科技部補助專題研究計畫報告

探討長鏈非編碼核醣核酸於口腔黏膜下纖維化症之致病機轉及臨床意涵(第3年)

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執 行 單 位 ： 中山醫學大學牙醫學系（所）

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本研究具有政策應用參考價值：■否 □是，建議提供機關
（勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
本研究具影響公共利益之重大發現：□否 □是

中 華 民 國 109 年 10 月 20 日

中 文 摘 要 ： 近年來發現，長鏈非編碼核醣核酸與癌症及纖維化等疾病調節相關，但其在口腔黏膜纖維化症發展中的影響仍未知。本研究初步結果發現長鏈非編碼核醣核酸H19會高度表現於口腔黏膜纖維化症組織及纖維化頰黏膜母細胞， H19可競爭性結合miR-29b增進COL1A1表現。H19表現抑制過度表現miR-29b可降低肌纖維母細胞活性如纖維化頰黏膜母細胞之膠體收縮能力、細胞移動性、細胞侵襲性及傷口癒合能力，口腔黏膜纖維化症臨床檢體中miR-29b與纖維化標記呈現負相關性，進而證實檳榔鹼可透過TGF-β訊息路徑而提昇H19表現促進口腔黏膜纖維化症病程。

中 文 關 鍵 詞 ： 長鏈非編碼核醣核酸; H19; 口腔黏膜纖維化症

英 文 摘 要 ： Long non-coding RNAs (lncRNAs) recently emerged as an important regulator of fibrosis and tumorigenesis, however, its role in regulating oral submucous fibrosis (OSF) remains unknown. In this study, we investigated the functional role of lncRNA H19 in myofibroblastic differentiation activity and identified its potential target. Our results showed that the expression of H19 was up-regulated in OSF specimen and fibrotic buccal mucosal fibroblasts (fBMFs). The upregulated H19 contributed to the higher myofibroblast activities through direct targeting miR-29b, which interfered with the direct binding of miR-29b to the 3´- untranslated region of type I collagen (COL1A1). We showed that ectopic expression of miR-29b ameliorated various myofibroblast phenotypes and the expression of α-smooth muscle actin (α-SMA) and COL1A1 in fBMFs. The reduced expression of miR-29b in OSF tissues was consistent with the finding of a negative correlation between miR-29b and several fibrosis markers. Lastly, we demonstrated that arecoline stimulated the upregulation of H19 through the TGF-β pathway.

英 文 關 鍵 詞 ： long noncoding RNA; H19; oral submucous fibrosis

科技部補助專題研究計畫成果報告

（□期中進度報告/■期末報告）

探討長鏈非編碼核醣核酸於口腔黏膜下纖維化症之致病機轉及臨床意涵

計畫類別：■個別型計畫　□整合型計畫
計畫編號：MOST 106-2314-B-040-001-MY3
執行期間：106 年 8 月 1 日至 109 年 7 月 31 日

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計畫主持人：張育超 教授
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計畫參與人員：博士後研究人員廖翊妏

本計畫除繳交成果報告外，另含下列出國報告，共 ___ 份：
□執行國際合作與移地研究心得報告
□出席國際學術會議心得報告
□出國參訪及考察心得報告

本研究具有政策應用參考價值：■否　□是，建議提供機關________
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本研究具影響公共利益之重大發現：■否　□是

中　華　民　國 109 年 10 月 20 日

中文摘要

近年來發現，長鏈非編碼核醣核酸與癌症及纖維化等疾病調節相關，但其在口腔黏膜纖維化症發展中的影響仍未知。本研究初步結果發現長鏈非編碼核醣核酸H19會高度表現於口腔黏膜纖維化症組織及纖維化頰黏膜母細胞， H19可競爭性結合miR-29b增進COL1A1表現。H19表現抑制過度表現miR-29b可降低肌纖維母細胞活性如纖維化頰黏膜母細胞之膠體收縮能力、細胞移動性、細胞侵襲性及傷口癒合能力，口腔黏膜纖維化症臨床檢體中miR-29b與纖維化標記呈現負相關性，進而證實檳榔鹼可透過TGF-β訊息路徑而提昇H19表現促進口腔黏膜纖維化症病程。

關鍵詞: 長鏈非編碼核醣核酸; H19; 口腔黏膜纖維化症

Abstract

Long non-coding RNAs (lncRNAs) recently emerged as an important regulator of fibrosis and tumorigenesis, however, its role in regulating oral submucous fibrosis (OSF) remains unknown. In this study, we investigated the functional role of lncRNA H19 in myofibroblastic differentiation activity and identified its potential target. Our results showed that the expression of H19 was up-regulated in OSF specimen and fibrotic buccal mucosal fibroblasts (fBMFs). The upregulated H19 contributed to the higher myofibroblast activities through direct targeting miR-29b, which interfered with the direct binding of miR-29b to the 3´- untranslated region of type I collagen (COL1A1). We showed that ectopic expression of miR-29b ameliorated various myofibroblast phenotypes and the expression of α-smooth muscle actin (α-SMA) and COL1A1 in fBMFs. The reduced expression of miR-29b in OSF tissues was consistent with the finding of a negative correlation between miR-29b and several fibrosis markers. Lastly, we demonstrated that arecoline stimulated the upregulation of H19 through the TGF-β pathway.

Key words: long noncoding RNA; H19; oral submucous fibrosis

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（一）研究計畫之背景及目的

Oral submucous fibrosis (OSF) is a precancerous disease characterized by epithelial atrophy and progressive accumulation of collagen and other extracellular matrix components in the lamina propria and submucosal layer of the oral cavity. The common clinical symptoms include blanched mucosa and stiffness of the mouth, leading to restriction of mouth opening and tongue movement, limitation of food consumption, impaired speaking ability and difficulty in maintaining oral health. Epidemiological evidence has indicated that areca nut chewing is the most significant risk factor for OSF [1]. Treatment of buccal mucosal fibroblasts (BMFs) with arecoline, the major areca nut alkaloid, has been found to increase collagen synthesis and expression of extracellular matrix-associated genes, such as tissue inhibitor of metalloproteinase-1 (TIMP-1) [2], plasminogen activator inhibitor-1 (PAI-1) [3] and connective tissue growth factor (CTGF) [4]. Inflammation also plays a role in the progression of OSF. Arecoline has been shown to upregulate the expression of αvβ6 integrin in oral keratinocytes, leading to activation of TGF-β [5]. And it has been found that inflammatory cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-6) or inflammation-associated mediators (cyclooxygenase (COX)-2, prostaglandin E_2) secreted by oral keratinocytes may contribute to oral fibrogenic condition [6]. Our previous studies have shown that up-regulation of vimentin [7], COX-2 [6,8], TIMP-1 [2], PAI-1 [3,9], IL-6 [6,10], keratinocyte growth factor-1 [11], insulin-like growth factor (IGF)-1 [12], nuclear factor-kappa B (NF-κB) [13], cystatin C [14], heat shock protein 47 [15], heme oxygenase-1 [16], S100A4 [17], or SSEA-4 [18] are implicated in the deposition of extracellular components in OSF. Nevertheless, the detailed molecular mechanisms underlying the pathogenesis of OSF still require further exploration.

The key effector cells of fibrosis are activated fibroblasts called myofibroblasts, a highly contractile phenotype characterized by the presence of well-developed microfilament bundles [19] and α-smooth muscle actin (α-SMA) [20]. Myofibroblasts have been recognized as the major cell type to secret collagen and remodel the ECM during tissue repair and even organ fibrosis. Local tissue fibroblasts are originally considered as the predominant source of myofibroblasts, however, it is now known that myofibroblasts can be derived from multiple sources, such as epithelial cells in a process termed epithelial–mesenchymal transition (EMT) [21,22]. Several studies have found deregulation of myofibroblast activity in a number of organ fibroses, such as heart [23], liver [24], kidney [25] and OSF [26]. Our previous researches have demonstrated

that various EMT-associated molecules, including PAI-1 [3,9], IGF-1 [12], vimentin [7] and NF-κB [13] were upregulated in human BMFs following arecoline treatment. Altogether, these data suggest a possibility that myofibroblast transdifferentiated from EMT cells may directly involve in the pathogical changes of OSF. As a result, efficiently abrogating myofibroblast activation is considered as a potential therapeutic strategy for OSF. After sequencing the human genome, it has been indicated that only approximately 2% are protein-coding transcripts and the remaining 98% of the genome sequences belong to noncoding RNAs (ncRNAs) [27]. NcRNAs include small non-coding RNAs and long non-coding RNAs (lncRNAs) with the length of less or greater than 200 nucleotides, respectively [28]. Majority of lncRNAs are derived from intergenic regions, and sense or antisense to other transcripts. LncRNAs are found both in the nucleus and the cytoplasm, and often transcribed by RNA polymerase from either strand within a coding locus [29,30]. LncRNAs can function by targeting either genomically local (*cis*-regulation) or genomically distant (*trans*-regulation) genes. Additionally, most transcriptional enhancer elements are transcribed to produce unspliced transcripts termed 'enhancer RNAs' (eRNAs), which are correlated with regulating the neighbouring protein coding genes [31]. More recently dozens of researches have emerged implicating lncRNAs in numerous cellular processes ranging from cell differentiation, proliferation, migration and diseases such as cancer [32,33]. It is increasingly evident that lncRNAs have crucial roles in the development of fibrotic diseases by exhibiting both pro- or anti-fibrotic properties. LncRNA maternally expressed 3 (MEG3) has been shown to correlate with several human cancers [34,35] and possess inhibitory effects on hepatic stellate cells (HSCs) activation and liver fibrogenesis [36]. MEG3 has been found downregulated in liver fibrosis [36] and to work in concert with anti-fibrogenic miR-29 to attenuate TGF-β-induced fibrosis [37]. Another lncRNA-p21 acts as an antifibrotic factor in fibrosis as well. It has been demonstrated that the expression of lincRNA-p21 was reduced in liver fibrosis, and lincRNA-p21induced a significant reduction in α-SMA and type I collagen and repressed liver fibrogenesis via p21 [38] or PTEN [39]. In contrast, the expression of lincRNA PVT1 was found increased in activated HSCs and fibrotic tissues, while depletion of PVT1 attenuated collagen deposits in CCl_4-induced liver fibrosis [40].

Moreover, functions of some lncRNAs remain elusive. For instance, a number of studies have shown that the level of lncRNA H19 was significantly increased in TGF-β-induced cardiac and renal fibroses [41,42]. However, another conflicting report indicated H19 was significantly downregulated in HSCs and fibrosis

tissues following CCl_4-induced liver fibrosis [43]. H19 is a maternally imprinted gene and can function as microRNA precursor [44]. It has been shown that H19 involve in both proliferation and differentiation processes, and play a role in orchestrating the EMT-MET decision [45-47]. Previous studies have initiated a debate on whether H19 acts as a tumor suppressor [48] or as a tumor promoter [45]. Controversy also remains in its role of regulating fibrosis. Recent experimental study has shown H19 promoted bleomycin-induced EMT of alveolar epithelial cell through interacting with miR-29b [49]. H19 inversely regulated anti-fibrotic miR-29b expression via directly binding to the 3'UTR. It has been shown that miR-29b inhibited COL1A1 expression, resulting in the attenuated pulmonary fibrosis [49]. Several studies also demonstrated that H19 promotes fibroblast proliferation [42], and inhibition of H19 reduced fibrosis [41]. Nevertheless, an opposite finding was reported in a study showing down-expression of H19 in liver fibrosis [43]. They found that overexpression of H19 in HSCs repressed the level of IGF1R and inhibited the TGF-β1-induced proliferation of HSCs [43].

Results

In our results, real-time RT-PCR analysis demonstrates that the expression level of H19 was higher in OSF tissues but lower in normal buccal mucosa subjects **(Figure 1)**. An increase of H19 in primary cultivated fibroblasts from OSF tissues in comparison with pair BMFs subjects **(Figure 2)**. Furthermore, H19 knockdown significantly reduced myofibroblast activation including collagen contraction and migration capacities in fibotic buccal mucosal fibroblasts **(Figure 2)**. Mechanistically, we demonstrated that H19 might function as ceRNA (competing endogenous RNA) for miR-29b interaction **(Figure 3)**. Our results demonstrated that COL1A1 was a direct target of miR-29b **(Figure 4)**. miR-29b was downregulated in OSF specimen and fBMFs **(Figure 5)**. SB431542 treatment significantly prevented the arecoline- or TGF-ß1-induced H19 expression in BMFs **(Figure 6).** These results showed that the up-regulation of H19 after arecoline stimulation was via TGF-ß signaling and may be associated with the OSF development. These findings support the crucial role of H19/miR-29b/COL1A1 axis in the pathogenesis of OSF **(Figure 7)**.

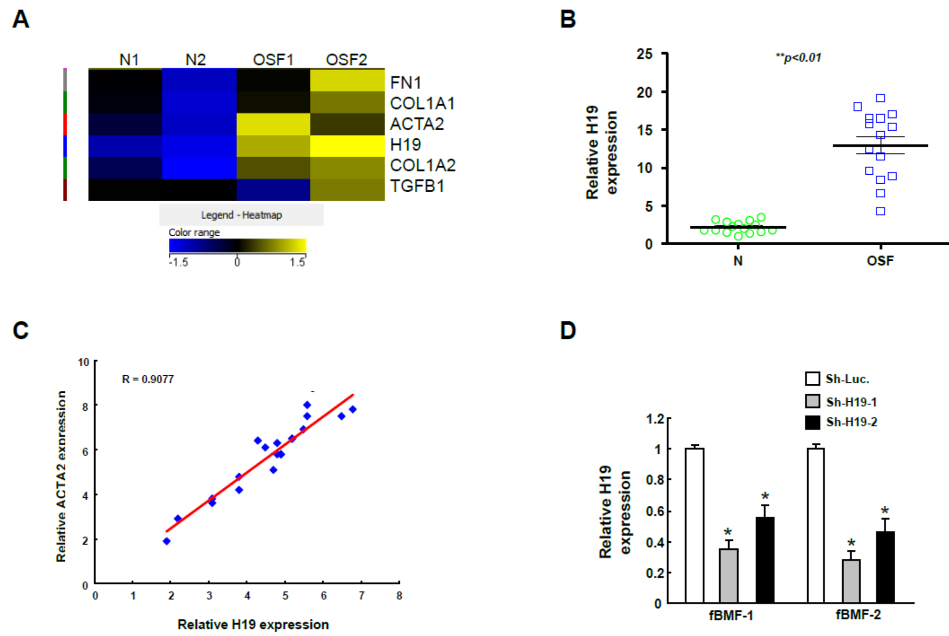

Figure 1. lncRNA H19 was significantly increased in OSF

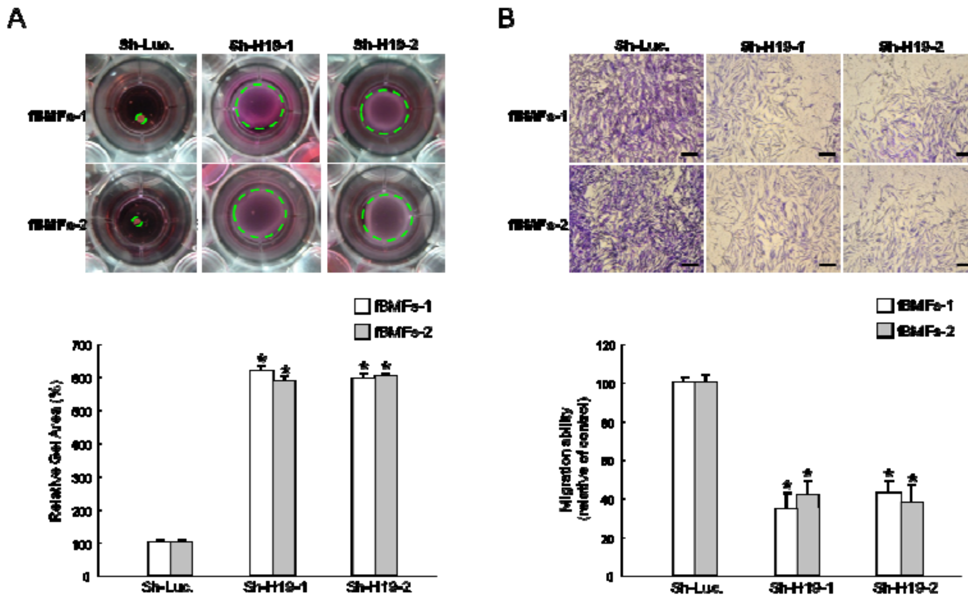

Figure 2. Depletion of H19 represses myofibroblastic differentiation activity in fBMFs

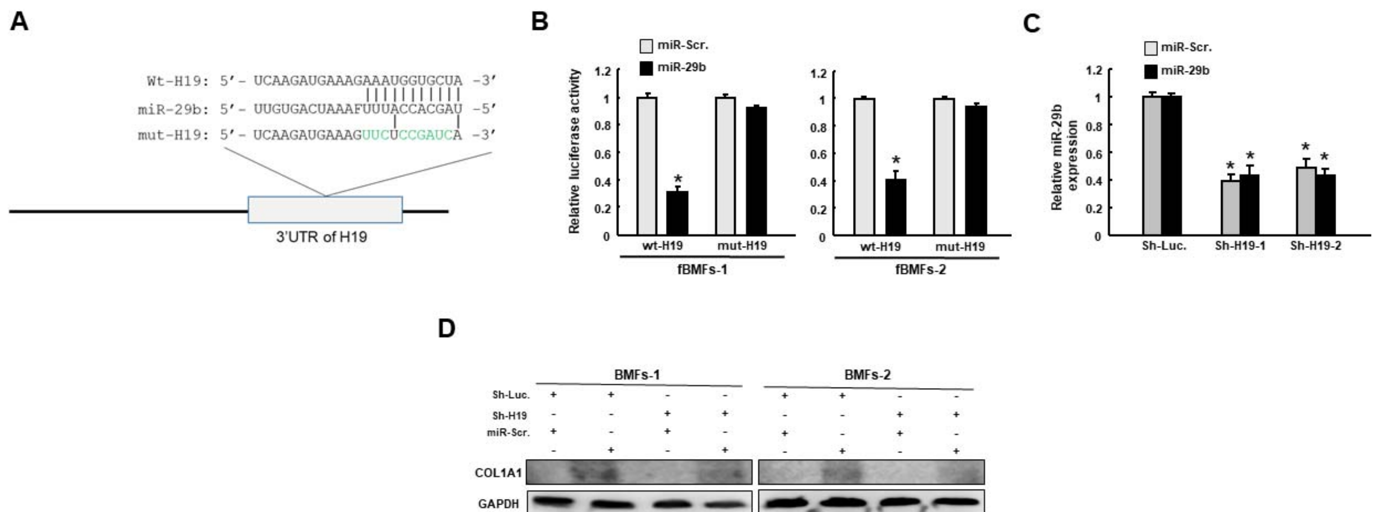

Figure 3. H19 functions as Competing endogenous RNA (ceRNA) in OSF through miR-29b interaction

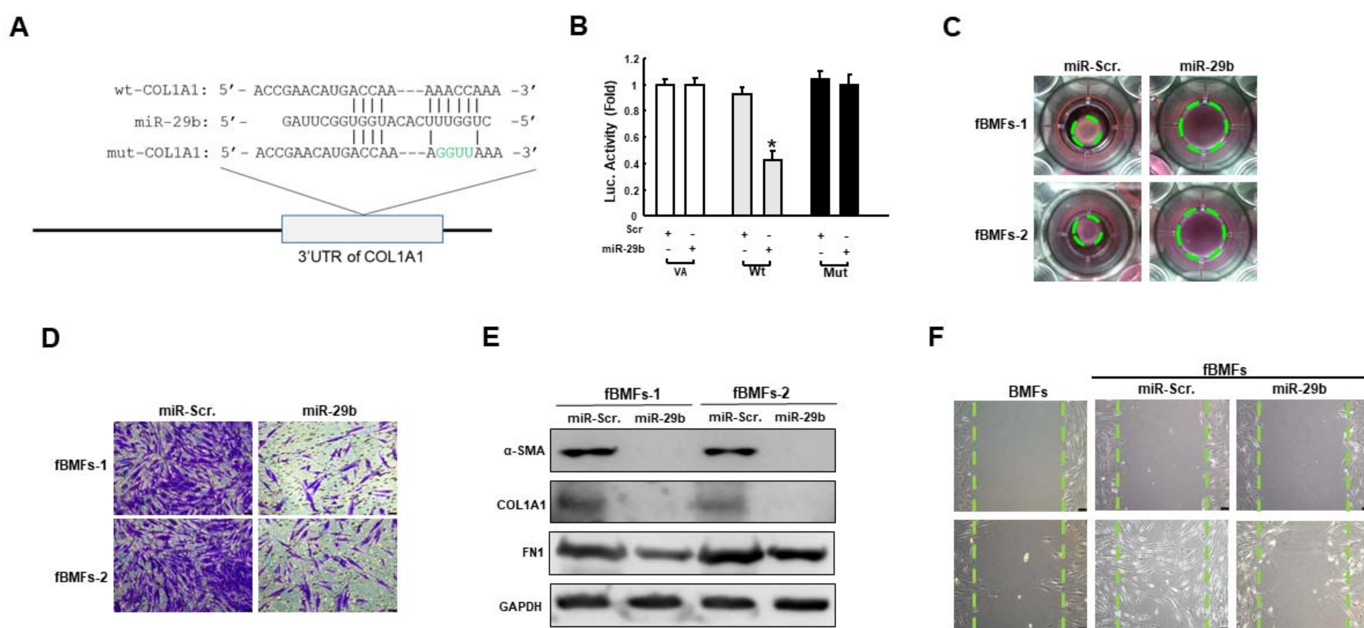

Figure 4. miR-29b directly targets COL1A1 and inhibits myofibroblasts activities in OSF

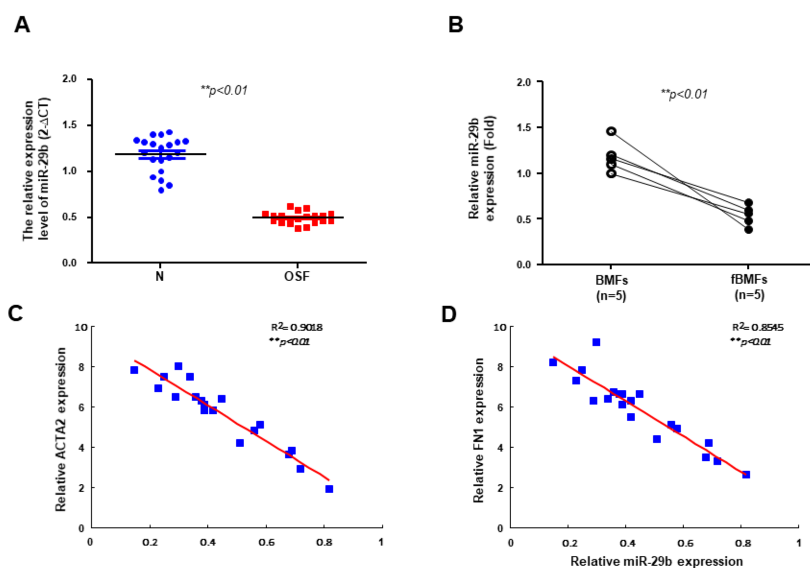

Figure 5. MiR-29b is downregulated in OSF tissues and fBMFs

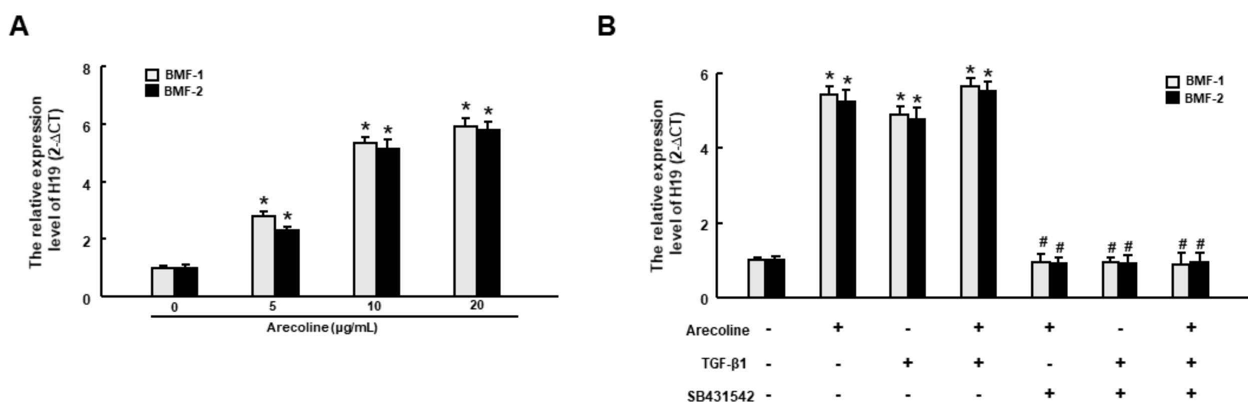

Figure 6. H19 is regulated by arecoline via TGF-ß signaling

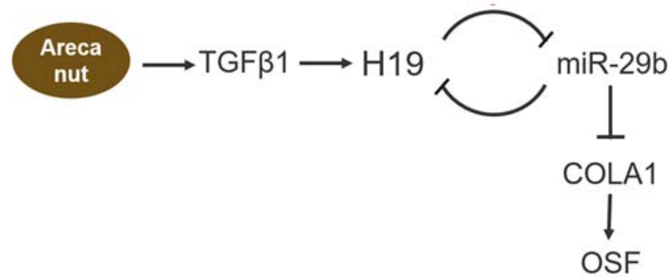

Figure 7. Graphical abstract of the present study

Materials and Methods

Oral mucosal tissues

Oral mucosal tissues will be obtained from 30 OSF patients with areca quid chewing habits during surgical biopsy and 10 health donors without areca quid chewing habits in Chung Shan Hospital. Clinical diagnosis will be confirmed by histopathological examination of the biopsy specimens. The samples will be fully encoded and examined under a protocol approved by the Institutional Review Board of Human Subjects Research Ethics Committee (Chung Shan Medical University, Taichung, Taiwan). RNA of normal or diseased oral mucosal tissues will be extracted by Trizol Reagent (Invitrogen) according the manufacturer's instructions.

Primary BMFs and fBMFs culture

All procedures of tissues acquirements have followed the tenets of the Declaration of Helsinki and are reviewed by Institutional Review Committee at Chung Shan Medical University. BMFs and fBMFs are cultivated as previously described. Cell cultures between the third and eighth passages were used in this study

Quantitative real-time PCR (qRT-PCR)

Total RNA is prepared from cells using Trizol reagent according to the manufacturer’s protocol (Invitrogen Life Technologies, Carlsbad, CA, USA). qRT–PCR of lncRNAs are reverse-transcribed using the Superscript III first-strand synthesis system for RT–PCR (Invitrogen Life Technologies, Carlsbad, CA, USA). qRT-PCR reactions on resulting cDNAs were performed on an ABI StepOne™ Real-Time PCR Systems (Applied Biosystems). The primer sequences of PR1 (H19) and internal control (GAPDH) listed below: H19: 5’-TGCTGCACTTTACAACCACTG-3’ and 5’- ATGGTGTCTTTGATGTTGGGC-3’;Gapdh: 5’-

CCGGGAAACTGTGGCGTGATGG-3' and 5'- AGGTGGAGGAGTGGGTGTCGCTGTT-3'

Lentiviral-mediated silencing H19

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 pLVRNAi to generate a lentiviral expression vector. The target sequences for are: Sh-H19-1: 5'-AAAAGCTTTCCTGTCTTTCCTTTATGGATCCATAAAGGAAAGACAGGAAAGC-3',Sh-H19-2:5'-A AAAGCTTTCCTGTCTTTCCTTTATGGATCCATAAAGGAAAGACAGGAAAGC -3.

Collagen contraction assay

Cells will be suspended in 0.5 ml of 2 mg/ml collagen solution (Sigma-Aldrich, St. Louis, MO, USA) and added into one well of 24-well-plate. Plate will be incubated at 37°C for 2 hours which caused polymerization of collagen cell gels. After detaching gels from wells, the gels will be further incubated in 0.5 ml medium for 48 h. Contraction of the gels will be photographed and measured using ImageJ software (NIH, Bethesda, MD, USA) to calculate their areas.

Transwell migration assays

1 x 10^5 cells will be plated into the top chamber of a transwell (Corning, Acton, MA) with a porous transparent polyethylene terephthalate membrane (8.0 μm pore size) with lower serum (0.5% FBS), and medium supplemented with higher serum will be used as a chemoattractant in the lower chamber. After 24 h incubation, cells that do not migrate through the pores will be removed by a cotton swab. Cells on the lower surface of the membrane will be stained with crystal violet. The number of migration cells in a total of five randomly selected fields will be counted.

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106年度專題研究計畫成果彙整表

計畫主持人：張育超	計畫編號：106-2314-B-040-001-MY3
計畫名稱：探討長鏈非編碼核醣核酸於口腔黏膜下纖維化症之致病機轉及臨床意涵	

成果項目			量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文	0	篇	
		研討會論文	0		
		專書	0	本	
		專書論文	0	章	
		技術報告	0	篇	
		其他	0	篇	
國外	學術性論文	期刊論文	0	篇	manuscript submission
		研討會論文	1		4th Meeting of the International Association for Dental Research Asia Pacific Region 2019 11/28-30
		專書	0	本	
		專書論文	0	章	
		技術報告	0	篇	
		其他	0	篇	
參與計畫人力	本國籍	大專生	1	人次	溫姿儀 107/08/15-108/07/31 108/09/17-109/07/31 收集文獻
		碩士生	1		胡潔仁 106/08/11-107/07/31實驗技術收集文獻
		博士生	2		游惠捷 106/08/11-107/07/31實驗技術、實驗數據分析 曾千芳 107/08/28-108/07/31 108/08/01-109/07/31 實驗技術、實驗數據分析、收集文獻
		博士級研究人員	1		廖翊妏博士後研究人員 實驗規劃與執行、實驗技術指導、實驗數據分析、收集文獻、撰寫論文報告及實驗室管理
		專任人員	2		張敦程 107/01/12-107/07/31 實驗數據分析 蘇淳絹 108/01/01-108/07/31 實驗執行
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士級研究人員	0		
		專任人員	0		
其他成果					

（無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）	