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ORIGINAL ARTICLE

Elevation of Twist expression by arecoline contributes to the pathogenesis of oral submucous fibrosis

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Background/purpose: Oral submucous fibrosis (OSF), a chronic progressive scarring disease, has been considered as a precancerous condition of oral mucosa. In this study, we investigated the functional role of Twist, an epithelial-mesenchymal transition (EMT) transcriptional factor, in myofibroblastic differentiation activity of OSF.
Methods: Arecoline, a major areca nut alkaloid, was used to explore whether expression of Twist could be changed dose-dependently in human primary buccal mucosal fibroblasts (BMFs). Collagen gel contraction and migration capability in arecoline-stimulated BMFs and primary oral submucous fibrosis-derived fibroblasts (OSFs) with Twist knockdown was presented.
Results: We observed that the treatment of arecoline dose-dependently increased Twist expression transcript and protein levels in BMFs. The myofibroblast activity including collagen gel contraction and migration capability also induced by arecoline, while knockdown of Twist reversed these phenomena. Importantly, inhibition of Twist led to the suppression collagen contraction and wound healing capability of primary cultivated OSFs. Clinically, Twist transcript and protein expression was higher in areca quid chewing-associated OSF tissues than in normal oral mucosa tissues.
Conclusion: This evidence suggests that upregulation of Twist might be involved in the pathogenesis of areca quid-associated OSF through dysregulation of myofibroblast activity.
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Conflicts of interest: The authors have no conflicts of interest relevant to this article.

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Introduction

Oral submucous fibrosis (OSF), a chronic progressive scarring disease, has been considered as a precancerous condition of oral mucosa.[1,2] OSF is characterized by the submucosal accumulation of dense fibrous connective tissue with inflammatory cell infiltration and epithelial atrophy.[1,2] Epidemiological evidence strongly indicates that OSF is highly associated with an areca quid chewing habit. However, the detailed molecular mechanisms involved in the pathogenesis of OSF are still poorly understood.

Myofibroblasts, the α-smooth muscle actin (SMA)-expressing contractile fibroblasts, contribute to wound healing repair processes and tissue fibrosis through the contraction, dysregulation, and secretion of extracellular matrix (ECM) protein.[3] Recent findings demonstrated that myofibroblasts may play the role of conductors in the pathogenesis of fibrosis.[3] High myofibroblastic differentiation activity has been reported by several groups of organ fibrosis, such as liver,[4] heart,[5] and lung.[6] The origin of myofibroblast is still being debated as it might be derived from other cell types such as resident stromal fibroblasts and endothelial cells or from terminally epithelial differentiated cells that undergo an epithelial-mesenchymal transition (EMT) process to transdifferentiate into myofibroblasts.[3] Several experimental and clinical studies have revealed that aberrant expression of several EMT-related molecules, such as plasminogen activator inhibitor-1,[7] insulin-like growth factor-1,[8] and NF-κB (nuclear factor kappa B),[9] vimentin,[10] S100A4,[1] or ZEB1[11] were detected in OSF. These findings suggest that EMT program may be involved in the pathogenesis of OSF.

Twist, a basic helix-loop-helix domain-containing transcription factor, functions as a transcription repressor to activate EMT traits by repressing the expression of epithelial marker E-cadherin.[12] *In vitro* and *in vivo* evidence supports major roles for Twist as a regulator of EMT.[13] Twist was observed to be upregulated in fibroblasts of lung tissue from idiopathic pulmonary fibrosis patients.[14,15] Twist was involved in bleomycin-induced pulmonary fibrosis.[16] Upregulation of Twist-positive cells is associated with liver and kidney fibrosis.[17,18] The role of twist in areca nut chewing-associated OSF also remains unknown. However, it is unclear whether Twist is involved in the pathogenesis of OSF.

In this study, we explore a possible role of Twist in the pathogenesis of areca quid-associated OSF. Targeting Twist attenuated arecoline-stimulated collagen gel contraction and migration capability in buccal mucosal fibroblasts (BMFs). Knockdown of Twist could abolish myofibroblastic activity in primary OSFs, supporting the clinical elevation of Twist expression in OSF tissues.

Materials and methods

Reagents and antibodies

Arecoline was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ploylonal antihuman Twist antibody was purchased from Santa Cruz Biotechnology, Inc. (sc-15393; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Primary BMFs and OSFs cell culture

All procedures of tissues acquirements have followed the tenets of the Declaration of Helsinki and are reviewed by the Institutional Review Committee at Chung Shan Medical University, Taichung. Taiwan. Primary BMFs and OSFs cells were established as previously described.[1] Fibroblast cultures were grown and maintained by using the explant method as described previously.[1] Cell cultures between the third and eighth passages were used in this study.

Western blot assay

The extraction of proteins from cells and western blot analysis were performed as described.[19] Samples (15 μL) were boiled at 95°C for 5 minutes and separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were wet-transferred to Hybond-ECL nitrocellulose paper (Amersham, Arlington Heights, IL, USA). The following primary antibodies were used: mouse antihuman Bcl-2 and rabbit antihuman Bax (Upstate Biotechnology, Charlottesville, VA, USA); and mouse anti-β-actin (Chemicon, Temecula, CA, USA). Immunoreactive protein bands were detected by the ECL detection system (Amersham Biosciences Co., Piscataway, NJ, USA).

Quantitative real-time reverse transcriptase-polymerase chain reaction

Total RNA was prepared from cells or tissues using Trizol reagent according to the manufacturer's protocol (Invitrogen, Calsbad, CA, USA). Quantitative real-time reverse transcriptase-polymerase chain reactions (qRT-PCR) of mRNAs were reverse-transcribed using the Superscript III first-strand synthesis system for RT-PCR (Invitrogen). qRT-PCR reactions on resulting cDNAs were performed on an ABI StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The end-point used in the real-time quantification was calculated with StepOne software (Applied Biosystems, Foster City, CA, USA), and the threshold cycle number (Ct value) for each analyzed sample was calculated. Each target gene was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to derive the change in Ct value ($\triangle$Ct). The primer sequences used in this study were listed as follows: *Twist*: 5′-GGGAGTCCGCAGTCTTACGA-3′ and 5′-AGACCGAGAAG GCGTAGCTG -3′; and *Gapdh*: 5′- CATCATCCCTGCCTCTACTG-3′ and 5′- GCCTGCTTCACCACCTTC-3′.

Lentivirus-based short hairpin RNA delivery

The pLV-RNAi vector was purchased from Biosettia Inc. (Biosettia, San Diego, CA, USA). The method of cloning the double-stranded short hairpin RNA (shRNA) sequence is described in the manufacturer's protocol. Lentiviral vectors expressing shRNA that targets human Twist were synthesized and cloned into pLVRNAi to generate a lentiviral expression vector. Lentivirus production was performed with

transfection of plasmid DNA mixture with lentivector plus helper plasmids (VSVG and Gag-Pol) into 293T cells using Lipofectamine 2000 (Invitrogen). Supernatants were collected 48 hours after transfection and then were filtered; the viral titers were then determined using fluorescence-activated cell sorting at 48 hours post-transduction. Subconfluent cells were infected with lentivirus in the presence of 8 μg/mL polybrene (Sigma-Aldrich). The green fluorescence protein, which was coexpressed in lentiviral-infected cells, was served as a selection marker to indicate the successfully infected cells.[20]

Collagen contraction assay

Cells were suspended in 0.5 mL of 2 mg/mL collagen solution (Sigma-Aldrich) and added into one well of 24-well-plate. The plate was incubated at 37°C for 2 hours which caused polymerization of collagen cell gels. After detaching the gels from the wells, the gels were further incubated in 0.5 mL MEMα medium for 48 hours. Contraction of the gels was photographed and measured using ImageJ software (National Institutes of Health, Bethesda, Maryland) to calculate their areas.[1]

Cell migration assay

For transwell migration assays, 1×10^5 cells were plated into the top chamber of a transwell (Corning, Acton, MA, USA) with a porous transparent polyethylene terephthalate membrane (8.0 μm pore size). Cells were plated in medium with lower serum (0.5% fetal bovine serum), and medium supplemented with higher serum was used as a chemoattractant in the lower chamber. The cells were incubated for 24 hours and cells that did not migrate through the pores were removed by a cotton swab. Cells on the lower surface of the membrane were stained with Giemsa (Sigma-Aldrich, Oakville, ON, Canada). The number of migration cells in a total of five randomly selected fields was counted.[21]

Wound healing assay

Cells were seeded into a 12-well culture dish, and then wounds were introduced to the confluent monolayer of cells with a sterile 200 μL plastic pipette tip to create a denuded area. Cell movement into the wound area was photographed at 0 hours and 24 hours under a microscope.[21]

Statistical analysis

Data are presented as mean ± SD. A Student *t* test or analysis of variance test was used to compare the continuous variables between groups, as appropriate. The Chi-square test or Fisher's exact test was used to compare the categorical variables. A p value < 0.05 was considered statistically significant.

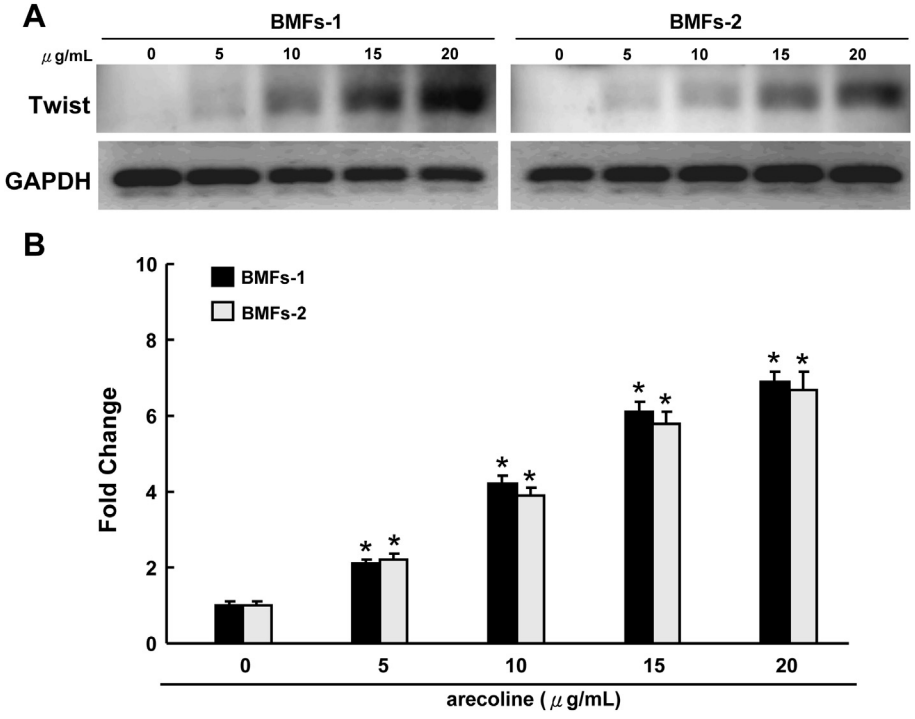

Figure 1 Arecoline dose-dependently induces the expression of Twist in BMFs. (A) BMFs were serum-starved (0.5% FBS (fetal bovine serum)) for 48 hours and treated with indicated concentration of arecoline for further 24 hours in serum-free medium. The expression of Twist was detected by western blot. GAPDH was used as protein loading control. (B) Protein levels of Twist stimulated by arecoline in BMFs were measured with a densitometer. The relative level of Twist protein expression was normalized against GAPDH signal and the control was set as 1.0. Optical density values represent the mean ± SD. * represents significant difference from control values with $p < 0.05$. BMF = buccal mucosal fibroblast; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

Results

Arecoline induces the expression of Twist in BMFs

OSF is highly associated with the habit of areca quid chewing based on the epidemiological evidences.[22] To examine the effect of arecoline, a major areca nut alkaloid, on the Twist expression in human primary BMFs, two BMFs strains were treated with dose-dependently increasing concentration of arecoline and the levels of Twist protein were measured using western blotting. Arecoline treatment dose-dependently elevated Twist expression in BMFs (Figures 1A and 1B).

Knockdown of Twist suppresses arecoline induced collagen contraction and migration capabilities of BMFs

Myofibroblasts are the contractile fibroblasts that contribute to tissue repair during wound healing but play a pathological role in OSF.[23,24] To further investigate whether Twist plays a role in maintaining myofibroblastic differentiation activity associated with OSF, the approach of loss-of-function of Twist was first conducted. Downregulation of Twist in arecoline-stimulated BMFs was achieved by viral transduction with lentiviral vector expressing shRNA targeting Twist (sh-Twist-1 and sh-Twist-2), and lentiviral vector expressing shRNA against luciferase (sh-Luc) was used as control. Real-time RT-PCR and immunoblotting analyses confirmed that lentivirus expressing both sh-Twist-1 and sh-Twist-2 markedly reduced the expression level of arecoline-induced Twist transcript (Figure 2A) and protein

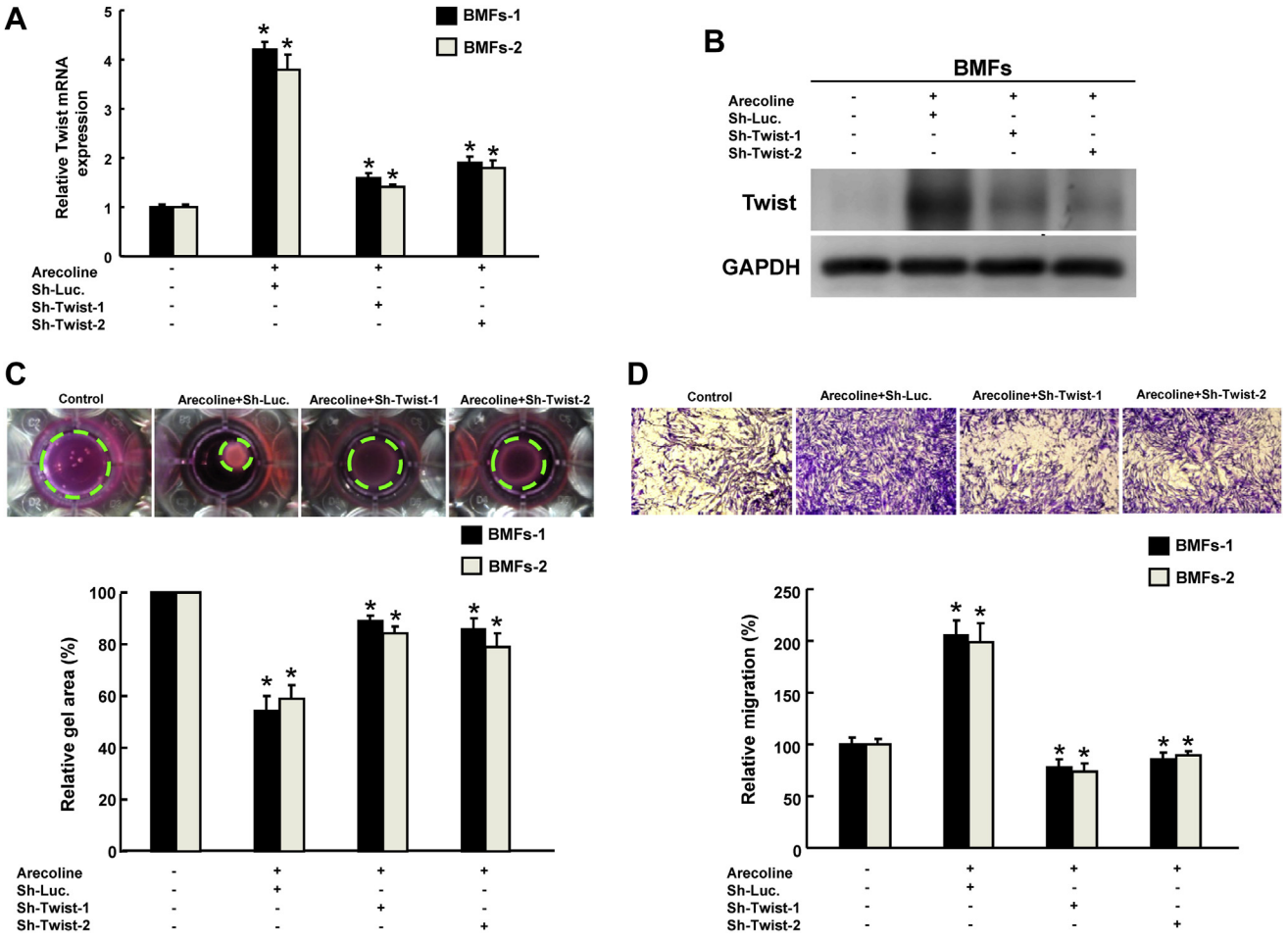

Figure 2 Silencing of Twist expression suppresses arecoline-induced collagen contraction and migration capabilities of BMFs. (A) The silencing effect of Twist shRNA in arecloine-treated BMFs was validated transcriptionally by real-time RT-PCR analysis. (B) Total proteins prepared from single cell suspension of BMF transduced with sh-Luc or sh-Twist entivirus, individually or concurrently, and treated with or without arecoline were analyzed for Twist expression by western blotting. (C) Single cell suspension of arecoline-treated BMFs infected with Twist-specific shRNA or control sh-Luc lentivirus was analyzed by collagen gel contraction assay. (D) The migration ability of the control or Twist-knockdown in arecoline-treated BMFs was evaluated. *$p < 0.05$ Sh-Luc. + arecoline group versus control group. **$p < 0.05$ Sh-Twsit-1+arecoline or Sh-Twist-2+ arecoline versus Sh-Luc. + arecoline group. BMF = buccal mucosal fibroblast; RT-PCR = reverse transcriptase-polymerase chain reaction; shRNA = short hairpin RNA.

(Figure 2B) expression in BMFs. Previously, we have demonstrated that arecoline could induce myofibroblast activities in BMFs.[1,11] Targeting sh-Twist-1 abrogated arecoline-induced collagen gel contraction (Figure 2C) and migration abilities (Figure 2D) in BMFs.

Twist mediates myofibroblast activities in OSFs

To validate the significance of Twist in clinical specimens, we collected five paired samples of normal buccal mucosa and fibrotic buccal mucosa from OSF patients for primary culture and real-time RT-PCR analysis (Figure 3A). Quantitative real-time RT-PCR analysis has confirmed an increase of Twist in primary oral submucous fibrosis-derived fibroblasts (OSFs) compared with BMFs samples (Figure 3A). Lentivirus expressing both sh-Twist-1 and sh-Twist-2 markedly reduced the expression level of Twist in OSFs (Figure 3B). The collagen gel contraction was elevated in OSFs compared with the BMFs. Notably, silencing Twist reversed these effects (Figure 3C). Similarly, targeting Twist also abolished wound healing capability in OSFs (Figure 3D). These results collectively suggest that Twist mediates the maintenance of myofibroblastic properties in OSF.

Twist is upregulated in OSF specimen tissues

To validate the significance of Twist in clinical specimens, we also compared the expression levels of Twist between normal buccal mucosa (N) and fibrotic buccal mucosa from OSF patients. In line with our previous data, the transcript level of Twist expression was higher in OSF sample tissues but lower in normal buccal mucosa subjects by real-time RT-PCR analysis (Figure 4A). Accordingly, the western blotting data showed that the protein levels of Twist in OSF tissues were also upregulated (Figure 4B).

Discussion

EMT, a dedifferentiation program converting epithelial cells phenotypes into mesenchymal traits, is involved in embryonic development in several cellular functions that positively affect tumor development and progression, and

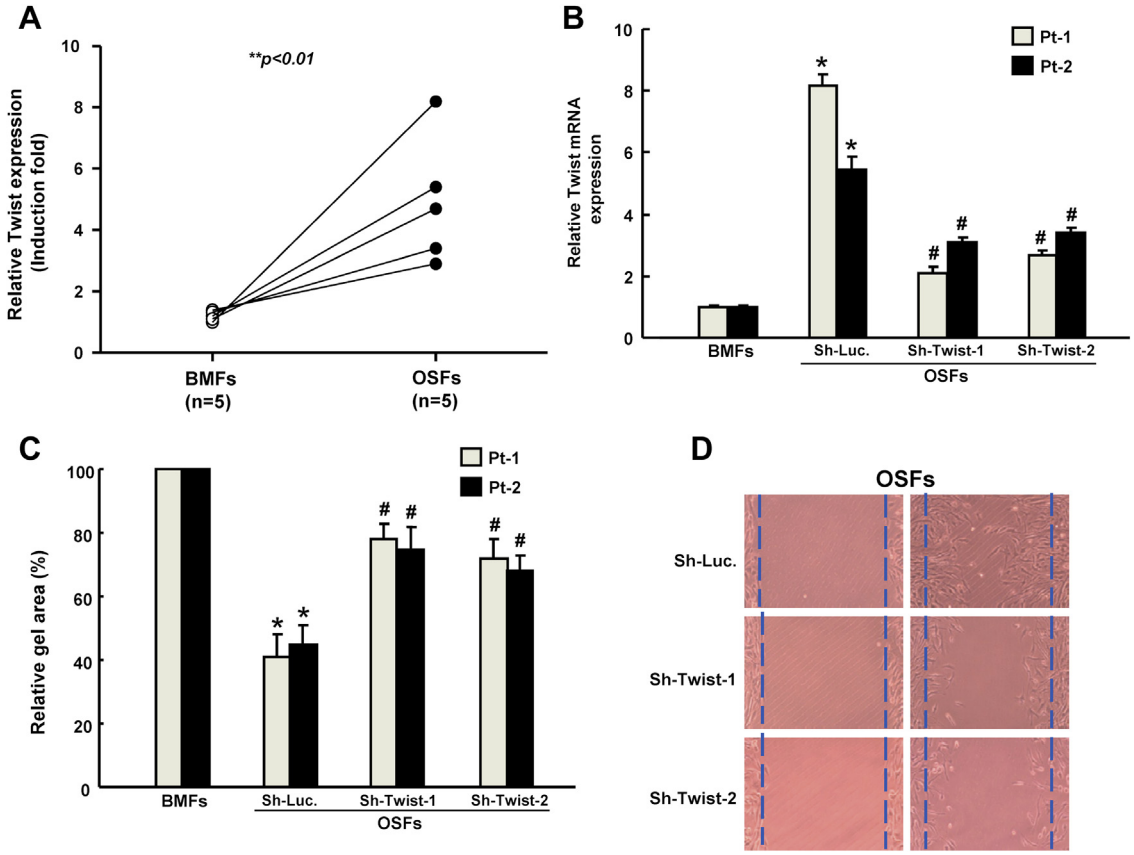

Figure 3 Depletion of Twist represses myofibroblastic differentiation activity in OSF. (A) Analysis of Twist transcript expression in independent pairs ($n = 5$) of normal buccal mucosa-derived fibroblasts (BMFs) and OSF specimens-derived fibroblasts by real-time RT-PCR analysis. (B) The silencing effect of Twist shRNA in primary OSFs was validated transcriptionally by real-time RT-PCR analysis. (C) OSFs were transduced with shRNA lentivirus and embedded into collagen gels. After 48 hours, contraction of the gels was photographed and measured using ImageJ software (NIH) to calculate their areas (lower panel). (D) Single cell suspension of OSFs infected with Twist-specific shRNA or control sh-Luc lentivirus was analyzed by wound healing assay. *$p < 0.05$ Sh-Luc. +OSFs group versus BMFs group. **$p < 0.05$ Sh-Twsit-1 or Sh-Twist-2 versus Sh-Luc group. OSF = oral submucous fibrosis; RT-PCR = reverse transcriptase-polymerase chain reaction; shRNA = short hairpin RNA.

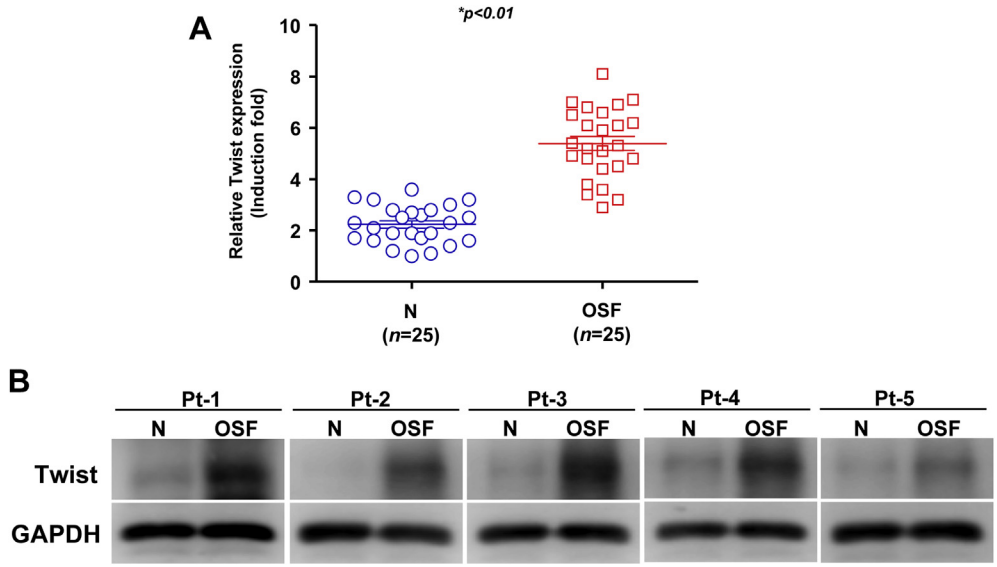

Figure 4 Clinical significance of Twist expression in OSF tissues. (A) Pairs of normal buccal mucosa (N) tissues and OSF specimen lesions were subjected to real-time RT-PCR analysis for the transcript expression levels of Twist; (B) the representative image of Twist expression in normal buccal mucosa (N) tissues and OSF specimens by western blotting. *$p < 0.01$; paired t test was used in this sample cohort. OSF = oral submucous fibrosis; RT-PCR = reverse transcriptase-polymerase chain reaction.

fibrosis.[3] Intensive studies revealed that transcriptional factors, such as ZEB1, Snail, Slug, and Twist activate the EMT process.[25] Myofibroblasts could also come from other cell types within tissues, such as epithelial cells, endothelial cells, and hepatocytes, through EMT process.[26] Our previous study has demonstrated that ZEB1 could bind to the E-box domain of α-SMA promoter to induce myofibroblast transdifferentiation activities in OSF.[11] However, the detailed molecular mechanisms involved in the regulatory links between Twist-mediated EMT and myofibroblast in OSF are still poorly understood. In this study, we attempted to investigate the role of Twist in the pathogenesis of areca quid chewing-associated OSF. We first found that Twist could be induced by arecoline in BMFs (Figure 1). Depletion of Twist by lentiviral-mediated knockdown reversed arecoline-induced myofibroblastic differentiation (Figure 2). The upregulation of Twist was also observed in OSF tissues from patients with areca quid chewing habit (Figure 4). Our data suggest that Twist expression is involved in the pathogenesis of OSF.

Transforming growth factor-β (TGF-β), a multifunction cytokine, is associated with the onset and progression of fibrosis in many human tissues, such as liver fibrosis,[27] renal fibrosis,[28] pulmonary fibrosisc,[6] and cardiac fibrosis.[29] TGF-β1 is also a well-known inducer of EMT program.[30] Arecoline could promote the transdifferentiation of human BMFs into myofibroblasts through activation integrin αvβ6/TGF-β1 signalings.[2] Areca nut extracts were found to induce TGF-β signaling in primary human gingival fibroblast.[31] During fibrogenesis in OSF, it could be possible that arecoline induced Twist is mediated by TGF-β signaling.

OSF is thought to be an oral precancerous condition with about 7.6% malignant transformation rates.[32] Currently, there is no specific and effective therapy for OSF treatment. Surgical intervention, as well as anti-inflammation drugs have been used to manage OSF, but the effect is limited.[33] The present findings provide the crucial role of Twist in areca quid-associated OSF. It is worth using Twist as a molecular target to select inhibitors or phytochemicals which can downregulate Twist expression for antifibrotic therapeutics in OSF. For example, (-)-epigallocatechin-3-gallate[34] and curcumin[35] have been reported to inhibit Twist expression in cancer cells.

Conclusively, we report a possible role of Twist in the pathogenesis of areca quid-associated OSF. Arecoline treatment enhanced Twist expression and myofibroblast transdifferentiation activities, while silencing Twist effectively reversed these phenomena. This study would greatly contribute to a deeper understanding of pathogenesis in OSF and promote the development of effective therapies for OSF through targeting Twist.

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