Journal of the Formosan Medical Association (2018) xx, 1–7

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.jfma-online.com

Original Article

miR-200c inhibits the arecoline-associated myofibroblastic transdifferentiation in buccal mucosal fibroblasts

Ming-Yi Lu [a,b,e], Cheng-Chia Yu [a,b,c,e], Pei-Yin Chen [a,b], Pei-Ling Hsieh [c], Chih-Yu Peng [a,b], Yi-Wen Liao [a], Chuan-Hang Yu [a,b,*], Kuo-Hua Lin [d,]**

[a] *School of Dentistry, Chung Shan Medical University, Taichung, Taiwan*
[b] *Department of Dentistry, Chung Shan Medical University Hospital, Taichung, Taiwan*
[c] *Institute of Oral Sciences, Chung Shan Medical University, Taichung, Taiwan*
[d] *Department of General Surgery, Changhua Christian Hospital, Changhua, Taiwan*

Received 29 April 2018; received in revised form 11 May 2018; accepted 24 May 2018

KEYWORDS
Oral submucous fibrosis;
Arecoline;
miR-200c;
ZEB1;
Myofibroblasts

Background/Purpose: MicroRNA-200c (miR-200c) recently emerged as an important regulator of tumorigenesis and cancer metastasis, however, its role in regulating oral submucous fibrosis (OSF) remains unknown. In this study, we investigated the functional role of miR-200c in myofibroblastic differentiation activity and identified its potential target.
Methods: qRT-PCR was applied to assess the expression of miR-200c in OSF tissues and fibrotic buccal mucosal fibroblasts (fBMFs). Arecoline, a major areca nut alkaloid, was utilized to explore whether the expression of miR-200c would alter following stimulation. Collagen gel contraction, migration and invasion capabilities were examined in arecoline-stimulated BMFs as wells as in fBMFs. Luciferase reporter assay was conducted to show the relationship between miR-200c and ZEB1.
Results: Our results showed that the expression of miR-200c was downregulated in OSF specimen and fBMFs. Arecoline treatment dose-dependently reduced the relative expression of miR-200c in normal BMFs. Overexpression of miR-200c impeded the arecoline-induced collagen gel contraction, migration, invasion and wound healing capacities. Moreover, ectopic expression of miR-200c in fBMFs successfully reduced the increased collagen gel contractility and invasion abilities. Our results demonstrated that ZEB1 was a direct target of miR-200c, and overexpression of miR-200c inhibited the expression of ZEB1 and α-SMA.

* Corresponding author. School of Dentistry, Chung Shan Medical University, No.110, Sec.1, Jianguo N. Rd., Taichung 40201, Taiwan. Fax: +886 4 24759065.
** Corresponding author. Fax: +886 4 7232942.
E-mail addresses: tao2008@csmu.edu.tw (C.-H. Yu), 120380@cch.org.tw (K.-H. Lin).
[e] Contributed equally to the results of this study.

https://doi.org/10.1016/j.jfma.2018.05.016

Conclusion: These findings suggest that downregulation of miR-200c in OSF may be involved in the pathogenesis of areca nut-associated OSF through regulation of ZEB1.

Introduction

Oral submucous fibrosis (OSF) is a chronic scarring disease and also an oral potentially malignant disorder.[1] This precancerous condition of the oral mucosa is associated with inflammation, epithelial atrophy, and accumulation of connective tissue,[2] leading to limited mouth opening. Although it has been known that areca nut chewing habit is a major etiological factor for OSF,[3,4] the detailed mechanism underlying the areca nut-associated OSF has not been completely unraveled.

Alpha-smooth muscle actin (α-SMA)-expressed myofibroblast is the principal cell type responsible for fibrogenesis.[5] The contractile and secretory myofibroblast plays a critical role in wound healing and tissue remodeling, but its persistent existence often results in excessive contraction and extracellular matrix protein secretion.[5] As in other fibrosis diseases, the presence of the myofibroblast was observed in OSF.[6] Among various origins, cells that undergo epithelial–mesenchymal transition (EMT) have been considered as one of the sources of myofibroblasts.[5] Our previous work has demonstrated that the areca nut induced the formation of α-SMA-positive stress fibers in buccal mucosal fibroblasts (BMFs) expressing nuclear zinc finger E-box binding homeobox 1 (ZEB1),[6] which is a transcription repressor to EMT and negatively regulate the expression of E-cadherin. After elucidating that ZEB1 binds to the E-box region in the α-SMA promoter to activate the activity of myofibroblasts,[6] we sought to further investigate the upstream factors that regulate ZEB1 in order to better understand the pathogenesis of OSF.

Emerging evidence has indicated that non-coding RNAs are the keys to the initiation and progression of fibrosis diseases. One of our recent work has shown that long non-coding RNAs (lncRNAs) GAS5-AS1 inhibits myofibroblasts activities of OSF.[7] In addition to lncRNAs, microRNAs (miRNAs) are another common non-coding RNAs of 21–25 nucleotides that mediate the post-transcriptional regulation of gene expression via binding to the 3'untranslated region (UTR) in target transcripts.[8] Indeed, it was reported that various miRNAs were differentially expressed in OSF tissue.[9] Moreover, several studies have shown that the interaction between miR-200 family and ZEB1 was critical in the regulation of EMT and invasion in cancer cells.[10–12] Therefore, it is imperative to examine whether miR-200 family is associated with the arecoline-induced ZEB1 upregulation in precancerous OSF.

In this study, we aimed to evaluate the critical role of miR-200c in the arecoline-induced transdifferentiation of BMFs and in the myofibroblast activities. We also examined the relationship between miR-200c and ZEB1 in order to gain a better insight into the pathogenesis of the arecoline-associated OSF.

Materials and methods

Reagents and cell culture

Arecoline and collagen solution from bovine skin were purchased from Sigma-Aldrich (St. Louis, MO, USA). OSF patients with areca nut chewing habit, attending the Oral Medicine Center (Chung Shan Medical University Hospital, Taichung, Taiwan), were enrolled with informed consents for this study. All procedures were carried out in accordance with the approved guidelines from the Institutional Review Board of Chung Shan Medical University Hospital. Cell cultures were grown and maintained as previously described.[13] Cells between the third and eighth passages were used in this study.

Quantitative real-time PCR (qRT-PCR)

Total RNA of tissues or cells was isolated using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA)[14] and reverse-transcribed to cDNA by Superscript III first-strand synthesis system (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. SYBR Green-based qRT-PCR reactions on resulting cDNAs were performed on an ABI StepOne™ Real-Time PCR Systems (Applied Biosystems).

Collagen gel contraction assay

2×10^5 BMFs were suspended in 2 mg/ml collagen solution (Sigma-Aldrich) and added into 24-well-plate. The plate was incubated at 37 °C for 2 h which caused polymerization of collagen cell gels. After dissociating gels from wells, the gels were further incubated in 0.5 ml MEMα medium with arecoline for 48 h. The area of collagen gel (contraction index) was recorded by a digital camera at a fixed distance above the gels and quantified by ImageJ software.

Migration and invasion assays

The 24-well Transwell system with a polycarbonate filter membrane of 8-μm pore size (Corning, United Kingdom) was utilized to evaluate the migration and invasion capacities. For invasion assay, the filter membrane was coated with Matrigel (BD Pharmingen, NJ, USA). In the upper chamber, cells were seeded at a cell density of 1×10^5 in serum-free medium followed by 24 h of incubation. Cells migrating to the lower chamber with serum-containing media were stained with crystal violet (Sigma-Aldrich) subsequent to the filter membrane fixation. These cells were counted

Please cite this article in press as: Lu M-Y, et al., miR-200c inhibits the arecoline-associated myofibroblastic transdifferentiation in buccal mucosal fibroblasts, Journal of the Formosan Medical Association (2018), https://doi.org/10.1016/j.jfma.2018.05.016