Oral Oncology 49 (2013) 1051–1058

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Transforming growth factor-β and epithelial–mesenchymal transition are associated with pulmonary metastasis in adenoid cystic carcinoma

Ling Dong [a,1], Xi-Yuan Ge [a,b,1], Yi-Xiang Wang [a,b], Lin-Qian Yang [b], Sheng-Lin Li [a,b,*], Guang-Yan Yu [c,*], Yan Gao [d], Jia Fu [b]

[a] Laboratory of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, 22 Zhongguancun Nandajie, Haidian District, Beijing 100081, PR China
[b] Central Laboratory, Peking University School and Hospital of Stomatology, 22 Zhongguancun Nandajie, Haidian District, Beijing 100081, PR China
[c] Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, 22 Zhongguancun Nandajie, Haidian District, Beijing 100081, PR China
[d] Department of Oral Pathology, Peking University School and Hospital of Stomatology, 22 Zhongguancun Nandajie, Haidian District, Beijing 100081, PR China

ARTICLE INFO

Article history:
Received 17 January 2013
Received in revised form 27 July 2013
Accepted 31 July 2013
Available online 17 August 2013

Keywords:
Adenoid cystic carcinoma
Pulmonary metastasis
Transforming growth factor-β1
Epithelial–mesenchymal transition
Gene therapy

SUMMARY

Objectives: Adenoid cystic carcinoma (ACC) is one of the most common malignancies of salivary glands, characterized by poor prognosis, particularly due to pulmonary metastasis. We previously reported that transforming growth factor (TGF)-β1 promoted ACC cell migration and invasion *via* the Smad pathway *in vitro*. The aim of this study was to establish the underlying mechanisms.
Materials and methods: TGF-β1, phospho-Smad2 and β-catenin expression in ACC tissues derived from patients was evaluated by immunohistochemistry. The role of TGF- β1 on the invasive capacity of ACC cells was determined by transwell assays in SACC-83 cells transfected with TGF-β1 and TGF-β type II dominant-negative receptor (TβRIIDN) plasmids or silenced by TGF-β1 siRNA. Expression of the epithelial–mesenchymal transition (EMT) markers, β-catenin, E-cadherin and Nectin-1, was determined by real-time PCR and immunochemistry. *In vivo* investigations were performed by inoculating nude mice with the transfected ACC cells and examining metastasis in bilateral lung tissues by immunohistochemistry.
Results: Overexpression of TGF-β1 and phospho-Smad2, and reduced expression of membrane β-catenin, were closely associated with lung metastasis in ACC. Furthermore, the EMT markers were downregulated. *In vitro*, cells transfected with TGF-β1 exhibited altered morphology and increased invasive capacity compared to TβRIIDN-transfected cells or TGF-β1 siRNA silenced cells. *In vivo*, mice inoculated with TGF-β1 transfected ACC cells exhibited more metastases than other cells.
Conclusion: TGF-β1, phospho-Smad2 and β-catenin were significantly correlated with ACC metastasis. Blockade of TGF-β signaling by TβRIIDN or siRNA may offer potential gene therapies against pulmonary metastasis in patients with ACC.

© 2013 Elsevier Ltd. All rights reserved.

Introduction

Adenoid cystic carcinoma (ACC) is one of the most common types of malignant tumors of salivary glands. ACC has several distinctive characteristics including a slow indolent growth pattern, multiple local recurrences, high incidences of metastasis and poor prognosis [1–3]. Despite investigations on the molecular mechanisms of ACC metastasis [4,5], further in-depth and systematic research is needed develop effective therapies to reduce morbidity and mortality in patients with ACC.

Transforming growth factor (TGF)-β1 is a pluripotent cytokine with dual roles in tumorigenesis [6]. Multiple *in vivo* studies have shown that TGF-β1 enhances the metastatic and invasive properties of various cancer types, suggesting that it induces epithelial–mesenchymal transition (EMT) in cancer cells [7]. In our previous study, we showed that TGF-β1 promoted cell migration and invasion in ACC *in vitro via* the Smad pathway [8].

EMT is considered to be a key step in embryonic development, chronic inflammation, cancer progression and metastasis. It is a complex phenomenon involved in epithelial plasticity and is typi-

Abbreviations: ACC, adenoid cystic carcinoma; β-catenin RM, membrane expression of β-catenin; CAMs, Ca^{2+}-independent immunoglobulin-like cell adhesion molecules; EMT, epithelial–mesenchymal transition; H-score, immunohistochemical score; p-Smad2, phospho-Smad2; ROC, receiver operating characteristic; TGF-β1, transforming growth factor.

* Corresponding authors. Address: Central Laboratory, Peking University School and Hospital of Stomatology, 22 Zhongguancun Nandajie, Haidian District, Beijing 100081, PR China. Tel.: +86 10 82195534; fax: +86 10 62173402 (S.-L. Li), Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, 22 Zhongguancun Nandajie, Haidian District, Beijing 100081, PR China. Tel.: +86 10 62191099; fax: +86 10 62173402 (G.-Y. Yu).
E-mail addresses: kqshlli@bjmu.edu.cn (S.-L. Li), gyyu@263.net (G.-Y. Yu).
[1] Co-first authors.

1368-8375/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.oraloncology.2013.07.012

cally characterized by loss of cell–cell adhesion [9]. Adherens junctions consist of a protein complexes in which β-catenin plays a central role by regulating the coordination of cell–cell adhesion. It controls cadherin-mediated cell adhesion at the plasma membrane by mediating the interplay of adherens junction molecules with the actin cytoskeleton [10]. It is commonly accepted that EMT induced by TGF-β1 is an important process during tumor metastasis, however the mechanism in ACC is poorly understood. The aim of this study was to evaluate expression of TGF-β1, phospho-Smad2 (p-Smad2) and β-catenin in ACC, and to establish the role of TGF-β1-induced EMT in ACC metastasis.

Patients, materials and methods

Patients and tissue specimens

The medical records of 50 patients diagnosed with ACC of the salivary glands between 1967 and 2008 at the Department of Oral and Maxillofacial Surgery, Peking University School of Stomatology, were reviewed retrospectively and confirmed by an experienced pathologist. The patient sample included 22 males and 28 females with a median age of 46.5 years. The mean follow-up period was 74.4 months and an X-ray was performed to determine if there was pulmonary metastasis. Clinicopathological data are summarized in Table 1. Informed written consent was obtained from all subjects, and the study was approved by the Peking University School of Stomatology Institutional Review Board.

Immunohistochemistry and semi-quantitative assessment

Paraffin-embedded tissue sections (4 μm thick) were deparaffinized in xylene and rehydrated in ethanol. The slides were subjected to antigen retrieval in 0.01 M citrate buffer (pH 6.0), followed by incubation in 3% hydrogen peroxide to block endogenous peroxidase activity. Non-specific binding was prevented by incubation in 10% normal goat serum. Slides were washed by PBS and incubated at 4 °C overnight with the following primary antibodies: mouse monoclonal antibody to TGF-β1 (1:50; Abcam, Cambridge, MA, USA); rabbit polyclonal antibodies to p-Smad2 and β-catenin (1:50; Cell Signaling Technology, Danvers, MA, USA); or IgG control. They were subsequently incubated with biotinylated secondary antibody (Zhongshan Golden Bridge Biological Technology, Beijing, China) in room temperature for 1 h. The immunocomplexes were visualized using diaminobenzidine (DAB) as a chromogenic substrate (Zhongshan Golden Bridge Biological Technology). The sections were lightly counterstained with hematoxylin.

Table 1
Demographic and clinical characteristics.

Classification	*N* (%)
Sex	
Male	22 (44)
Female	28 (56)
Age, y	
⩽45	23 (46)
>45	27 (54)
Tumor location	
Major salivary gland	32 (64)
Minor salivary gland	18 (36)
Clinical stage	
I + II	24 (48)
III + IV	26 (52)
Histological	
Tubular and/or cribriform	43 (86)
Solid	7 (14)
Perineural invasion	
Negative	34 (68)
Positive	16 (32)
Recurrence	
Absent	20 (40)
Present	30 (60)
Pulmonary metastasis	
Absent	23 (46)
Present	27 (54)

The nuclear and cytoplasmic staining of TGF-β1 and p-Smad2 were analyzed semi-quantitatively by assigning an immunohistochemical score (H-score) that defined both the distribution and intensity of specific labeling. The H-score is evaluated by the formula HS = $\sum$(Pi × *i*)/100, where Pi is the percentage of labeled cells and *i* is the labeling intensity ranging from 1 to 3 [11]. A tumor with a score >0.5 is considered strongly positive and ⩽0.5 is considered weakly positive. To evaluate the loss of membrane β-catenin expression, the score was defined as the percentage of cells without membrane staining. Three areas, each containing a high number of cells, were randomly selected by microscopy at 40× magnification.

Cell lines and cell culture

The SACC-83 cell line was derived from a patient pathologically diagnosed in 1983 with ACC in the sublingual gland [12]. Cells were grown in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco) at 37 °C in a humidified atmosphere of 5% CO_2.

Plasmid construction, transfection and siRNA knockdown

The plasmid pcDNA3-TGF-β1 containing full-length human TGF-β1 cDNA was kindly donated by Dr. Matthias Löhr (Mannheim University of Heidelberg, Mannheim, Germany). This plasmid was used to construct a pcDNA3-TGF-β1-m dominant-active form by mutation at sites 223 and 225. The TGF-β type II dominant-negative receptor plasmid (pcDNA3-TβRIIDN) was generated by PCR from the cDNA of SACC-83 cells, which contained the extracellular and transmembrane domains of the receptor but lacked the cytoplasmic kinase domain.

These plasmids were transfected into SACC-83 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After incubation in 37 °C for 48 h, the cells were treated with 600 μg/mL of the selective antibiotic, G418 (Sigma–Aldrich, St. Louis, MO, USA), and cells resistant to G418 were collected after 4 weeks. The cells transfected with pcDNA3 were labeled as EV (empty vector); the cells transfected with pcDNA3-TGF-β1-m were labeled as Tm1,2,...,12; and the cells transfected with pcDNA3-TβRIIDN were labeled as DN1,2,...,6.

Two small interfering RNAs (siRNA) specific for TGF-β1 were purchased from Guangzhou RiboBio Co., Ltd. After transfection with Lipofectamine RNAiMAX (Invitrogen) for 72 h, the level of TGF-β1 mRNA expression was examined by real-time PCR. These are labeled as TGFsi1, TGFsi2.

The morphologic features of the cells were photographed at 40× magnification (TE-2000 U; Nikon, Japan).

Western blot analysis

To confirm integration of pcDNA3-TβRIIDN, the cells were incubated with 0.5 ng/mL TGF-β1 (R&D systems, Minneapolis, MN, USA) for 1 h and the expression of p-Smad2 was detected by Western blotting. In brief, whole cell protein extracts were separated by 10% SDS–PAGE and transferred to polyvinylidene difluoride

(PVDF) membranes (Millipore, MA, USA). The membranes were probed with anti-total-Smad2/3 and anti-phospho-Smad2 antibodies (1:1000; Cell Signaling, Danvers, MA, USA) at 4 °C overnight. The immunocomplexes were detected using an enhanced chemiluminescence kit (Applygen Technologies, Beijing, China).

Real-time PCR

Total mRNA was isolated from the cells by Trizol reagent (Invitrogen). The cDNA was obtained using a two-step M-MLV Reverse Transcription kit (Promega, Madison, WI, USA). Real-time PCR was performed using FastStart Universal SYBR Green Master (Rox; Roche, Indianapolis, IN, USA) with an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. The primers are given in Table 2.

Transwell invasion assays

Cells were seeded at 2×10^5 cells/well in the upper chambers of the Transwell inserts. After incubation for 16 h, cells on the upper surface of the membrane were wiped off with a cotton swab, and the membranes were fixed with 95% ethanol and stained with 1% crystal violet (Sigma–Aldrich, St. Louis, MO, USA). The cells that invaded the lower surface were photographed at 20× magnification.

Immunocytochemistry

Cells seeded on glass coverslips were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. After incubation with 3% hydrogen peroxide and10% normal goat serum to prevent non-specific binding, the cells were incubated with primary antibodies against β-catenin, E-cadherin and Nectin-1 at 4 °C overnight. Negative control cells were prepared by immunostaining duplicate samples without exposure to primary antibodies. The slides were treated with biotinylated secondary antibody and visualized using DAB, as described for immunohistochemistry, before being mounted and photographed using a DP71 camera (Olympus, Japan).

Animal model of lung metastasis in ACC

To establish an *in vivo* model of lung metastasis in ACC, 3×10^6 cells from each of the different SACC-83 transfected clones were injected into the tail veins of 4-week old female BALB/c nu/nu mice ($n = 10$ mice per group). After 55 days, the mice were sacrificed and the bilateral lung tissues were removed and fixed in formalin. The maximal cross-sectional areas were prepared for hematoxylin and eosin staining. All appropriate measures were taken to minimize any pain, distress or discomfort to the mice, and all procedures were approved by the Institutional Animal Care Committee of Peking University.

Table 2
The primers for real-time PCR.

Genes	Primer sequence (5′-3′)
Human TGFB1 (TGF-β1)	5′-GACTACTACGCCAAGGAGGTCA-3′ 5′-AGTCAATGTACAGCTGCCGCAC-3′
Human CDH1 (E-cadherin)	5′-GCAGGATGTACTATTCTCGGC-3′ 5′-CCTGGATGGTGTTCTGGTTCT-3′
Human CTNNB1 (β-catenin)	5′-ATGGAACCAGACAGAAAAGC-3′ 5′-CAGGATTGCCTTTACCACTCA-3′
Human PVRL1 (Nectin 1)	5′-TG GAG GATGAGGGTGTC TAC-3′ 5′-CCCATTGGCTGAGGTGC-3′
Human GAPDH	5′-TGATGACATCAAGAAGGTGGTGAAG-3′ 5′-TCCTTGGAGGCCATGTAGGC-3′

Statistical analysis

All statistical analyses were carried out using SPSS v. 16.0 software (SPSS Inc., Chicago, IL, USA). Quantitative data were expressed as means ± SD and compared by Student's t test. Analysis of categorical variables was carried out by Pearson's chi-square test to assess the association between protein expression and selected clinicopathological characteristics, and to analyze the association between TGF-β1 and metastasis in the animal experiments. To visualize the efficacy of TGF-β1, p-Smad2 and β-catenin as candidate biomarkers, we summarized the data using receiver operating characteristic (ROC) curves and performed Spearmean's Rank correlation tests to determine bivariate correlations between the markers. $P < 0.05$ was considered statistically significant.

Results

Overexpression of cytoplasmic TGF-β1and p-Smad2 and reduced expression of membrane β-catenin were associated with pulmonary metastasis in ACC cells

TGF-β1, p-Smad2 and β-catenin expression was detected by immunohistochemistry in ACC tissue samples from patients with and without pulmonary metastasis (denoted the metastasis-group and non-metastasis group, respectively). TGF-β1 and p-Smad2 were either absent or weakly expressed in the non-metastasis group; whereas, both were strongly expressed in the cytoplasm and nuclei of the metastasis-group. In contrast, β-catenin showed reduced expression at the cell membranes in the non-metastasis group and was further decreased in the metastasis group (Fig. 1A). Representative samples with scores of 0, 1, 2 and 3, are shown in Fig. 1B, indicating no (<10%), weak, moderate and strong immunoreactivity, respectively.

In accord with these observations, semi-quantitative analysis by Pearson's chi-square test showed that TGF-β1, p-Smad2 were strongly overexpressed (H-score > 0.5); whereas expression of membrane β-catenin (denoted β-catenin RM) was strongly reduced (H-score > 0.5). Furthermore, all were associated with occurrence of pulmonary metastasis ($P < 0.001$; Table 3). To test the efficiency of these three proteins for diagnosis or prognosis of pulmonary metastasis, ROC curve analyses were performed (Fig. 1C). The areas under curve (AUC) were calculated as a measure of the discriminative efficacy of a biomarker, giving values of 0.836 ($P < 0.001$), 0.915 ($P < 0.001$) and 0.842 ($P < 0.001$) for TGF-β1, p-Smad2 and β-catenin RM, respectively. None of the proteins were found to be associated with the other clinicopathological variables given in Table 1 (data not shown). Based on these results, it is suggested that TGF-β1, p-Smad2 and β-catenin RM may be suitable biomarkers of pulmonary metastasis in ACC.

Correlations between TGF-β1, p-Smad2 and β-catenin RM expressions in ACC cells

Correlations between TGF-β1, p-Smad2 and β-catenin RM were determined by Spearman's Rank correlation coefficient tests. A coefficient of +1 or −1 indicates maximum correlation between two variables; a coefficient of 0 indicates no correlation. Our results showed that all were significantly correlated ($P < 0.05$; Table 4). The correlation coefficients between TGF-β1 and p-Smad2 or β-catenin RM were 0.618 and 0.649, respectively; the correlation coefficient between p-Smad2 and β-catenin RM was 0.425. These suggested that TGF-β1 expression was closely correlated with overexpression of p-Smad2 and reduced expression of membrane β-catenin in ACC.

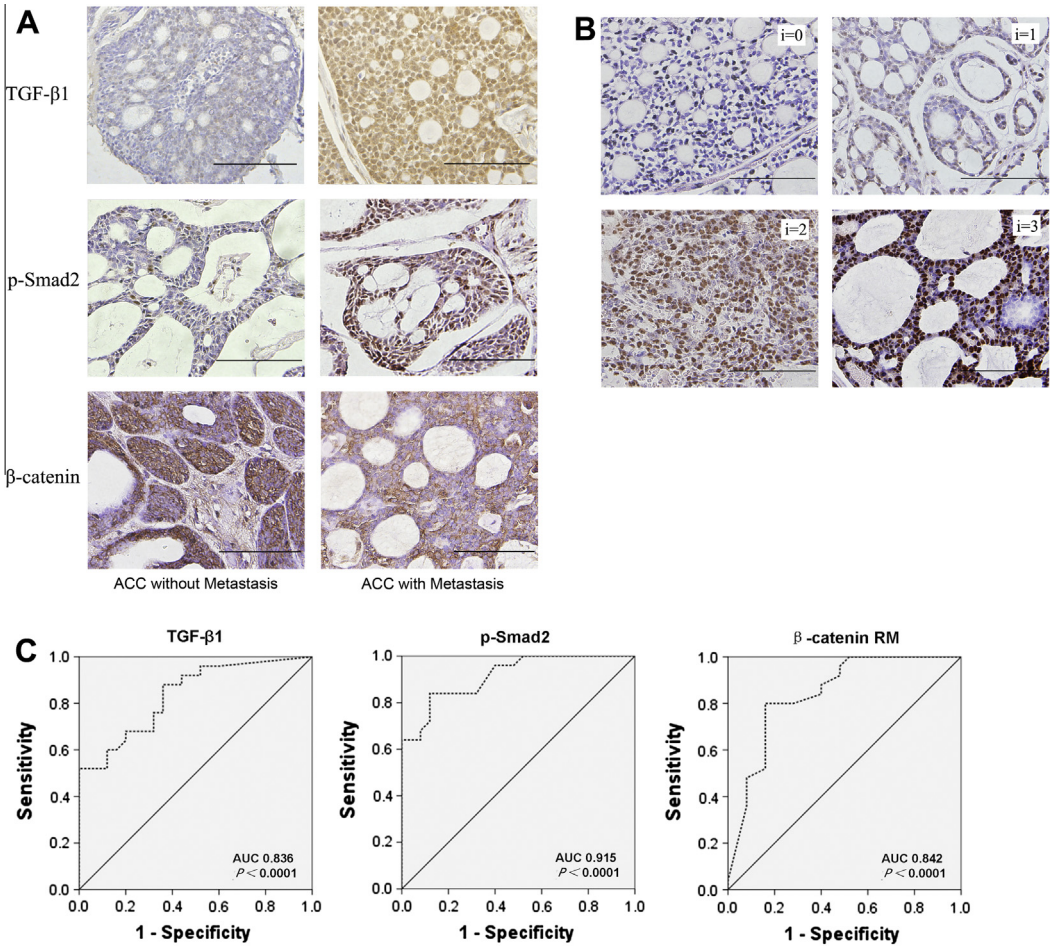

Figure 1. (A) Representative immunohistochemical staining of protein markers in ACC tissues show increased expression of TGF-β1, p-Smad2 and β-catenin in ACC tissues with metastasis (right panel) compared to those without metastasis (left panel); bar = 100 μm. (B) Different immunoreactivity scores for positive TGF-β1 staining in ACC tissues: *i* = 0 (<10%), 1 (weak), 2 (moderate) and 3 (strong); bar = 100 μm. (C) The effectiveness of TGF-β1, p-Smad2 and β-catenin RM as markers for the diagnosis or prognosis of pulmonary metastasis in ACC is determined by ROC curve analysis.

Table 3
Correlation between protein expression and metastasis of ACCs.

H-score or score	Total	Absent	Present	*P*-Value
TGF-β1				
⩽0.5	18	15	3	<0.001
>0.5	32	8	24	
p-Smad2				
⩽0.5	22	18	4	<0.001
>0.5	28	5	23	
β-catenin RM				
⩽0.5	26	20	6	<0.001
>0.5	24	3	21	

Table 4
Correlation analysis of TGF-β1, p-Smad2 and β-catenin expression.

	TGF-β1	p-Smad2	β-catenin RM
TGF-β1			
Correlation coefficient	1	0.618**	0.649**
P-value		<0.001	<0.001
p-Smad2			
Correlation coefficient	0.618**	1	0.425**
P-value	<0.001		0.002
β-catenin RM			
Correlation coefficient	0.649**	0.425**	1
P-value	<0.001	0.002	

** Correlation is significant at the 0.01 level (2-tailed).

Transformation of cellular morphologic features and promotion of cell invasion by TGF-β1 in vitro

Real-time PCR was performed to confirm the expression of TGF-β1 in TGF-β1-overexpressed clones and TGF-β1 siRNA-transfected cells. The clones, Tm9 and Tm11, exhibited higher levels of TGF-β1 mRNA expression compared to EV controls ($P < 0.05$; Fig. 2A). In contrast, the TGF-β1 siRNA transfected cells, TGFsi1 and TGFsi2, exhibited lower expression levels of TGF-β1 mRNA than control siRNA cells ($P < 0.05$; Fig. 2B). To confirm dominant-negative pcDNA3-TβRIIDN function, we carried out Western blot analysis on the transfected cells. The results showed a distinct increase in phosphorylation of Smad2 in EV cells after treatment with TGF-β1; whereas phosphorylation by TGF-β1 was blocked in the DN1 and DN2 cells, thereby confirming integration and biofunction of pcDNA3-TβRIIDN. In addition, phosphorylation of Smad2 was higher in TGF-β1-overexpressed clones compared to EV controls (Fig. 2C).

EMT frequently causes cells to undergo changes in cellular morphology and develop stronger invasive capabilities. Here, SACC-83 cells transfected with EV, pcDNA3-TβRIIDN or TGF-β1 siRNA were almost exclusively polygonal, with little or no evidence of the spindle-cell phenotype. Conversely, cells transfected with TGF-β1-overexpressed clones, Tm9 and Tm11, were dominated by spindle cells (Fig. 2D). Transwell invasion assays revealed that Tm9 and

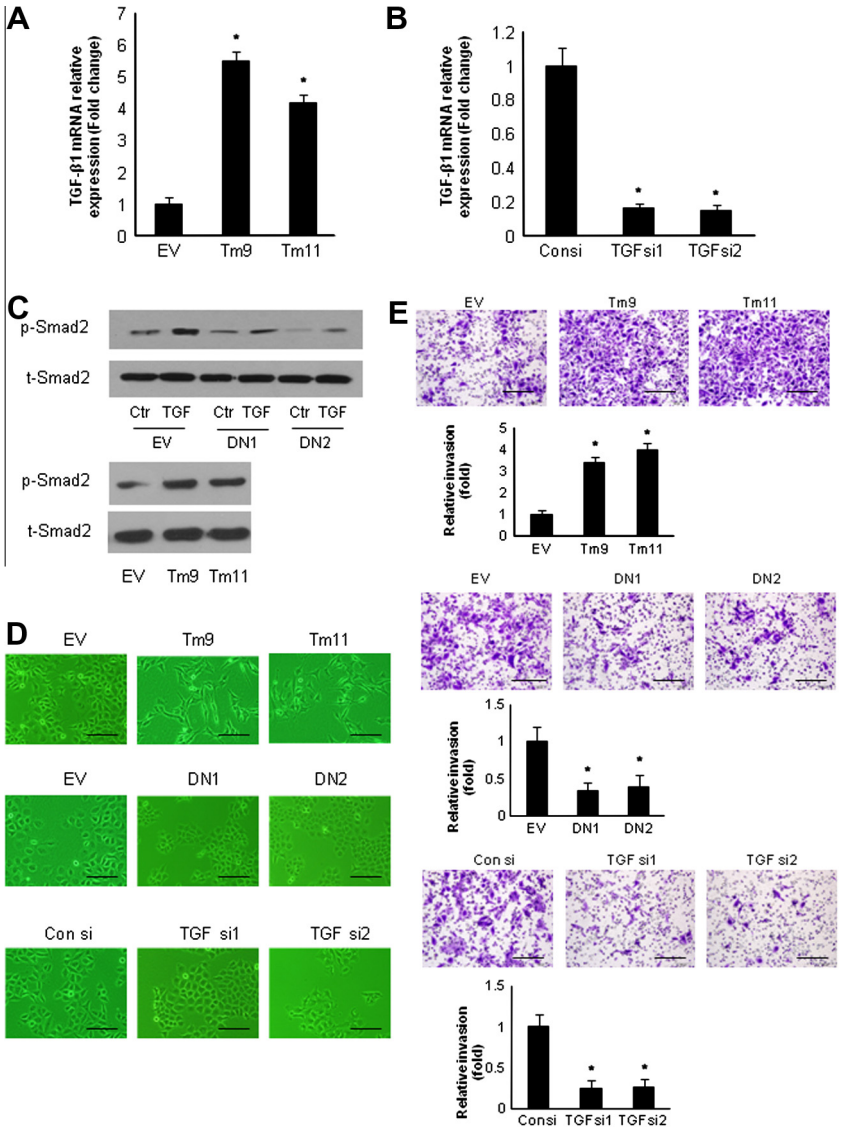

Figure 2. TGF-β1 expression and TGF-β1-induced EMT in ACC cells. (A) Real-time PCR analysis of TGF-β1 mRNA levels in TGF-β1 overexpressed clones, Tm9 and Tm11 compared to EV (empty vector) cells; $^{*}P < 0.05$. (B) Real-time PCR analysis of TGF-β1 mRNA levels in TGF-β1 siRNA1 cells, TGFsi1 and TGFsi2, compared to Consi (Control siRNA) cells; $^{*}P < 0.05$. (C) Western blot analysis of phospho-Smad2 levels in SACC-83 cells. (D) Cell morphologic features in transfected cells; bar = 100 μm. (E) Transwell invasion assay showing increased invasion of ACC cells in the metastasis group compared to the control group; $^{*}P < 0.05$; 20× magnification; bar = 100 μm.

Tm11 cells exhibited higher invasive capabilities than EV controls; whereas the invasive capabilities of cells transfected with pcDNA3-TβRIIDN or TGF-β1 siRNA were reduced ($P < 0.05$; Fig. 2E). These results suggested that EMT might be induced by TGF-β1 in ACC SACC-83 cells *in vitro*.

Expression of β-catenin, E-cadherin and Nectin-1 was regulated by TGF-β1 in ACC cells

To further verify EMT in TGF-β1 transfected SACC-83 cells, the expression levels of established EMT markers, β-catenin and E-cadherin, and a recently discovered marker, Nectin-1, were determined by real-time PCR. This revealed that mRNA expression of E-cadherin and Nectin-1 decreased in the TGF-β1-overexpressed clones, Tm9 and Tm11, ($P < 0.05$, Fig. 3A) but increased in TGF-β1 siRNA silenced cells ($P < 0.05$, Fig. 3B). This was confirmed by immunocytochemistry (Fig. 3C). Taken together, these results suggested that expression of the EMT markers, β-catenin, E-cadherin and Nectin-1, was regulated by TGF-β1 in ACC cells.

EMT increased lung metastasis in vivo

An animal model was designed to confirm our findings*in vivo*. Although metastases in the lung tissues of treated mice were not visible to the naked eye, microscopy revealed tiny metastatic foci in tissues from the Tm9 group (Fig. 4A and B). None of the mice in the DN1 group exhibited metastasis in the lung. The metastatic ratios (metastasis number/number of animals × 100%) for EV, Tm9 and DN1 cells were 30% (3/10), 70% (7/10) and 0% (0/10), respectively (Table 5). However, these results were not statistically significant.

Discussion

ACC is associated with multiple types of metastasis, leading to poor prognosis [13], therefore the discovery of effective markers to predict the risk of malignancy, and elucidation of the underlying molecular mechanisms, are essential to understand the clinical behavior and facilitate the management of ACC. In this study, we found that overexpression of TGF-β1, activation of Smad2 phos-

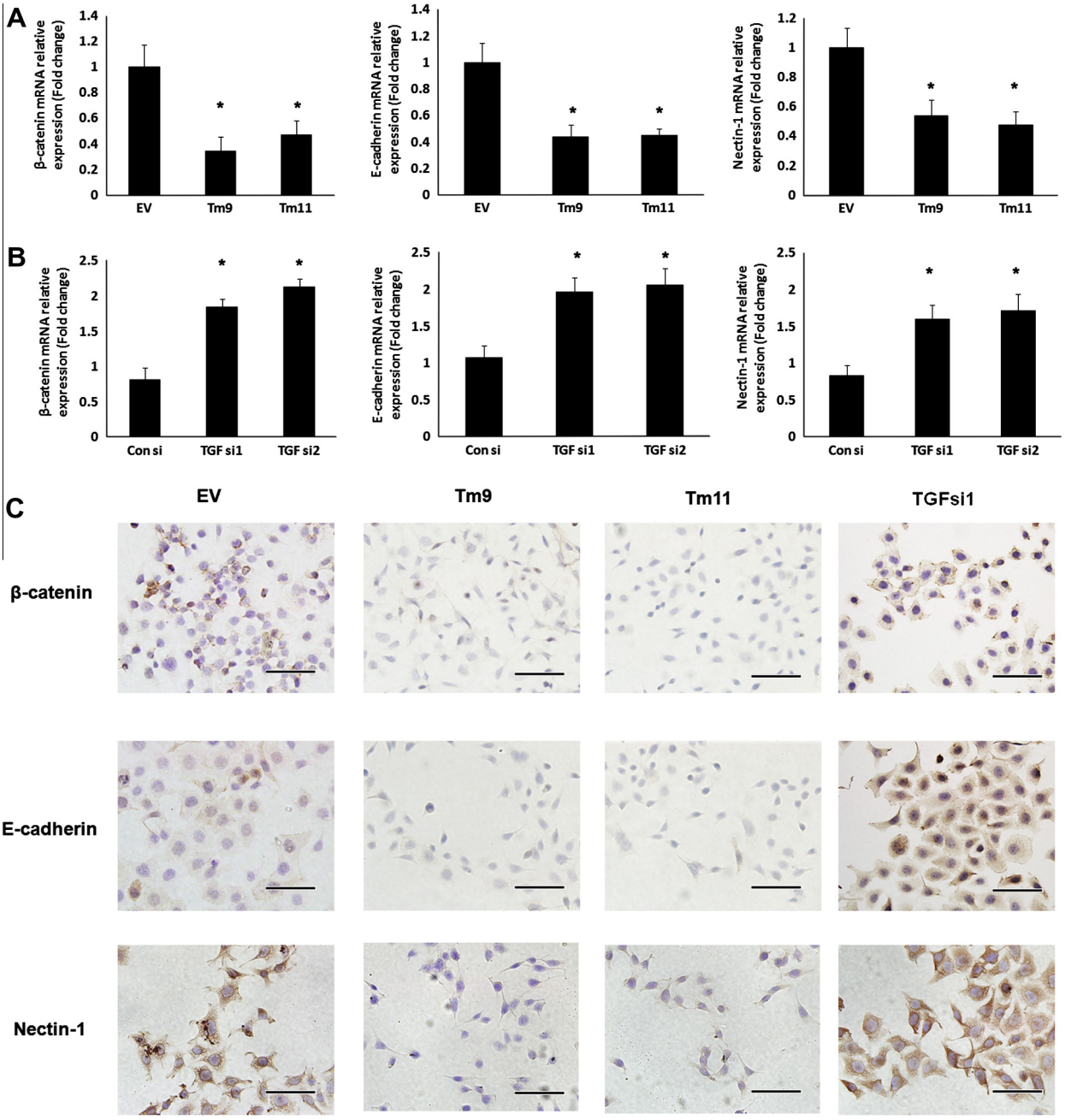

Figure 3. Expression of EMT markers, β-catenin, E-cadherin and Nectin-1 in ACC cells. (A) Real-time PCR analysis of TGF-β1 mRNA expression in ACC cells compared to EV cells; $^{*}P < 0.05$. (B) Real-time PCR analysis of mRNA expression in ACC cells compared to Consi (Control siRNA) cells; $^{*}P < 0.05$. (C) Protein localization and expression in ACC cells observed by immunocytochemistry; bar = 100 μm.

phorylation and reduced expression of membrane β-catenin were closely associated with lung metastasis in ACC, and that TGF-β1-induced EMT may underlie ACC metastasis.

Previous studies have revealed that TGF-β1 contributes to the invasive and metastatic capability of malignant tumor cells [14–16], and may be dependent upon an intact Smad pathway [17,18]. In agreement with these reports, our study has shown that both TGF-β1 and p-Smad2 were significantly increased, and highly correlated, in ACC with pulmonary metastasis, suggesting that the TGF-β1/Smad pathway may participate in invasion and metastasis of ACC *in vivo*. This was also consistent with our previous *in vitro* study which had shown that TGF-β1 promoted migration and invasion of ACC in cell lines *via* the Smad pathway [8].

TGF-β1 plays a central role in EMT, which involves a series of events leading to tumor invasion and metastasis including stress fiber formation and complex changes in cell architecture. It is also accepted that the E-cadherin/β-catenin complex provides the physical structure for cell–cell attachment [19]. The critical importance of this complex was demonstrated by Birchmeier et al. who showed that downregulation of any of its components resulted in the loss of the tumor-suppressive actions of adherens junctions, thereby enabling cancer cells to escape from the primary tumor and migrate to other sites [20]. Subsequent studies have reported that the addition of TGF-β1 to a proximal tubular cell line reduced levels of E-cadherin and membrane β-catenin, resulting in a loss of cell–cell contacts and dissociation of adherens junctions [21]; and

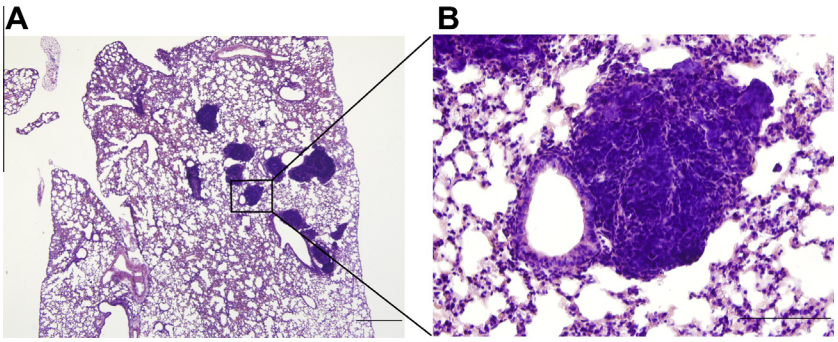

Figure 4. Lung tissue of mice injected with Tm9 cells (dominant-active pcDNA3-TGF-β1-m plasmids) visualized after hematoxylin and eosin staining. (A) Lung tissue showing multiple tiny metastatic foci; 4× magnification; bar = 500 μm. (B) A single metastatic focus magnified from the image on the left; 40× magnification; bar = 100 μm.

Table 5
Metastasis occurrence in different animal groups.

Groups	Total	Absent	Present	*P*-Value
EV	10	7	3	
Tm9	10	3	7	0.07
EV	10	7	3	
DN1	10	10	0	0.06

that loss of the E-cadherin/β-catenin complex was related to tumor progression in salivary glands [22]. A further study of ACC confirmed the occurrence of EMT by demonstrating the positive expression of Slug, which is a transcription factor of EMT and associated with metastasis of patients with ACC [23]. In our study, the expression of membrane β-catenin in ACC with metastasis was significantly higher in ACC with metastasis than in ACC without metastasis, and that loss of β-catenin was associated with expression of TGF-β1.

Based on these findings, we hypothesized that EMT induced by TGF-β1 and mediated by Smad2 was characterized by a decrease in membrane β-catenin expression, thereby contributing to ACC metastasis.

To validate our hypothesis, we transfected the recombinant eukaryotic expression plasmid, pcDNA3-TGF-β1-m, into SACC-83 cells *in vitro*. Our investigations revealed morphological changes, enhanced invasive capacity, and downregulation of both β-catenin and E-cadherin. Furthermore, we found that Nectin-1, a novel cell–cell adhesion effect or in ACC cells, was also regulated by TGF-β1. Nectins are a family of Ca^{2+}-independent immunoglobulin-like cell adhesion molecules (CAM), consisting of four members. Whereas cadherins are linked to the actin cytoskeleton *via* α- and β-catenin [24], nectins are associated with the actin cytoskeleton by binding to afadin, an actin filament-binding protein. The nectin–afadin complex plays an important role in the formation of the junctional complex. In contrast, we were able to interrupt the TGF-β pathway by transfecting SACC-83 cells with TGF- β1 pcDNA3-TβRIIDN or by silencing TGF- β1 expression with siRNA.

To validate our hypothesis *in vivo*, we established a lung metastasis model of ACC in mice and applied Pearson's chi-square test to analyze our findings. A greater number of mice developed metastasis after being inoculated with pcDNA3-TGF-β1-m cells compared to those inoculated with EV cells, furthermore, none of the mice inoculated with pcDNA3-TβRIIDN cells developed metastasis. Although these differences were not statistically significant, which may have been a consequence of the small sample size or the characteristics of individual mice, these observations supported our theory.

In conclusion, overexpression of TGF-β1 and p-Smad2, with reduced expression of membrane β-catenin, was significantly associated with pulmonary metastasis in patients with ACC. Furthermore, this study is the first to confirm EMT in ACC cells. As TGF-β1 induces EMT and alters cellular morphology in ACC cells, silencing TGF-β1 expression or blockading TGF-β pathways might be promising strategies for the prevention of ACC metastasis.

Conflict of interest statement

None declared.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (Nos. 30228028, 30572050, and 39470754) and Beijing Natural Science Foundation (No. 7062069).

References

[1] Spiro RH. Salivary neoplasms: overview of a 35-year experience with 2,807 patients. Head Neck Surg 1986;8(3):177–84.
[2] Matsuba HM, Simpson JR, Mauney M, et al. Adenoid cystic salivary gland carcinoma: a clinicopathologic correlation. Head Neck Surg 1986;8(3):200–4.
[3] Ciccolallo L, Licitra L, Cantu G, et al. Survival from salivary glands adenoid cystic carcinoma in European populations. Oral Oncol 2009;45(8):669–74.
[4] He JF, Ge MH, Zhu X, et al. Expression of RUNX3 in salivary adenoid cystic carcinoma: implications for tumor progression and prognosis. Cancer Sci 2008;99(7):1334–40.
[5] Muller A, Sonkoly E, Eulert C, et al. Chemokine receptors in head and neck cancer: association with metastatic spread and regulation during chemotherapy. Int J Cancer 2006;118(9):2147–57.
[6] Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 2003;425(9):577–84.
[7] Wendt MK, Tian M, Schiemann WP. Deconstructing the mechanisms and consequences of TGF-beta-induced EMT during cancer progression. Cell Tissue Res 2012;347(1):85–101.
[8] Dong L, Wang YX, Li SL, et al. TGF-beta1 promotes migration and invasion of salivary adenoid cystic carcinoma. J Dent Res 2011;90(6):804–9.
[9] Du F, Nakamura Y, Tan TL, et al. Expression of snail in epidermal keratinocytes promotes cutaneous inflammation and hyperplasia conducive to tumor formation. Cancer Res 2010;70(24):10080–9.
[10] Brembeck FH, Rosário M, Birchmeier W. Balancing cell adhesion and Wnt signalin, the key role of β-catenin. Curr Opin Genet Dev 2006;16(1):51–9.
[11] Huang AH, Pettigrew NM, Watson PH. Immunohistochemical assay for oestrogen receptors in paraffin wax sections of breast carcinoma using a new monoclonal antibody. J Pathol 1996;180(1):223–7.
[12] Li SL, Liu XP, Zhang KH. Establishment of a human cancer cell line from adenoid cystic carcinoma of the minor salivary gland. Chin J Stomatol 1990;25:29–31.
[13] Kokemueller H, Eckardt A, Brachvogel P, et al. Adenoid cystic carcinoma of the head and neck – a 20 years experience. Int J Oral Maxillofac 2004;33(1):25–31.
[14] McEarchern JA, Kobie JJ, Mack V, et al. Invasion and metastasis of a mammary tumor involves TGF-beta signaling. Int J Cancer 2001;91(1):76–82.
[15] Tang B, Vu M, Booker T, et al. TGF-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. J Clin Invest 2003;112(7):1116–24.
[16] Janda E, Lehmann K, Killisch I, et al. Ras and TGF-beta cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. J Cell Biol 2002;156(2):299–313.

[17] Oft M, Akhurst RJ, Balmain A. Metastasis is driven by sequential elevation of H-ras and Smad2 levels. Nat Cell Biol 2002;4(7):487–94.
[18] Tian F, Byfield SD, Parks WT, et al. Reduction in Smad2/3 signaling enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. Cancer Res 2003;63(23):8284–92.
[19] Knust E, Bossinger O. Composition and formation of intercellular junctions in epithelial cells. Science 2002;298(5600):1955–9.
[20] Birchmeier W, Hulsken J, Behrens J. E-Cadherin as an invasion suppressor. Ciba Found Symp 1995;189:124–41. discussion 136–41, 174–6.
[21] Tian YC, Phillips AO. Interaction between the transforming growth factor-beta type II receptor/Smad pathway and beta-catenin during transforming growth factor-beta 1-mediated adherens junction disassembly. Am J Pathol 2002;160(5):1619–28.
[22] Furuse C, Cury PR, Altemani A, et al. Beta-catenin and E-cadherin expression in salivary gland tumors. Int J Surg Pathol 2006;14(3):212–7.
[23] Tang YL, Liang XH, Zheng M, et al. Expression of c-kit and Slug correlates with invasion and metastasis of salivary adenoid cystic carcinoma. Oral Oncol 2010;46(4):311–6.
[24] Takai Y, Shimizu K, Ohtsuka T. The roles of cadherins and nectins in interneuronal synapse formation. Curr Opin Neurobiol 2003;13(5):520–6.