行政院國家科學委員會專題研究計畫 成果報告

S100A4 調控傳導路徑對於口腔黏膜下纖維化致病機轉之研究
研究成果報告(精簡版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 100-2314-B-040-008-
執 行 期 間 ：100 年 08 月 01 日至 101 年 07 月 31 日
執 行 單 位 ：中山醫學大學醫學研究所

計 畫 主 持 人：蔡崇弘
共 同 主 持 人：張育超、余承佳

公 開 資 訊 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 101 年 10 月 24 日

中 文 摘 要 ： 口腔黏膜纖維化(oral submucous fibrosis)為一種口腔癌發生之癌前病變，主要與檳榔嚼食習慣相關，然而口腔黏膜纖維化症之致病分子機轉尚未清楚。S100A4 鈣結合蛋白在黏膜纖維化中扮演著非常重要的角色，鮮少有研究探討 S100A4 與口腔黏膜纖維化間的關聯性。實驗首次發現臨床口腔黏膜下纖維化母組織 S100A4 會過度表現。正常頰黏膜纖維母細胞在處理不同 arecoline 處理下會增強 S100A4 的表現，加入 mTOR， ERK，或 NF-KB 抑制劑會抑制 arecoline 所誘發之 S100A4 表現。利用 shRNAi 抑制 S100A4 會降低 arecoline 促進之膠原蛋白收縮力。本研究成果能希望利用 S100A4 傳遞路徑當作口腔黏膜下纖維化治療標靶。

中文關鍵詞： 口腔黏膜纖維化, S100A4

英 文 摘 要 ： S100A4, a member of calcium-binding proteins (CBPs), is dramatically elevated involved in a variety of fibrotic diseases. Areca nut chewing is the most important etiological factor in the pathogenesis of oral submucous fibrosis (OSF). OSF has been considered as pre-cancerous condition of oral mucosa. The aim of this study was to determine the critical role of S100A4 expression in the pathogenesis of OSF in vitro and in vivo. Initially, S100A4 expression was higher expression in areca quid chewing-associated OSF specimens than that of normal buccal mucosa fibroblasts (BMF) specimens ($p=0.001$). Arecoline, a major areca nut alkaloid, led to dose- and time-dependent elevation S100A4 expression in BMF cells ($p<0.05$). The additions of pharmacological agents including rapamycin (mTOR inhibitor), PD98059 (ERK inhibitor), and Bay117082 (NF-KB inhibitor) were found to inhibit the arecoline-induced S100A4 expression ($p < 0.05$) in BMF cells. These results suggest that S100A4 expression is significantly up-regulated in OSF specimens. Arecoline-induced S100A4 expression was down-regulated by Rapamycin, PD98059, and Bay117082 treatment. Targeting S100A4 might be a potential therapeutic target for OSF.

英文關鍵詞： Oral submucous fibrosis, S100A4

Introduction

Oral submucous fibrosis (OSF) is a chronic progressive scarring disease which characterized by the submucosal accumulation of dense fibrous connective tissue with inflammatory cell infiltration and epithelial atrophy and has been considered as pre-cancerous condition of oral mucosa [1]. The fibro-elastic changes are almost entirely due to abnormal accumulation of collagen in the subepithelial layers, resulting in dense fibrous bands in the mouth [2]. A number of epidemiological evidence, case-series reports, large sized cross sectional surveys, case-control studies, cohort and intervention studies provide over whelming evidence that areca nut is the main etiological factor for OSF [3].

Arecoline, a major areca nut alkaloid, was found to stimulate human (buccal mucosa fibroblasts) BMF proliferation and collagen synthesis *in vitro* [4]. Up-regulation of vimentin [5], cyclooxygenase-2 [6], tissue inhibitor metalloproteinase-1 (TIMP1)[7], plasminogen activator inhibitor-1[8], interleukin-6 [9], keratinocyte growth factor-1[10], insulin-like growth factor-1, nuclear factor-kappa B [11], cystatin C [12], and heme oxygenase-1[13], may contribute to the extracellular components accumulation in OSF, but the pathologic mechanism(s) of OSF needs to be further clarified.

S100A4, a member belongs to S100 super family of calcium-binding proteins (CPB), is associated with the onset and progression of fibrosis in many human tissues, such as liver fibrosis, kidney fibrosis, pulmonary fibrosisc, and cardiac fibrosis [6, 14-16]. S100A4 is consistently and dramatically upregulated expression in carbon tetrachloride (CCl4)-induced hepatic fibrosis and functions as a marker of primary biliary cirrhosis [14]. Up-regulation of S100A4-positive cells is associated with kidney fibrosis [15]. TGF-β, an inducer of endothelial–mesenchymal transition, induced S100A4 expression in cardiac fibrosis mouse model [6]. Nevertheless, the S100A4 mediated molecular mechanisms in regulating OSF pathogenesis is still unclear.

More specifically, we have therefore measured the relative levels of S100A4 in OSF compared with normal buccal mucosa and the effects of arecoline, a major areca nut alkaloid, on S100A4 in normal human buccal mucosa fibroblasts (BMFs) in vitro. Ultimately, we demonstrated the significance of S100A4-mediated signaling on OSF process.

Purpose

The aim of this study was to determine the critical role of S100A4 expression in the pathogenesis of OSF in vitro and in vivo

Materials and Methods

OSF patient subjects and immunohistochemistry

This research follows the tenets of the Declaration of Helsinki and all samples were obtained after informed consent from the patients. OSF patients' tissue samples with different stages of oral cancer were spotted on glass slides for immunohistochemical stainings. After deparaffinization and rehydration, the tissue sections were processed with antigen retrieval by1X Trilogy diluted in H2O (Biogenics) and heat. The slides were immersed in 3% H2O2 for 10 minutes and washed with PBS 3 times. The tissue sections were then blocked

with serum (Vestastain Elite ABC kit, Vector Laboratories, Burlingame, CA) for 30 minutes, and followed by incubating with the primary antibody and anti-S100A4 (code no. A5114; Dako) in PBS solution at room temperature for 2 hours in a container. Tissue slides were washed with PBS and incubated with biotin-labeled secondary antibody for 30 minutes and then incubated with streptavidin-horse radish peroxidase conjugates for 30 minutes and washed with PBS 3 times. Afterwards, the tissue sections were immersed with chromogen 3-3'-diaminobenzidine plus H2O2 substrate solution (Vector® DBA/Ni substrate kit, SK-4100, Vector Laboratories, Burlingame, CA) for 10 minutes. Hematoxylin was applied for counter-staining (Sigma Chemical Co., USA). Finally, the tumor sections were mounted with a cover slide with Gurr® (BDH Laboratory Supplies, U.K.) and examined under a microscope. Pathologists scoring the immunohistochemistry were blinded to the clinical data. The interpretation was done in five high-power views for each slide, and 100 cells per view were counted for analysis.

Reagents

Arecoline was purchased from Sigma (St Louis, MO, USA). Rapamycin, PD98059, or Bay117082 were obtained from Merck (Merck Biosciences, Darmstadt, Germany)**.** All pharmacologic agents were first dissolved in dimethyl sulfoxide and then diluted with the culture medium. The final concentration of solvent in the medium did not exceed 0.25% (v⁄v). At these concentrations the solvents used were not cytotoxic to BMFs. The final concentrations of Rapamycin, PD98059, or Bay117082 used in this study were 100 nM, 10 μM, and 1 μM, respectively.

Cell cultivation of BMFs

BMFs were cultured by using an explant technique as described previously. Two healthy individuals were selected from the crown lengthening procedure for this study. The normal buccal mucosa tissue samples were minced using sterile techniques and washed twice in phosphate buffer saline (PBS) supplemented with antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml of fungizone). Explants were placed into 60 mm Petri dishes and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10 % fetal calf serum (FCS) (Gibco Laboratories, Grand Island, NY, USA) and antibiotics as described above. Cell cultures between the third and eighth passages were used in this study.

Quantitative real-time reverse-transcriptase (RT)-PCR

Total RNA was prepared from cells or tissues using Trizol reagent according to the manufacturer's protocol (Invitrogen). qRT–PCRs 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 Systems (Applied Biosystems). Primer sequences are listed in **Table 1**.

Effect of arecoline on S100A4 expression in BMFs by Western blot

Cells arrested in G_0 by serum deprivation (0.5 % fetal calf serum; 48 h) were used in the experiments. Nearly confluent monolayers of BMFs were washed with serum-free Dulbecco's modified Eagle's medium and immediately thereafter exposed to various concentrations (0, 5, 15, and 20 μg/mL) of arecoline (Sigma, St Louis, MO, USA) after 24 h incubation period. Cells were solubilized with sodium dodecyl

sulfate-solubilization buffer (5 mM EDTA, 1 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.5 and 0.5 % Trition X-100, 2 mM phenylmethylsulfonyl fluoride, and 1 mM N-ethylmaleimide) for 30 min on ice. Then, cell lysates were centrifuged at 12,000 g at 4 °C and the protein concentrations determined with Bradford reagent using bovine serum albumin as standards. Equivalent amounts of total protein per sample of cell extracts were run on a 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immediately transferred to nitrocellulose membranes. The membranes were blocked with phosphate-buffered saline containing 3 % bovine serum albumin for 2 h, rinsed, and then incubated with primary antibodies anti-S100A4 (1:500) in phosphate-buffered saline containing 0.05 % Tween 20 for 2 h. After three washes with Tween 20 for 10 min, the membranes were incubated for 1 h with biotinylated secondary antibody diluted 1:1000 in the same buffer, washed again as described above and treated with 1:1000 streptavidin-peroxidase solution for 30 min. After a series of washing steps, protein expression was detected by chemiluminescence using an ECL detection kit (Amersham Biosciences UK Limited, England), and relative photographic density was quantitated by scanning the photographic negatives on a gel documentation and analysis system (AlphaImager 2000, Alpha Innotech Corp., San Leandro, CA). Each densitometric value was expressed as the mean ± standard deviation (SD).

S100A4 knockdown in arecoline-treated BMF cells by Lentiviral-mediated shRNAi

The pLV-RNAi vector was purchased from Biosettia Inc. (Biosettia, San Diego, CA). The method of cloning the double-stranded shRNA sequence is described in the manufacturer's protocol. Lentiviral vectors expressing short hairpin RNA (shRNA) that targets human S100A4 (oligonucleotide sequence: Sh-S100A4-1:5'-AAAAGGTGTCCACCTTCCACAAGTATTGGATCCAATACTTGTGGAAGGTGGACAC C-3';Sh-S100A4-2:5'-AAAAGAAGCTGATGAGCAACTTGGATTGGATCCAATCCAAGTTGCTCATCAG CTTC-3') were synthesized and cloned into pLVRNAi to generate a lentiviral expression vector. Lentivirus production was performed by transfection of plasmid DNA mixture with lentivector plus helper plasmids (VSVG and Gag-Pol) into 293T cells using Lipofectamine 2000 (Invitrogen, Calsbad, CA). Supernatants were collected 48 hours after transfection and then were filtered; the viral titers were then determined by FACS at 48 hours post-transduction. Subconfluent cells were infected with lentivirus in the presence of 8 μg/ml polybrene (Sigma-Aldrich). The red fluorescence protein (RFP), which was co-expressed in lentiviral-infected cells, was served as a selection marker to indicate the successfully infected cells.

Collagen gel contraction assays

The bioactivity of myofibroblast function will be performed by collagen contraction assay kit (Cell BioLabs, Inc., San Diego, CA, USA). Cells will be prepared as 2×10^5 cells /ml and mixed with cold collagen solution at ratio of 1:4. Cell/collagen mixture will be loaded into wells of 24-well-plate at 0.5ml/well and cover with 1ml of cell culture medium after polymerization of collagen. To initiate contraction, collagen gels will be gently released from the sides of the culture dishes with a sterile spatula. The collagen gel size change (contraction index) will be pictured at various times and quantified by IamgeJ software.

Statistical analysis

Statistical Package of Social Sciences software (version 13.0) (SPSS, Inc., Chicago, IL) was used for statistical analysis. Student's t test was used to determine statistical significance of the differences between

experimental groups; p values less than 0.05 were considered statistically significant. The level of statistical significance was set at 0.05 for all tests.

Results

Figure 1. S100A4 is significant up-regulated of in OSF specimens

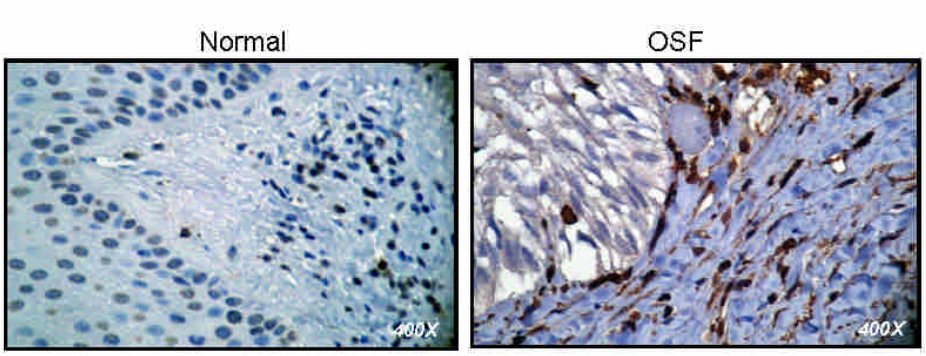


Figure 2. Arecoline dose-dependently and time-dependently increased S100A4 in BMF cells

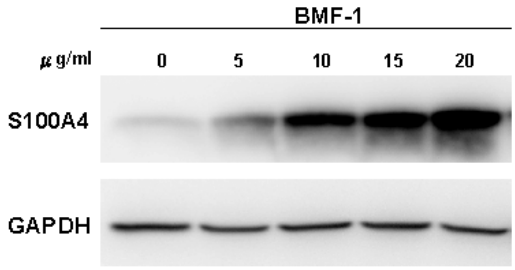


Figure 3. Arecoline time-dependently increased S100A4 in BMF cells

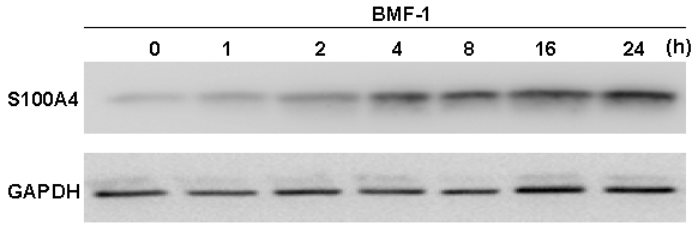


Figure 3. NF-KB, ERK, or mTOR signaling pathway is involved in arecoline-induced S100A4 up-regulation

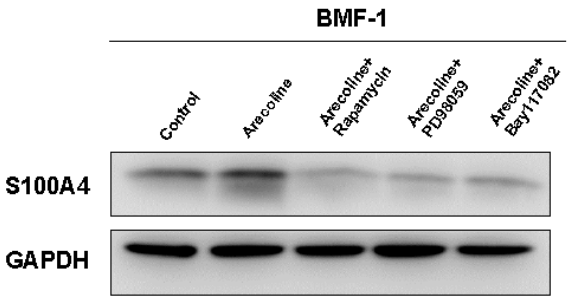
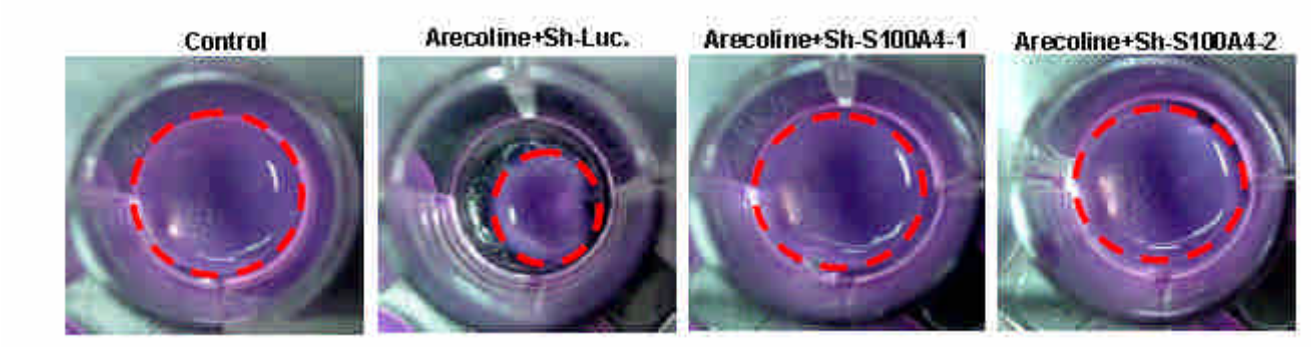


Figure 4. Knockdown of S100A4 repressed arecoline-induced collagen gel contraction



References:

1. Chiu CJ, Chiang CP, Chang ML, Chen HM, Hahn LJ, Hsieh LL, Kuo YS, Chen CJ (2001) Association between genetic polymorphism of tumor necrosis factor-alpha and risk of oral submucous fibrosis, a pre-cancerous condition of oral cancer. J Dent Res 80:2055-2059
2. More CB, Das S, Patel H, Adalja C, Kamatchi V, Venkatesh R (2012) Proposed clinical classification for oral submucous fibrosis. Oral Oncol 48:200-202
3. Chole RH, Gondivkar SM, Gadbail AR, Balsaraf S, Chaudhary S, Dhore SV, Ghonmode S, Balwani S, Mankar M, Tiwari M, Parikh RV (2012) Review of drug treatment of oral submucous fibrosis. Oral Oncol 48:393-398
4. Kuo MY, Chen HM, Hahn LJ, Hsieh CC, Chiang CP (1995) Collagen biosynthesis in human oral submucous fibrosis fibroblast cultures. J Dent Res 74:1783-1788
5. Chang YC, Tsai CH, Tai KW, Yang SH, Chou MY, Lii CK (2002) Elevated vimentin expression in buccal mucosal fibroblasts by arecoline in vitro as a possible pathogenesis for oral submucous fibrosis. Oral Oncol 38:425-430
6. Zeisberg EM, Tarnavski O, Zeisberg M, Dorfman AL, McMullen JR, Gustafsson E, Chandraker A, Yuan X, Pu WT, Roberts AB, Neilson EG, Sayegh MH, Izumo S, Kalluri R (2007) Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nat Med 13:952-961
7. Chang YC, Yang SF, Tai KW, Chou MY, Hsieh YS (2002) Increased tissue inhibitor of metalloproteinase-1 expression and inhibition of gelatinase A activity in buccal mucosal fibroblasts by arecoline as possible mechanisms for oral submucous fibrosis. Oral Oncol 38:195-200
8. Yang SF, Hsieh YS, Tsai CH, Chou MY, Chang YC (2003) The upregulation of type I plasminogen activator inhibitor in oral submucous fibrosis. Oral Oncol 39:367-372
9. Tsai CH, Yang SF, Chen YJ, Chu SC, Hsieh YS, Chang YC (2004) Regulation of interleukin-6

expression by arecoline in human buccal mucosal fibroblasts is related to intracellular glutathione levels. Oral Dis 10:360-364

10. Tsai CH, Yang SF, Chen YJ, Chou MY, Chang YC (2005) Raised keratinocyte growth factor-1 expression in oral submucous fibrosis in vivo and upregulated by arecoline in human buccal mucosal fibroblasts in vitro. J Oral Pathol Med 34:100-105
11. Ni WF, Tsai CH, Yang SF, Chang YC (2007) Elevated expression of NF-kappaB in oral submucous fibrosis--evidence for NF-kappaB induction by safrole in human buccal mucosal fibroblasts. Oral Oncol 43:557-562
12. Chung-Hung T, Shun-Fa Y, Yu-Chao C (2007) The upregulation of cystatin C in oral submucous fibrosis. Oral Oncol 43:680-685
13. Tsai CH, Yang SF, Lee SS, Chang YC (2009) Augmented heme oxygenase-1 expression in areca quid chewing-associated oral submucous fibrosis. Oral Dis 15:281-286
14. Robertson H, Kirby JA, Yip WW, Jones DE, Burt AD (2007) Biliary epithelial-mesenchymal transition in posttransplantation recurrence of primary biliary cirrhosis. Hepatology 45:977-981
15. Zeisberg M, Hanai J, Sugimoto H, Mammoto T, Charytan D, Strutz F, Kalluri R (2003) BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. Nat Med 9:964-968
16. Greenway S, van Suylen RJ, Du Marchie Sarvaas G, Kwan E, Ambartsumian N, Lukanidin E, Rabinovitch M (2004) S100A4/Mts1 produces murine pulmonary artery changes resembling plexogenic arteriopathy and is increased in human plexogenic arteriopathy. Am J Pathol 164:253-262

國科會補助計畫衍生研發成果推廣資料表

日期:2012/10/16

國科會補助計畫	計畫名稱: S100A4調控傳導路徑對於口腔黏膜下纖維化致病機轉之研究
	計畫主持人: 蔡崇弘
	計畫編號: 100-2314-B-040-008- 學門領域: 牙醫學

無研發成果推廣資料

100 年度專題研究計畫研究成果彙整表

計畫主持人：蔡崇弘	計畫編號：100-2314-B-040-008-
計畫名稱：S100A4 調控傳導路徑對於口腔黏膜下纖維化致病機轉之研究	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	0%	篇	
		研究報告/技術報告	0	0	0%		
		研討會論文	0	0	0%		
		專書	0	0	0%		
	專利	申請中件數	0	0	0%	件	
		已獲得件數	0	0	0%		
	技術移轉	件數	0	0	0%	件	
		權利金	0	0	0%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	0%	人次	
		博士生	0	0	0%		
		博士後研究員	0	0	0%		
		專任助理	0	0	0%		
國外	論文著作	期刊論文	0	1	100%	篇	投稿 SCI 雜誌中
		研究報告/技術報告	0	0	0%		
		研討會論文	0	0	0%		
		專書	0	0	0%	章/本	
	專利	申請中件數	0	0	0%	件	
		已獲得件數	0	0	0%		
	技術移轉	件數	0	0	0%	件	
		權利金	0	0	0%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	0%	人次	
		博士生	0	0	0%		
		博士後研究員	0	0	0%		
		專任助理	0	0	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。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 □未發表之文稿 ■撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

本計劃原申請 3 年期, 通過核準 1 年期, 原計劃 1 年期在探討 S100A4 在口腔黏膜下纖維症表達, 不管體內或體外, 表達均上升, 加上調控檳榔鹼的濃度變化及加入阻斷藥物, 探討其在各種不同路徑的角色, 已得到某種程度上證明, 已撰寫論文投稿中, 靜候審查的建議及修改.

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

口腔黏膜下纖維化症是台灣重要的口腔疾病, 本團隊研究室常年從事其致病機轉探討, 申請本計劃, 欲了解在上皮間質轉化占重要角色的 S100A4, 依以前所建立的模式外, 引進 knockdown 及探討膠質收縮的方法, 使研究更往前, 得到結果, 顯見 S100A4 在口腔黏膜下纖維症有其地位, 往後繼續探討上皮間質轉化在此疾病所伴演的角度.