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Original Article

LncRNA GAS5-AS1 inhibits myofibroblasts activities in oral submucous fibrosis

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KEYWORDS
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Background/Purpose: Emerging research findings suggest that long non-coding RNAs (lncRNAs) are key regulators to fibrosis formation. Nevertheless, the role of lncRNA GAS5-AS1 in the progression of precancerous oral submucous fibrosis (OSF) remains to be elucidated.
Methods: Quantitative real-time PCR were used to examine the expression of GAS5-AS1 in OSF tissues. The activities of myofibroblasts, including collagen contractility and cell migration, as well as the marker α-smooth muscle actin (SMA) were assessed following overexpression of GAS5-AS1. Also, we analyzed the expression of Smad activity in order to gain insight into the downstream regulator.
Results: The level of GAS5-AS1 was found significantly downregulated in the OSF tissues and fibrotic buccal mucosal fibroblasts (fBMFs). Ectopic expression of GAS5-AS1 significantly reduced the abilities of collagen gel contraction and migration in fBMFs or arecoline-treated BMFs. Moreover, we have shown that overexpression of GAS5-AS1 inhibited the expression of p-Smad and the marker of myofibroblasts.
Conclusion: We showed the reduced expression of GAS5-AS1 in OSF tissues and demonstrated its effect on the myofibroblast activities and the level of p-Smad and α-SMA, indicating its potential contribution in OSF pathogenesis.

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Introduction

Oral submucous fibrosis (OSF) is a chronic inflammatory and potentially malignant disorder[1] characterized by the gradually accumulated dense fibrous connective tissue, which results in marked rigidity and restricted mouth opening. It has been indicated that OSF is associated with areca quid chewing habit[2] and could progress to neoplasia,[1] particularly oral squamous cell carcinoma (OSCC), with a transformation rate about 7—13%.[3,4] And this may lead to increased mortality since oral cancer is the fifth leading cause of cancer death in Taiwan[5] and the 3-year overall survival rate of advanced OSCC patients was less than 30%.[6] Hence, there is an urgent need to better understand the etiological mechanisms of OSF in order to relieve patients' burden and prevent the malignant transformation.

Over the past few decades, non-coding RNAs (ncRNAs) have emerged as important regulators in diverse biological functions.[7] It is known now that around 98% of transcripts are non-protein coding RNAs and can be divided into small ncRNAs (such as microRNAs) or long non-coding RNAs (lncRNAs) based on their length of less or more than 200 nucleotides, respectively.[8] The functions of microRNAs have been relatively well-studied, whereas the roles of lncRNAs remain to be unraveled in detail. To date, a limited number of lncRNAs implicated in pathophysiology of fibrosis have been found, such as maternally expressed 3 (MEG3). It has been demonstrated that MEG3 regulated the transforming growth factor (TGF)-β pathway genes[9] and overexpression of MEG3 in TGF-β1-treated hepatic stellate cells significantly decreased the mRNA and protein levels of the myofibroblast marker, α-smooth muscle actin (SMA).[10]

LncRNA growth arrest-specific 5 (GAS5) is approximately 630 nt in length and was first identified in a search aimed to screen for novel tumor suppressors.[11] GAS5, as its name suggests, has been proven to be associated with the cell cycle progression and critical to normal growth arrest.[12] GAS5 was reported to be down-regulated in various cancers[13—15] and could repress liver fibrogenesis by competing with miR-222[16] or act as a negative modulator of profibrogenic miR-21.[17,18] Nonetheless, the expression and function of GAS5-AS1, the antisense RNA of GAS5, in OSF progression remains unknown. In the current study, we investigated the effect of GAS5-AS1 on myofibroblast activities and the expression of downstream Smad to elucidate its role in OSF pathogenesis.

Materials and methods

Tissue samples, cell culture and arecoline

All procedures of tissues acquirements have followed the tenets of the Declaration of Helsinki and were approved by the Institutional Review Committee at Chung Shan Medical University, Taichung, Taiwan. Primary normal buccal mucosal fibroblasts (BMFs) and human fibrotic buccal mucosal fibroblasts (fBMFs) from OSF tissue were established and cultivated as previously described.[19] Arecoline was purchased from Sigma (St Louis, MO, USA).

Quantitative real-time RT-PCR

Total RNA was prepared from cells or tissues using Trizol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). For detection of GAS5-AS1, qRT-PCR of mRNAs was reverse-transcribed using the Superscript III first-strand synthesis system (Invitrogen). qRT-PCR reactions on resulting cDNAs were performed on an ABI StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The expression of GAPDH was used for normalization. The threshold cycle (Ct) method was used to quantitate the relative changes of GAS5-AS1 expression. The primer sequences used in this study were listed as follows: GAS5-AS1: 5′-TGCCTTAAACCAGTTGTGCC-3′ and 5′-TTCATAGGCCCCTGTGCTAA-3′; and GAPDH: 5′-CTCATGACCACAGTCCATGC-3′ and 5′-TTCAGCTCTGGGAT-GACCTT-3′.

Overexpression of GAS-AS1

GAS-AS1 cDNA were cloned into pLV-EF1a-MCS-IRES-Puro (BioSettia, Cat. No: cDNA-pLV01; San Diego, CA, USA). Lentivirus production were performed by co-transfection of plasmid DNA mixture with lentivector plus helper plasmids (VSVG and Gag-Pol) into 293T cells (American Type Culture Collection, Manassas, VA, USA) using Lipofectamine 2000 (LF2000, Invitrogen, Carlsbad, CA, USA).

Lentiviral-mediated silencing Smad2

The pLV-RNAi vector is purchased from Biosettia Inc. (Biosettia, San Diego, CA, USA). The method of cloning the double-stranded shRNA sequence is described in the manufacturer's protocol. Oligonucleotide sequence of lentiviral vectors expressing shRNA that targets selected lncRNAs will be synthesized and cloned into Smad2 to generate a lentiviral expression vector. The target sequences for are: Sh-Smad2: 5′-AAAAGGACTGAGTACACCAAATATTGGATCCAA-TATTTGGTGTACTCAGTCC-3′.

Collagen gel contraction assay

BMFs or fBMFs (2×10^5) were suspended in 2 mg/ml collagen solution (Sigma—Aldrich, St. Louis, MO, USA) and added into 24-well plate followed by incubation at 37 °C for 2 h. After polymerization, the gels were further incubated within 0.5 ml minimum essential medium eagle-α medium with or without arecoline for 48 h. The collagen gel size change was quantified using ImageJ software (National Institutes of Health, Bethesda, MA, USA).

Cell migration assay

On the migration assay, cells were used to prevent proliferation in the presence of 2 mM hydroxyurea in both chambers to prevent cell proliferation. For transwell migration assays, 1×10^5 cells in a medium with lower serum were plated into the upper chamber of a transwell (Corning, Acton, MA, USA) with a porous transparent polyethylene terephthalate membrane (8.0 μm pore size) and medium supplemented with higher serum were used as a

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