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Short Communication

Inhibition of HIF1A-AS1 impedes the arecoline-induced migration activity of human oral mucosal fibroblasts

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Long non-coding RNA hypoxia-inducible factor 1α-antisense RNA 1 (HIF1A-AS1) has been known to participate in various types of malignancies, but its role in the development of precancerous oral submucous fibrosis (OSF) has not been investigated. In the current study, we first observed the aberrant upregulation of HIF1A-AS1 in OSF tissues and fibrotic buccal mucosal fibroblasts (fBMFs) isolated from OSF specimens. Next, we demonstrated that administration of arecoline, a natural alkaloid that is found in areca nut, induced the elevation of HIF1A-AS1 in BMFs. This finding showed that the habit of areca nut chewing may lead to an increase of HIF1A-AS1 in oral mucosa. Moreover, we found that knockdown of HIF1A-AS1 hindered the arecoline-stimulated migration capacity in BMFs, suggesting HIF1A-AS1 was critical to the transdifferentiation of BMFs into myofibroblasts. Altogether, our results demonstrated that overexpression of HIF1A-AS1 in OSF tissues may result from the use of areca nut and lead to activation of BMFs, which contribute to the progression of OSF.

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Introduction

Oral submucous fibrosis (OSF) is a premalignant disease of oral cavity featured with epithelial atrophy and accumulation of extracellular matrix (ECM) components in the lamina propria and submucosal layer. Like other fibrosis diseases, the key effector cells of OSF are activated fibroblasts called myofibroblasts with a highly contractile phenotype characterized by the presence of well-developed microfilament bundles[1] and α-smooth muscle actin (α-SMA).[2] Upon activation, these cells will migrate into the provisional matrix, proliferate, and produce more ECM components. Epidemiological evidence has indicated that the habit of areca nut chewing is the most important etiologic factor for OSF.[3] One constituent of areca nut, arecoline, has been employed to induce myofibroblast activation of buccal mucosal fibroblasts (BMFs).[4]

Over the past decades, various factors that regulate the activation of myofibroblasts have been studied. Among these factors, non-coding RNAs, including small non-coding RNAs (e.g. miR) and long non-coding RNAs (lncRNAs) with length of less or greater than 200 nucleotides,[5] have been reported to play crucial roles in the development of OSF. Non-coding RNAs, such as miR-1246,[6] LINC00974,[7] and miR-200c,[8] were found to increase the myofibroblast activities and contribute to the oral fibrogenesis. In the current study, we evaluated the expression of lncRNA hypoxia-inducible factor 1α-antisense RNA 1 (HIF1A-AS1) in OSF tissues and whether it affect the transdifferentiation of BMFs. A couple of studies have revealed that lncRNA HIF1A-AS1 was dysregulated in various cancers,[9,10] but whether it was also aberrantly expressed and implicated in the precancerous fibrosis condition was rarely mentioned. Therefore, we collected the OSF samples and examined the expression of lncRNA HIF1A-AS1 to determine its abnormal expression and effect on the myofibroblast activation.

Materials and methods

OSF tissue acquisition and chemicals

Arecoline (an alkaloid from areca nut) was purchased from Sigma–Aldrich (St. Louis, MO, USA). For tissue acquisition, all procedures have followed the tenets of the Declaration of Helsinki and were reviewed by Institutional Review Committee at Chung Shan Medical University, Taichung, Taiwan. Specimens of histologically normal or fibrotic mucosa from 20 normal subjects and OSF patients were collected in Department of Dentistry, Chung Shan Medical University Hospital. Normal BMFs and fibrotic BMFs (fBMFs) were cultivated as previously described. Cell cultures between the third and eighth passages were used in this study.[6]

Quantitative real-time PCR (qRT-PCR)

Trizol reagent was used for total RNA isolation and Superscript III first-strand synthesis system (Invitrogen Life Technologies, Carlsbad, CA, USA) was employed to reverse-transcribe the mRNAs according to the manufacturer's instruction. qRT-PCR reactions on resulting cDNAs were performed on an ABI StepOne™ Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). Fifty nanograms of cDNA sample were used in a SYBR Green-based qPCR reaction (Kapa Biosystems, Inc., Wilmington, MA, USA); the cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 sec and 60 °C for 1 min. The end-point used in the real-time quantification was calculated by the StepOne software, and the threshold cycle number (Ct value) for each analyzed sample was calculated.[6]

Silencing HIF1A-AS1 by lentiviral-mediated knockdown

The pLV-RNAi vector was purchased from Biosettia Inc. (Biosettia, San Diego, CA, USA). The method of cloning the double-stranded shRNA sequence was described in the manufacturer's protocol. Oligonucleotide sequence of lentiviral vectors expressing shRNA that targets HIF1A-AS1 was synthesized and cloned into pLVRNAi to generate a lentiviral expression vector. The target sequences for are: Sh-HIF1A-AS1-1: 5′- AAAAGGCGAAAAGGAGGAAAATTTTGGATC-CAAAATTTTCCTCCTTTTCGCC-3'; The target sequences for are: Sh-HIF1A-AS1-2: 5′- AAAAGGGAGAAGAAATGTTCCATT TGGATCCAAATGGAACATTTCTTCTCCC-3.

Migration assays

Cells were used to prevent proliferation in the presence of 2 mM hydroxyurea in both chambers to prevent cell proliferation.The Transwell system with a polycarbonate filter membrane of 8-μm pore size (Corning, United Kingdom) was utilized to assess the migration capacities. Serum-containing media (10%FBS) was used as the chemoattractant in the lower chamber. Cells on the other side of the membrane were stained with 0.1% crystal violet (Sigma–Aldrich) subsequent to fixation. These cells were counted from five different visual areas of 100-fold magnification under a microscope.[6,11]

Statistical analysis

Data were presented as mean ± SD. Statistical differences were evaluated by Student's t test and $p < 0.05$ will be considered significant.

Results

To determine the relative expression level of lncRNA HIF1A-AS1 in OSF specimens, we conducted RT-PCR to examine the gene expression of lncRNA HIF1A-AS1 in OSF tissues ($n = 20$) and fibrotic buccal mucosal fibroblasts (fBMFs) isolated from OSF specimens. Our results showed that lncRNA HIF1A-AS1 was overexpressed in both OSF tissues (Fig. 1A) and fBMFs (Fig. 1B) compared to normal tissue counterparts.

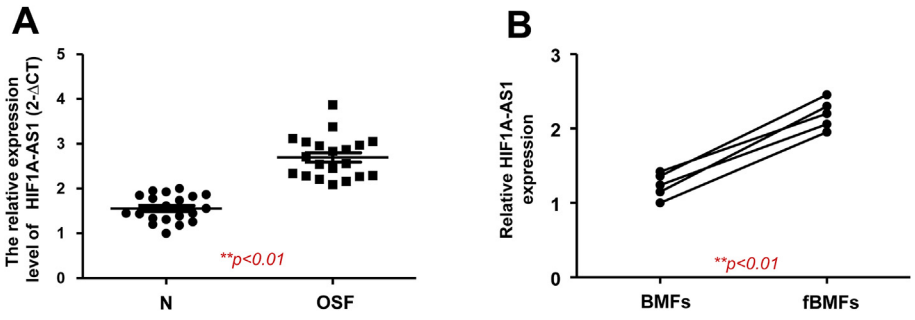

Figure 1 The expression of lncRNA HIF1A-AS1 is overexpressed in OSF. RT-PCR analysis of the expression level of lncRNA HIF1A-AS1 in OSF tissues (n = 20) (A) and fBMFs (n = 5) (B) isolated from OSF specimen. *$p < 0.01$ compared with their normal counterparts.

Subsequently, we examined whether this upregulation of lncRNA HIF1A-AS1 was due to the stimulation of the constituents of areca nut. Normal BMFs were treated with various concentration of arecoline, a major alkaloid in areca nut, and we observed that the expression of HIF1A-AS1 was significantly increased at concentrations of 10 μg/mL or more (Fig. 2A). We then evaluated the significance of lncRNA HIF1A-AS1 in arecoline treated BMFs by knockdown its expression and confirmed the knockdown efficiency using RT-PCR (Fig. 2B). As shown in Fig. 2C, arecoline incubation enhanced the migration ability of BMFs, while knockdown of lncRNA HIF1A-AS1 significantly abolished this

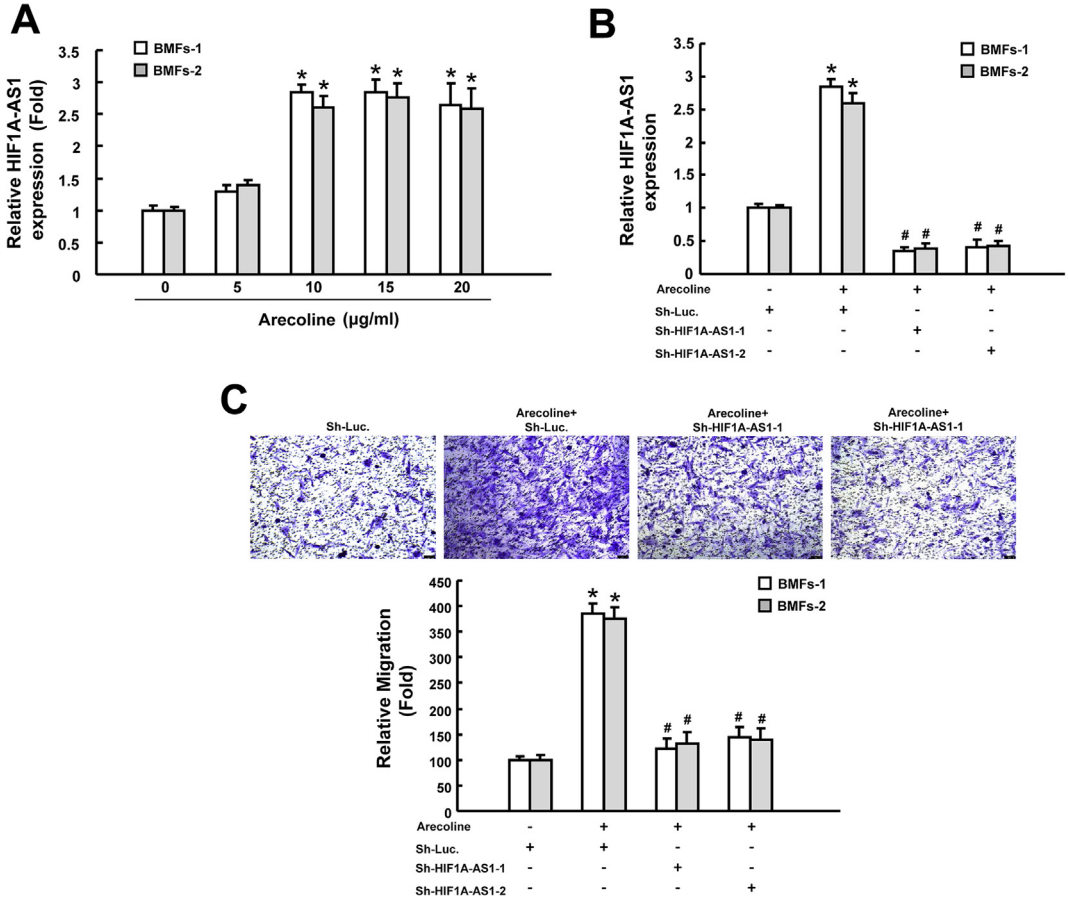

Figure 2 Inhibition of lncRNA HIF1A-AS1 hinders the arecoline-enhanced migration ability in BMFs. (A) The expression of lncRNA HIF1A-AS1 in BMFs treated with various concentration of arecoline; (B) Silencing efficiency of sh-HIF1A-AS1 following arecoline treatment was verified by qRT-PCR; (C) Migration ability was evaluated using transwell assay to examine the effect of sh-HIF1A-AS1 on arecoline (μg/ml)-induced myofibroblast activity. *$p < 0.05$ compared with no treatment control group. #$p < 0.05$ compared with arecoline only group.

elevation. Taken together, these findings suggested that the composition of areca nut, such as arecoline, may lead to an increase of lncRNA HIF1A-AS1 in OSF tissues, resulting in activation of myofibroblasts.

Discussion

Hypoxia-inducible factor 1-alpha (HIF-1α) is a master transcriptional factor in response to hypoxia and regulates various downstream target genes that contribute to fibrosis, such as NF-κB, TGF-β[12] or GLUT1.[13] Various studies have demonstrated that HIF-1α promoted inflammation, fibrosis,[12–15] and was necessary for collagen cross-linking in an *in vitro* model of fibrosis.[16] In OSF tissues, HIF-1α has been reported to be upregulated and considered as an important regulator for malignant transformation.[17] LncRNA HIF1A-AS1 is a natural antisense transcript derived from HIF-1α gene sequences encoding the 3′ untranslated region of HIF-1α mRNA, which was first found overexpressed in nonpapillary clear-cell renal carcinomas.[10] It has been revealed that HIF1A-AS1 may be a novel modulator of HIF-1α under oxidative stress conditions and for TGF-β3, as its expression was directly correlated with HIF-1α expression.[18] Oxidative stress and TGF-β signaling both have been considered to participate in the aetiopathogenesis of OSF.[19] As such, evaluation of the expression of HIF1A-AS1 in OSF tissues could give insight into whether HIF1A-AS1 was involved in the progression of malignant transformation of oral cavity.

In recent years, accumulating studies have revealed that dysregulation of lncRNA HIF1A-AS1 was functionally involved in several human disorders, including cancers and vascular diseases. For instance, lncRNA HIF1A-AS1 was reported to be implicated in the regulation of vascular smooth muscle cells (VSMCs) dysfunction and the pathogenesis of thoracoabdominal aortic aneurysms.[20,21] It also has been proven to affect numerous apoptosis proteins in aortic VSMCs.[21] Another study showed that HIF1A-AS1-siRNA lowered the myocardial fibrosis degree following myocardial ischemia/reperfusion injury in mice.[22] The serum level of lncRNA HIF1A-AS1 has been demonstrated to be associated with clinicopathological features and prognosis in colorectal cancer.[9] Nevertheless, its role in fibrogenesis or carcinogenesis has not been defined yet.

In the present study, we demonstrated that HIF1A-AS1 was upregulated in both precancerous OSF samples and the fBMFs. Our results showed that arecoline stimulation may be one of the reasons that we observed the elevation of HIF1A-AS1 in OSF. Besides, we demonstrated that inhibition of HIF1A-AS1 impeded the arecoline-induced myofibroblast activity, suggesting that targeting HIF1A-AS1 may be a potential treatment for OSF patients.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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