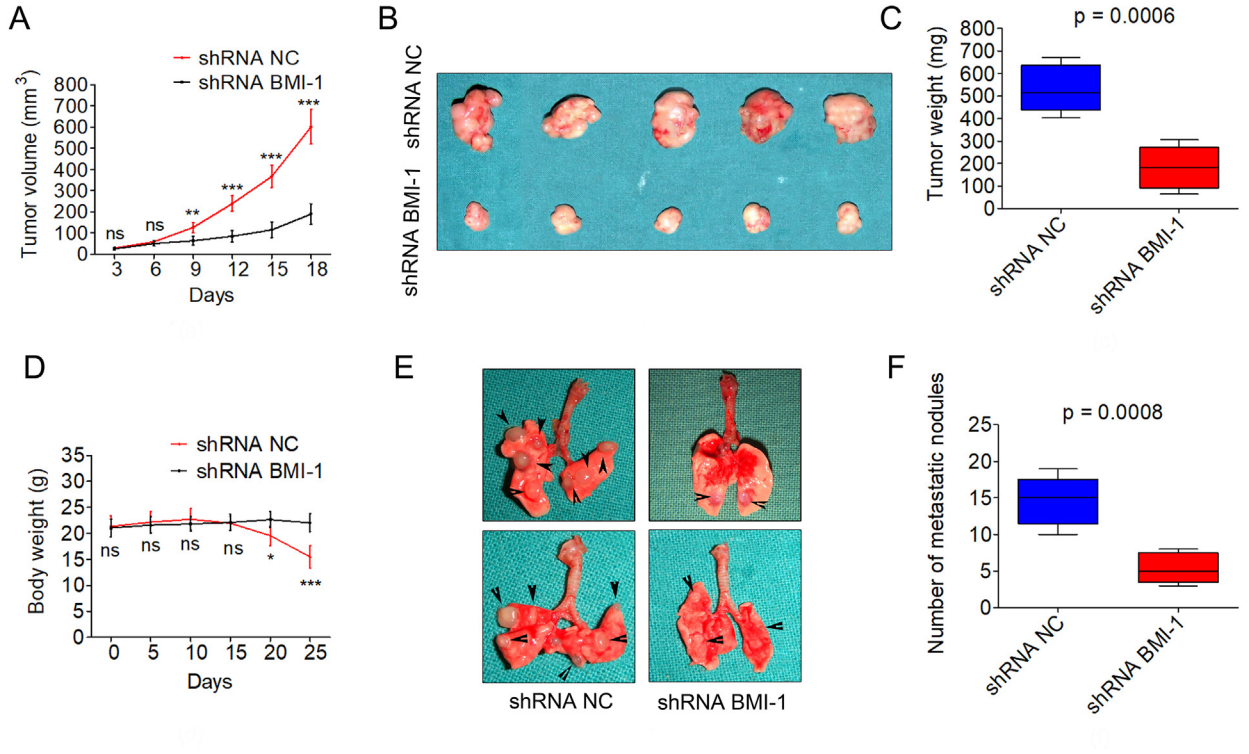

Fig. 6 Downregulating BMI-1 inhibits tumorigenicity and lung metastasis of SACC cells *in vivo*. (A) The tumor volume changes after the subcutaneous injection of SACC-LM cells stably transfected with shRNA BMI-1 or shRNA NC lentivirus (n = 5). (B, C) A comparison of the size and weight of xenografted tumors formed by SACC-LM cells stably transfected with shRNA BMI-1 or shRNA NC lentivirus (n = 5). (D) The changes in the weights of the mice after injection of SACC-LM cells stably transfected with shRNA BMI-1 or shRNA NC lentivirus (n = 5). (E, F) The number of metastatic nodules of the lungs from the mice injected with SACC-LM cells stably transfected with shRNA BMI-1 or shRNA NC lentivirus (n = 5) (ns, no significant difference; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

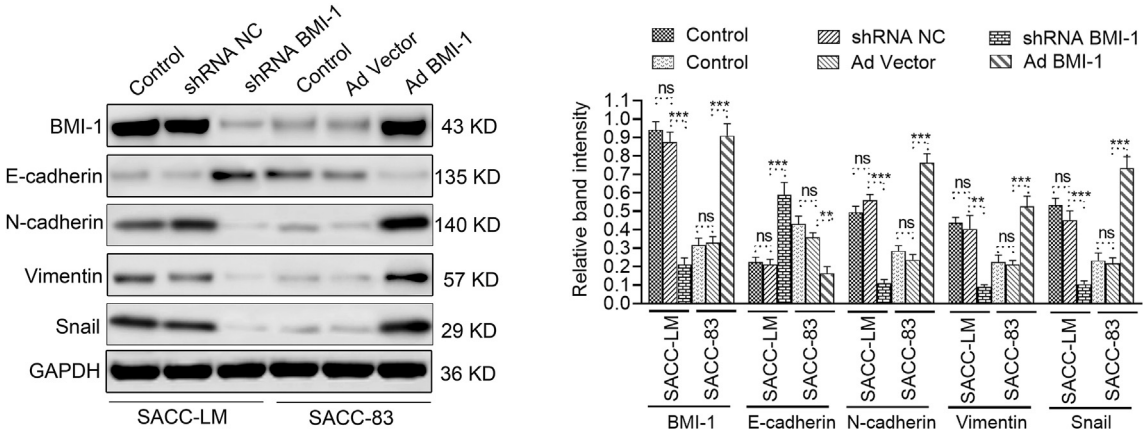

Fig. 7 BMI-1 silencing or overexpression affects the expression of EMT-related proteins in SACC cells. Western blotting shows the effect of BMI-1 downregulation or overexpression on the EMT-related proteins E-cadherin, N-cadherin, Vimentin, and Snail in SACC-LM or SACC-83 cells (ns, no significant difference; **$P < 0.01$, ***$P < 0.001$).

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2023.06.014.

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