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Deregulation of Bmi-1 is associated with enhanced migration, invasion and poor prognosis in salivary adenoid cystic carcinoma

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ABSTRACT

Background: Bmi-1 had been found to involve in self renewal of stem cells and tumorigenesis in various malignancies. In this study, we investigated the role of Bmi-1 in the development of salivary adenoid cystic carcinoma (SACC).

Methods: At first, we confirmed that the deregulation of Bmi-1 was a frequent event in SACC; up-regulation of Bmi-1 was correlated with clinical stages, vital status and distant metastasis and associated with reduced overall survival and disease free survival. SACC-LM cells, higher migration and invasion abilities, elevated the expression of Bmi-1 protein, epithelial-mesenchymal transition (EMT) related proteins (Snail, Slug and Vimentin) and cancer stem cells (CSCs) related proteins ($ABCG_2$, Notch, ALDH-1, Oct-4, Nanog and Epcam) compared to the SACC-83 cells (lower migration and invasion abilities). The migration and invasion abilities were inhibited in SACC-LM cells upon Bmi-1 knockdown. Meanwhile, Bmi-1 knockdown resulted in simultaneous loss of stem cell markers and EMT markers in SACC-LM cells.

Conclusion: Our studies confirm that Bmi-1 deregulation plays an important role in the development of SACC and contributes to the migration and the invasion abilities of SACC, which is involved in EMT and CSCs.

General significance: To our knowledge, this is the first study revealing that Bmi-1 deregulation is associated with enhanced migration, invasion and poor prognosis in salivary adenoid cystic carcinoma.

1. Introduction

Salivary adenoid cystic carcinoma (SACC), comprises approximately 7.5–10% of all salivary malignancies, is characterized by high incidence of local recurrence and distant metastasis (lung, bone and liver) [1,2]. Despite the advance in local treatments for primary cancer using surgical excision with and without post-operative radiotherapy, dealing with recurrences and metastases has become a formidable issue. To date, several studies have attempted to uncover the molecular mechanism underlying the distinctive biological behaviors of SACC. A number of signaling molecules have been implicated in the metastasis of SACC, including snail homolog 2 (Snail2, a.k.a., Slug) [3], mitogen-activated protein kinase 1/2 (MAPK1/2) [3] as well as matrix metalloproteinases (MMPs) [4]. Other non-coding genes (e.g.microRNA) have also been found to correlate with metastasis of SACC [3]. Our previous study found that the MicroRNA-181a suppresses the invasion and metastasis of SACC by targeting MAPK-Snai2 pathway [3].

Bmi-1(B-cell specific Moloney murine leukemia virus insertion site 1),a transcriptional repressor of polycomb group of transcription factors, had been found to involve in self renewal of hematopoietic [5], neuronal [6] and mammary [7] stem cells, and has been implicated in the tumorigenesis of various malignancies, including leukemia [8], lymphomas [9], non-small cell lung cancer [10], breast cancer [11] and nasopharyngeal carcinoma [12]. Moreover, Bmi-1 was also found to play a key role in tumor metastasis [13]. Chou found that Bmi-1 overexpression drive stem-like properties associated with the induction of the EMT that promotes migration, invasion and poor prognosis in head and neck cancer [14]. These observations led us to hypothesize that Bmi-1 deregulation may induce migration and invasion of SACC.

In this study we investigated the relationship between Bmi-1 deregulation and the migration and invasion of SACC. At first, we compared the Bmi-1 expression between SACC and adjacent normal salivary gland tissues by immunohistochemistry. Then, we investigated the function of Bmi-1 in the migration and invasion of SACC. We found the Bmi-1 deregulation in the development of SACC and play an important role in the migration and invasion of SACC.

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2. Materials and methods

2.1. Patient and sample

A total of 50 SACC tissues samples without preoperative chemotherapy or radiotherapy and 20 normal salivary glands tissues were collected from the surgically treated patients in the Cancer Center, Sun Yat-sen University between 1998 and 2010. All clinicopathologic characteristics of SACC patients were presented in Supplementary Table S1. The histological normal salivary gland tissues used in this study were obtained from patients undergoing surgery for other diseases (shown in Supplementary Table S2). The tumor staging was assessed according to UICC staging system. Survival was calculated from diagnosis to the date of latest follow-up (or death). Median duration of follow-up was 64 months (range 12–139 months). For the use of these clinical materials for research purposes, the approval from the Institute Research Ethics Committee was obtained (B2013-045-01).

2.2. Immunohistochemical staining

Immunohistochemistry was performed on 5 mm sections of formalin-fixed, paraffin-embedded tissue samples as previous description [15]. Briefly, the paraffin section was deparaffinized with xylene and rehydrated in alcohol. Antigen retrieval was treated with boiling citrate buffer (pH 6.0) for 4 min in a pressure cooker. Endogenous peroxidase activity was blocked by 3% H_2O_2 for 15 min followed by staining with anti- Bmi-1 antibody (1:500) (Cell Signaling Technology) for overnight at 4°C. After washing, the sections were incubated with the MaxVision™ HRP-Polymer anti-Rabbit IHC Kit for 15 min at room temperature (Maixin Co., Fuzhou, China); and then developed with the DAB Horseradish Peroxidase Color Development Kit (Maixin Co., Fuzhou, China) and counterstained with hematoxylin. Rabbit (DA1E) mAb IgG XP (Cell Signaling Technology) was used as isotype control and the results were presented in Fig. S1. The degree of immunostaining was scored independently by two observers according to both the proportion of positively stained tumor cells and the intensity of staining. The proportion of tumor cells was scored as follows: 0 (no positive tumor cells), 1 (<30% positive tumor cells), 2 (30–60% positive tumor cells), and 3 (>60% positive tumor cells). The intensity of staining was graded as following criteria: 0 (no staining); 1 (weak staining = light yellow), 2 (moderate staining = yellow brown), and 3 (strong staining = brown). The staining index was calculated as staining intensity score × proportion of positive tumor cells. Using this method of assessment, we evaluated the expression of Bmi-1 by determining the staining index, which scores as 0, 1, 2, 3, 4, 6, and 9. An optimal cutoff value (median) was identified: the staining index score of >4 was used to define tumors as high Bmi-1 expression and ≤4 as low expression of Bmi-1.

2.3. Cell culture and transfection

The SACC-LM/SACC-83, kindly presented by Dr. Shenglin Li, is paired cell lines generated from a SACC patient. The SACC-LM cell line is more aggressive than SACC-83 in terms of lung-metastatic rate [16,17]. Dr. Li had confirmed that SACC-83 and SACC-LM are authentic ACC cell lines [17]. Cells were maintained in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified incubator with 5% CO2. For transfection, cells were seeded in 6-well plates and transfected with Bmi-1 siRNA or control siRNA using Lipofectamine™ RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested for functional analysis and protein extraction after 24 h. Bmi-1 siRNA sequences (sense: CCA GAC CAC UAC UGA AUA UTT). Control siRNA sequences (sense: UUC UCC GAA CGU GUC ACG UTT).

2.4. Western blot analysis

Western blots were performed as described previously [18], using antibodies specific to extracellular signal- regulated kinase (ERK) 1/2, pERK1/2, Snail family members (Snai1 and Snai2), Bmi-1, E-cadherin, Vimentin, ALDH-1, ABCG2, Notch-1, Nanog, Epcam, Oct-4, and β-tubulin as a control (Cell Signaling Technology).

2.5. In vitro cell migration and invasion assays

Transwell assays were performed to assess cell migration and invasion ability using BD BioCoat Control Cell Culture Inserts or BD BioCoat BD MatrigelTM Invasion Chamber, respectively [3]. In brief, cells were seeded in the upper Boyden chambers of the cell culture inserts. After 24 h of incubation (for migration) or 48 h of incubation (for invasion), cells remaining in the upper chamber (for migration) or on the upper membrane (for invasion) were carefully removed. Cells adhering to the lower membrane were stained with DAPI in the dark, imaged and counted using an inverted microscope equipped with a digital camera. Five random fields were captured at 200× magnification under microscope. The number of cells on the bottom surface was compared between groups. Experiments were performed in triplicate.

2.6. Cell proliferation assays

Cell proliferation was measured by Cell Counting Kit-8 assay according to the manufacturer's instructions. In brief, cells were seeded in 96-well plates at the density of 5×10^3 cells per well after incubation for 24 h. Then added 10ul of the CCK-8 solution to each well of the plate and incubate the plate for 2 h in the incubator. The absorbance value of each well was assayed using a plate reader at a wavelength of 450 nm and cell inhibition rate was calculated as $(1\text{-}A_{treated}/A_{control}) \times 100\%$.

2.7. Clone forming assay

For colony formation assays, cells were plated at approximately 200 cells per well in 6-well, coated plates in RPMI-1640 supplemented with 10% FBS. The culture medium was changed twice per week. Fourteen days later, the cells were fixed in 4% formaldehyde and stained with Giemsa. Colonies larger than 1 mm (>50cells/clone) in diameter were counted.

2.8. Statistical analysis

Date was expressed as mean ± SD and all experiment were performed in triplicate. All statistical analyses were carried out using the Statistical Package for the Social Science (SPSS, Chicago, IL), Version 13.0. Student's T test was used to compare the difference between groups. χ^2 test was used to analyze the correlation between gene expression and the clinic pathologic characteristics. Survival curves were plotted using the Kaplan–Meier method and compared with the log-rank test. Cox regression was used for both univariate and multivariate analysis. $P < 0.05$ in all cases was considered statistically significant.

3. Results

3.1. Bmi-1 deregulation in the development of SACC

To confirm the relationship between Bmi-1 deregulation and the development of SACC, Bmi-1 expression was examined by immunohistochemistry (IHC) in 50 cases of SACC and 20 normal salivary gland tissues. As illustrated in Fig. 1, Bmi-1 staining was located in cell nucleus. Bmi-1 was rarely detectable in normal salivary gland tissues (Fig. 1A and Supplementary Fig. S2), but pronounced enhanced expression in SACC samples from patients with (Fig. 1B) or without (Fig. 1C) lung

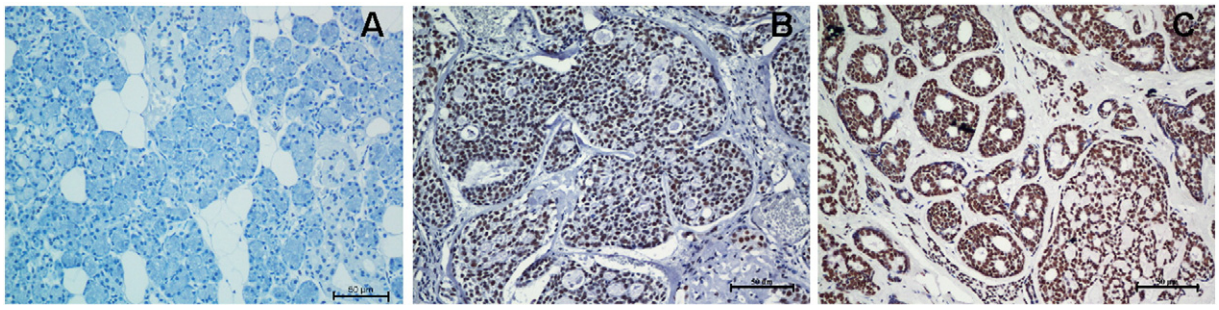

Fig. 1. Immunohistochemistry analyses of Bmi-1 expression in normal salivary gland and SACC tissue samples. Immunohistochemistry analyses for Bmi-1 were performed as described in material and methods on A: normal salivary gland; B: salivary gland adenoid cystic carcinoma without lung metastasis; C: salivary gland adenoid cystic carcinoma with lung metastasis. Scale bar: 50 μm.

metastasis. The Bmi-1 expression was significantly increased in primary cancer tissues compared with normal salivary gland tissues (Fig. 2A). Among SACC cases, Bmi-1 levels were significantly higher in pT_{1+2} than in pT_{3+4}, in late clinical stage (stage III and IV) than in early clinical stage (stage I and II) (Fig. 2B and C). Statistical significant increased Bmi-1 expression was also observed in SACC samples with positive distant metastasis status (pM+) as compared to those with negative status (pM-) (Fig. 2D).

3.2. Correlations between Bmi-1 expression and clinicopathologic characteristics of SACC patient

The correlation between Bmi-1 expression and clinicopathologic variables of patients with SACC were shown in Table 1. These observations show that high levels of Bmi-1 expression were associated with pT ($p < 0.001$), clinical stages ($p = 0.002$), vital status ($p = 0.005$) as well as the presence of distant metastasis ($p = 0.047$). No relationship could be found between Bmi-1 expression and age ($p = 0.643$), gender ($p = 0.405$), recurrence ($p = 0.676$) and tumor site ($p = 0.114$).

3.3. The prognostic value of Bmi-1 deregulation in SACC patients

To elucidate the prognostic role of Bmi-1 expression in SACC patients, we examined the relationship between Bmi-1 expression and patient outcome with long-term follow-up. As illustrated in Fig. 3A, a striking difference in overall survival (OS) was observed between the high Bmi-1 expression group (mean survival = 98.7 months) and the low Bmi-1 expression group (mean survival = 135.2 months) ($p = 0.004$). A statistically significant difference in survival was also observed between disease free survival (PFS) and Bmi-1 expression ($p = 0.007$) (Fig. 3B).

To further evaluate the impact of Bmi-1 expression and clinicopathological factors on the prognosis of SACC patients, univariate and multivariate analyses were carried out. The clinical parameters for univariate analysis included gender, age, clinical stage, T classification, distant metastasis and recurrence. When the *P* value of factors was less than 0.1 in the univariate analyses, the factors were then analyzed by the multivariate analyses. As illustrated in Table 2, for the 5-years overall survival, both univariate and multivariate analysis indicated that only Bmi-1 expression was a significant prognostic factor for patients with SACC. But for 5-years disease free survival, both univariate and multivariate analyses indicated that Bmi-1 expression, distant metastasis and recurrence were independent prognostic factor. Thus, our findings indicate that Bmi-1 expression level has a significant correlation with the prognosis of SACC.

3.4. Bmi-1 overexpression is related to the migration and invasion of SACC

To examine whether Bmi-1 could promote migration and invasion of SACC, transwell migration assay and invasion assay were applied. As shown in Fig. 4A and B, SACC-LM exhibited higher migration and invasion abilities than SACC-83. Meanwhile, the protein level of Bmi-1 in SACC-83 was significantly lower than SACC-LM as we detected by western blot (Fig. 4C). We also found that several metastasis-related and

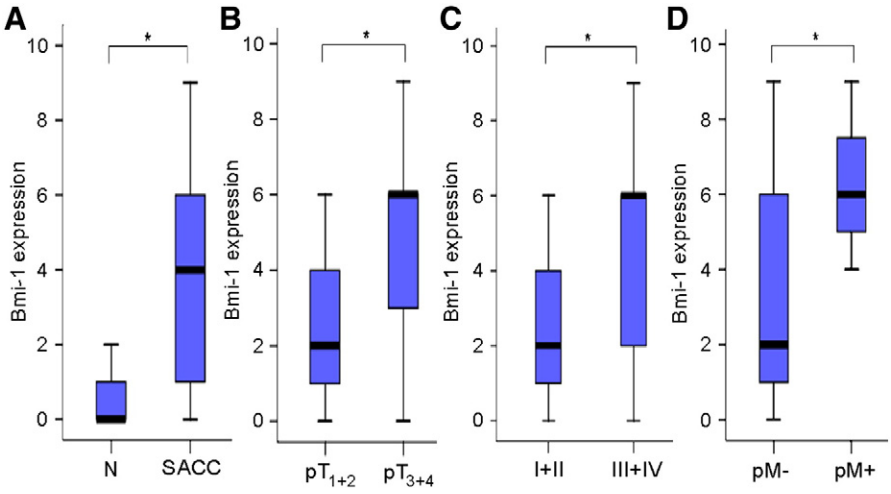

Fig. 2. The deregulation of Bmi-1 in the development of SACC tissue. Box plots were presented for comparing the expression of Bmi-1 between normal salivary gland and SACC cases (A), and in SACC cases with different Tumor (T) stages (B), different clinical stages (C), with or without distant metastasis (D). The *P*-values were computed using Student's T test. The boxes represent 25th to 75th percentile of the observations, and the lines in the middle of the box represent the median. *$P < 0.01$.

Table 1
Correlations between Bmi-1 expression and clinicopathologic characteristics of SACC patients.

Characteristics		BMI-1		*P*-value*
		Low	High	
Gender	Male	10	9	0.405
	Female	20	11	
Age(years)	≤40	12	6	0.470
	>40	18	14	
Tumor stage	$T_1 + T_2$	24	7	0.001
	$T_3 + T_4$	6	13	
Distant metastasis	Negative	28	14	0.047
	Positive	2	6	
Recurrence	Negative	24	15	0.676
	Positive	6	5	
Tumor site	Parotid	15	8	0.114
	Submandibular	11	12	
	Sublingual	4	0	
Vital status	Alive	29	13	0.005
	Death (tumor-related)	1	7	

* Chi-square test.

Table 2
Univariate and multivariate analyses for 5-year overall survival and 5-year disease-free survival in SACC patients.

	Univariate analysis		Multivariate analysis	
	P Regression coefficient(SE)		P Relative risk(95%CI)	
Overall survival				
Clinical stage	0.156	1.187(0.837)	–	–
Expression of Bmi-1	0.023	2.466(1.083)	0.023	11.770(1.410–98.257)
T classification	0.079	1.479(0.840)	0.406	–
Distant metastasis	0.074	1.372(0.767)	0.422	–
Gender	0.367	−0.745(0.825)	–	–
Age	0.213	3.812(3.062)	–	–
Recurrence	0.835	0.175(0.842)	–	–
Disease-free survival				
Clinical stage	0.116	0.694(0.442)	–	–
Expression of Bmi-1	0.010	1.160(0.453)	0.010	3.191(1.312–7.762)
T classification	0.061	0.822(0.438)	0.298	–
Distant metastasis	<0.001	1.867(0.470)	<0.001	9.551(3.504–26.035)
Gender	0.642	−0.215(0.464)	–	–
Age	0.843	−0.090(0.453)	–	–
Recurrence	<0.001	1.994(0.471)	<0.001	9.873(3.734–26.105)

Note: When the *P* value of factors was less than 0.1 in the univariate analyses, the factors were then analyzed by the multivariate analyses.

EMT-related proteins (Snail, Slug, ERK1/2, pERK1/2 and Vimentin) were elevated in SACC-LM than in SACC-83 cells and E-cadherin protein level was found to decrease in SACC-LM than in SACC-83 cells (Fig. 4C). The expression levels of CSCs markers ($ABCG_2$, Notch, ALDH-1, Oct-4, Nanog and Epcam) in SACC-LM cells were significant higher than those in SACC-83 cells (Fig. 4C). Moreover, we found that there had significantly lower clone-forming capacity (Fig. 4D) and proliferation capacity (Fig. 4D) in SACC-83 cells than in SACC-LM cells.

3.5. Bmi-1 siRNA inhibits the migration and invasion of SACC

To further investigate the role of Bmi-1 in aiding metastasis, we knocked down the expression of Bmi-1 by RNA interference. The protein level of Bmi-1 was significantly decreased in SACC-LM cells after transfection with the Bmi-1 siRNA (Fig. 5A). SACC-LM cells transfected with Bmi-1 siRNA displayed decreased migration and invasion abilities compared to the control transfected cells (Fig. 5B and C). After knockdown of the expression of Bmi-1 in SACC-LM cells, several metastasis-related protein (Snai, Slug, Vimentin, ERK1/2 and pERK1/2) were significantly decreased, and the protein levels of E-cadherin were significantly increased (Fig. 5A). Furthermore, Bmi-1 knockdown resulted in reduced the cell proliferation rate (Fig. 5D) and clone-forming capacity (Fig. 5E) in SACC-LM cells. We also found that several CSCs markers, including $ABCG_2$, ALDH-1, Nanog, Notch and Epcam, were dramatically reduced in SACC-LM cells after knockdown of Bmi-1 (Fig. 5A).

4. Discussion

Salivary adenoid cystic carcinoma (SACC), an uncommon malignant tumor, is prone to perineural and metastasis to distant organs. To develop novel treatments and cures, it is imperative to address the factors underlying tumorigenesis, invasion and metastasis. In this study, we identified and functionally characterized Bmi-1 as an important factor

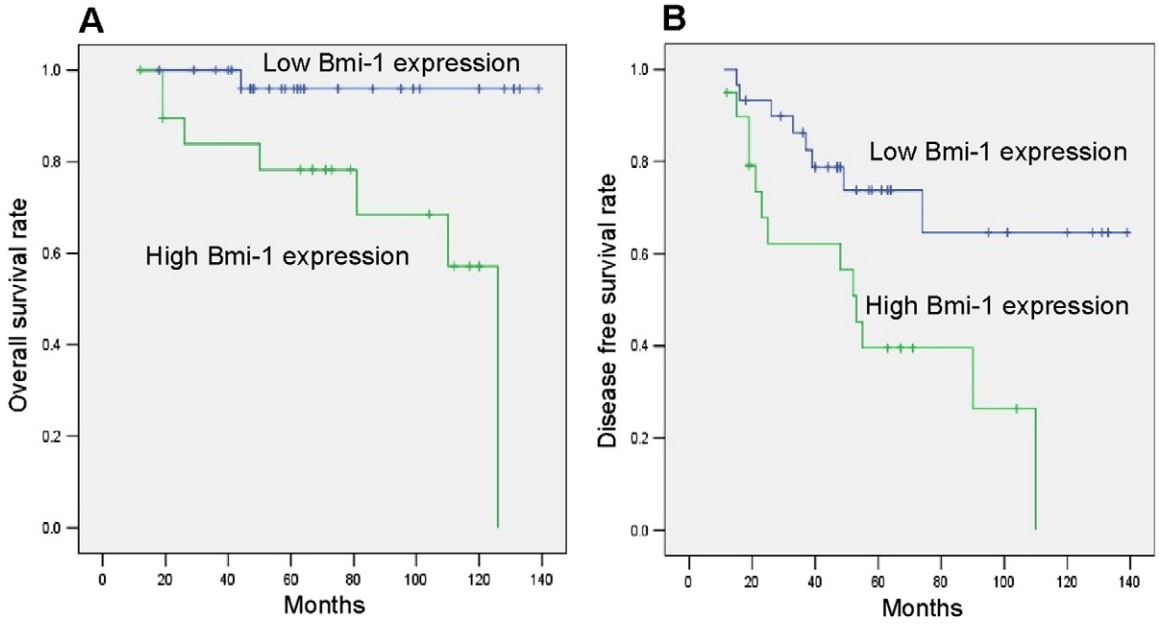

Fig. 3. The effects of Bmi-1 expression on prognosis. Kaplan–Meier plots of 5-years overall survival (A) and 5-years disease free survival (B) in patient groups defined by Bmi-1 immunohistochemistry. The differences in survival rates are statistically significant ($P < 0.05$).

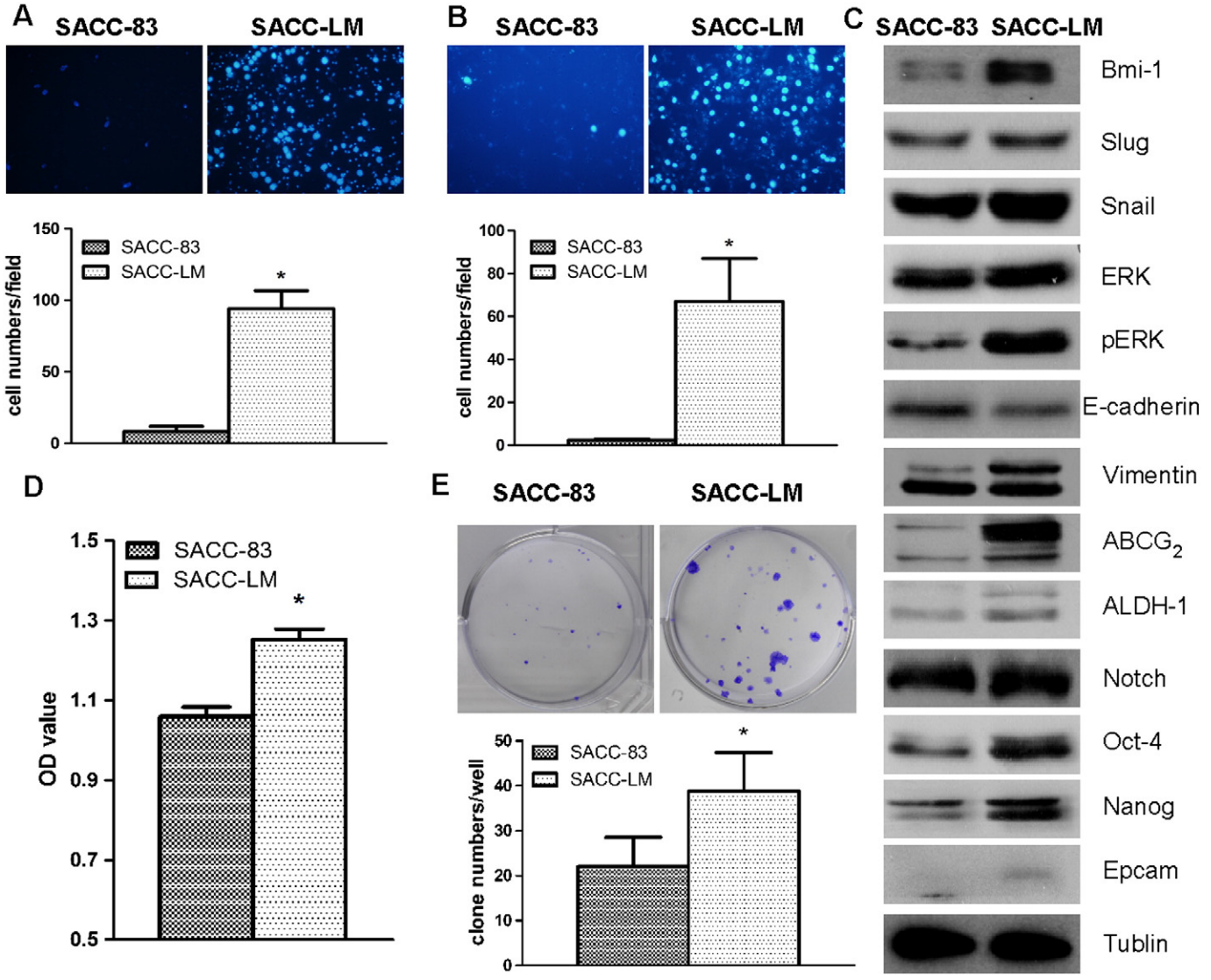

Fig. 4. Bmi-1 overexpression is related to the migration and invasion of SACC. (A, B)The migration and invasion ability of SACC cells was assessed by a transwell migration and invasion assay. SACC-LM cells displayed significantly higher migration and invasion ability than SACC-83 cells. $^{*}P < 0.05$. (C) Bmi-1 regulates EMT markers and cancer stem cell markers in the SACC. Western blotting shows the expression of EMT markers (E-cadherin, Vimentin, Slug and Snail), MAPK pathway proteins (ERK and PERK) and cancer stem cell markers ($ABCG_2$, ALDH-1, Notch, Oct-4,Nanog and Epcam) for SACC-LM and SACC-83 cells. (D)Cell proliferation was measured using an CCK-8 assay. The cell proliferation rate of SACC-LM was significantly higher than that of SACC-83. $^{*}P < 0.05$. (E)Representative pictures of colony formation assay of SACC-83 and SACC-LM cells. SACC-83 cells demonstrated weak clone-forming capacity than SACC-LM. $^{*}P < 0.05$.

in the development and progression of SACC. We first illustrated that the expression of Bmi-1 was more pronouncedly increased in SACC tissues compared with normal salivary gland tissues, followed by demonstrating that up-regulation Bmi-1 correlates with pT, clinical stage and distant metastasis. Recently, several researchers demonstrated that statistically significant correlation between Bmi-1expression and poor prognosis in many cancers [19,20]. Similarly, in our study, up-regulation of Bmi-1 was associates with 5-years overall survival and disease free survival of the SACC patients. Thus, these findings underscore the critical contribution of Bmi-1 deregulation in the tumorigenesis and metastasis of SACC.

Clinically significant elevations of Bmi-1 expression are associated with increased tumor invasion and metastasis in certain cancer types [12,21,22].In colon cancer and gastric cancer, Bmi-1 expression is significantly correlated with lymph nodal involvement, distant metastasis and clinical stage [20]. In addition, a high Bmi-1expression pattern was a significant predictor of metastasis in melanoma [22]. Moreover, knockdown of Bmi-1 contributes to decreased invasiveness of cervical cancer cells [23]. These studies indicate that Bmi-1 contributes to increased aggressive behavior of cancer cells. Similarly, in this study we found that SACC-LM cells displayed significantly higher migration and invasion abilities and higher levels of Bmi-1 protein, compared to the SACC-83 cell lines; Moreover, the migration and invasion abilities were inhibited in SACC-LM cells upon Bmi-1 knockdown. These findings suggest that increased Bmi-1 expression contributed to both the invasive and the migratory capacities of SACC.

Epithelial-mesenchymal transition (EMT), in which epithelial cells acquire mesenchymal-like properties, is thought to be a critical step in the induction of tumor metastasis [24]. Bmi-1 had been found to be related with EMT process. Loss of E-cadherin and gain of Vimentin are hallmark of the invasive phase of cancer [13,19]. In our study, Overexpression of Bmi-1 increases the motility and invasive properties of SACC cells, which is concurrent with the increased expression of mesenchymal markers (Vimentin), the decreased expression of epithelial markers (E-cadherin). Snail, a member of the zinc-finger transcription factor family, is one of the master regulators that promotes EMT and mediates invasiveness as well as metastasis in many different types of malignant tumors [25,26]. We found that both Snai1 and Snai2 were

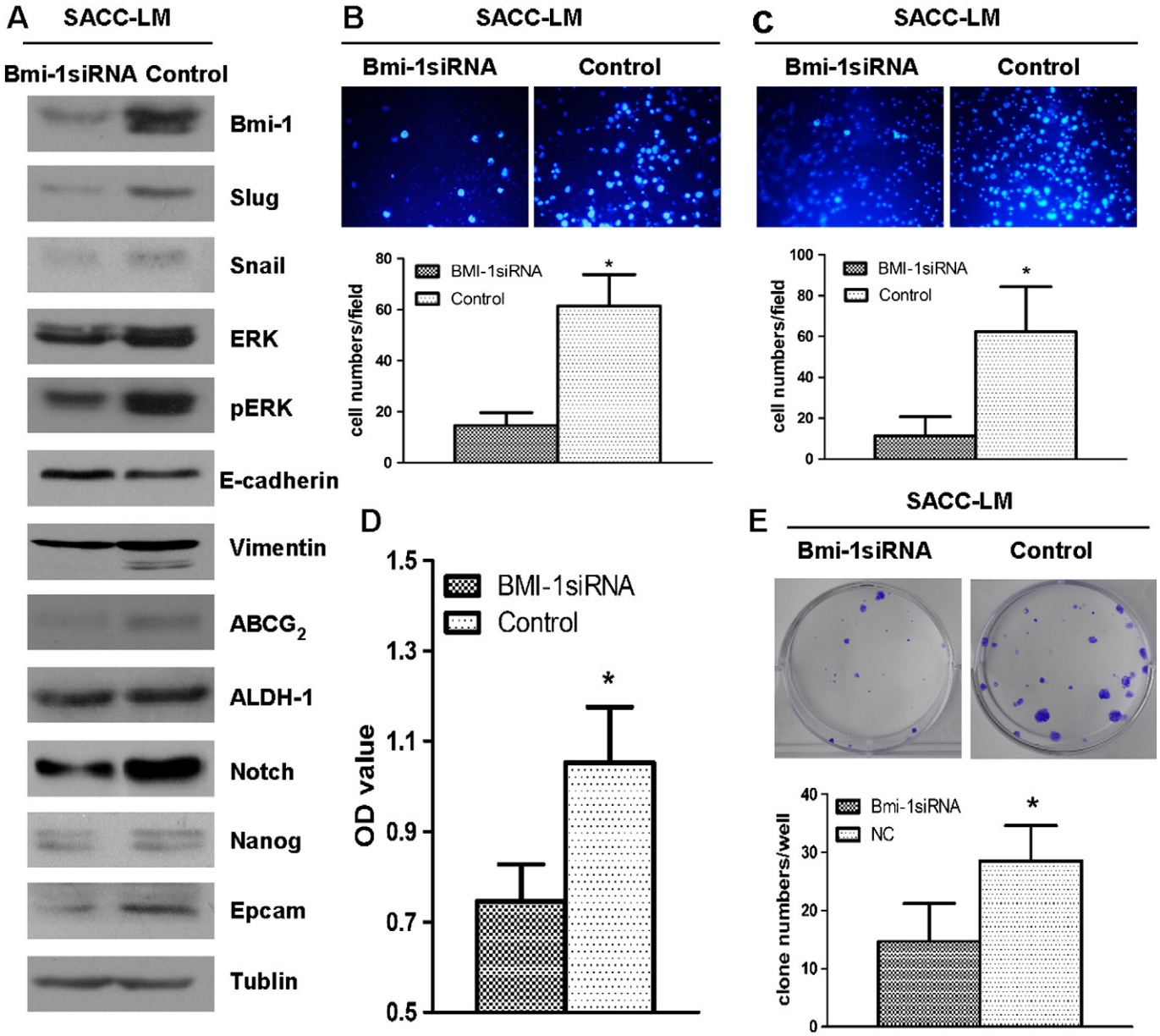

Fig. 5. Bmi-1 knockdown inhibits the migration and the invasion abilities of SACC. (A) Significant reductions in Bmi-1 protein levels were observed in the Bmi-1 siRNA-transfected SACC-LM cells compared to the negative control-transfected cells. SACC-LM cells displayed decreased Snail, Slug, Vimentin, ERK1/2, pERK1/2, $ABCG_2$, ALDH-1, Notch, Nanog and Epcam protein levels and increased E-cadherin protein levels upon Bmi-1 knockdown. (B, C) Bmi-1 knockdown inhibited the migration and invasion of SACC-LM cells. $^*P < 0.05$. (D) The cell proliferation rate of the SACC-LM line was significantly inhibited after transfection with the Bmi-1siRNA. $^*P < 0.05$. (E) Bmi-1 knockdown resulted in reduced the clone-forming capacity in SACC-LM. $^*P < 0.05$.

highly expressed in SACC-LM cells compared with SACC-83 cells. Upon knockdown Bmi-1 in SACC-LM cells, the expression of both Snai1 and Snai2 were inhibited. Furthermore, the protein level of Bmi-1 was positively correlated with the protein levels of ERK1/2 and pERK1/2, which can mediate cell proliferation, cell cycle arrest, apoptosis and metastasis [27–29].

During the process of tumor metastasis, which is often enabled by EMT, disseminated cancer cells presumably require a self-renewal capability similar to that exhibited by stem cells in order to spawn macroscopic metastases [30]. This phenomenon raises the possibility that the EMT process may also impart a self-renewal capability to disseminating cancer cells. Indeed, emerging evidence of a direct interaction between EMT and CSCs has been recently reported [31,32]. In this study, we identifies Bmi-1 as a regulator for both EMT and CSC characteristics based on the observation that Bmi-1 knockdown resulted in simultaneous loss of stem cell markers and EMT markers as well as clone forming ability and cell proliferation rate.

From above our study confirmed that Bmi-1 deregulation plays an important role in the development of SACC and contributes to the migration and the invasion abilities of SACC, which was involved in EMT and CSC.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.08.005.

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