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ORIGINAL ARTICLE

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

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KEYWORDS
arecoline;
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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.

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