科技部補助專題研究計畫報告

GAS5/GAS5-AS1透過競爭性結合RNA功能化於口腔黏膜下纖維化症之機制剖析及治療意涵(第3年)

報 告 類 別 ： 精簡報告
計 畫 類 別 ： 個別型計畫
計 畫 編 號 ： MOST 106-2314-B-040-004-MY3
執 行 期 間 ： 108年08月01日至109年07月31日
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 24 日

中 文 摘 要 ： 本研究鑑定出GAS5及GAS5-AS1於口腔頰黏膜纖維組織及其初代培養纖維化頰黏膜纖維母細胞低表達。GAS5-AS1過度表現可降低初代培養纖維化頰黏膜纖維母細胞及檳榔素誘發肌纖維母細胞活性。而TGF-β1為調控口腔黏膜下纖維化之重要細胞激素，可活化肌纖維母細胞特性、間質轉化而導致纖維化。GAS5過度表現可以明顯抑制TGF-β1誘導正常頰黏膜纖維母細胞之膠體收縮能力及抑制TGF-β/Smad訊號路徑。後繼之研究發現GAS5可競爭性結合促纖維化微型RNA如miR-222。部分研究成果已發表於J Formos Med Assoc. 2018;117(8):727-733.

中 文 關 鍵 詞 ： 口腔黏膜纖維化症; GAS5; GAS5-AS1; 肌纖維母細胞

英 文 摘 要 ： Our data has found the downregulation of GAS5 and GAS-AS1 in fibrotic buccal mucosal fibroblasts (fBMFs) using real-time PCR and next-generation sequencing technologies. Ectopic expression of GAS5-AS1 significantly reduced the abilities of collagen gel contraction and migration in fBMFs or arecoline-treated BMFs. It is well known that TGF-β1 involves in crucial events in pathogenesis of OSF, including activation of myofibroblasts and mesenchymal trans-differentiation. Overexpression of GAS5 and GAS-AS1 significantly inhibited the collagen gel contractility in TGF-β1-treated BMFs and suppressed TGF-β1/ Smad signaling pathway. Apart from that, GAS5/GAS-AS1 may act as a competing endogenous RNAs and interact with pro-fibrogenic miR-222 in OSF.

英 文 關 鍵 詞 ： oral submucous fibrosis; GAS5; GAS5-AS1; myofibroblasts

（一）研究計畫之背景及目的

Oral submucous fibrosis (OSF) is a chronic inflammatory and potentially malignant disorder [1] characterized by the progressive accumulation of dense fibrous connective tissue, which results in rigidity and restricted mouth opening. It has been indicated that OSF is associated with areca quid chewing habit [2] and a high risk of malignant transformation [1]. Several studies have shown that 7–13% of OSF patients progressed to have oral squamous cell carcinoma (OSCC) [3,4], the fifth leading cause of cancer death in Taiwan [5]. The current therapies, such as anti-inflammatory/anti-oxidant injections [6-8], physiotherapy [9] or surgery [8], are symptomatic treatments, not acting on its etiology. In addition, it has been indicated that radiotherapy aggravates preexisting fibrosis and adversely affects the prognosis of OSCC occurring in the background of OSF [10]. As such, we sought to decipher the mechanism underlying OSF pathogenesis as well as identify the critical biomarkers in order to develop effective treatment modalities, leading to the reduction in cancer transformation from OSF.

Similar to fibrosis in other tissues, imbalance between synthesis and degradation of extracellular matrix (ECM) components resulted in OSF [11]. And the activated myofibroblasts have been recognized as the key pathogenic cells that attributed to excessive deposition of ECM [12]. Increased activity of myofibroblast has been found in multiple tissue fibroses, such as heart [13], liver [14], lung [15], and OSF [16]. It is well known that pro-fibrogenic cytokines are critical mediators of fibrosis by differentiating fibroblasts to myofibroblast phenotype. One of the typical features of the fully differentiated myofibroblasts is the expression of α-smooth muscle actin (α-SMA) [17], which is positively correlated with severity of OSF [16]. The localized mucosal inflammation caused by areca quid leads to an increase in pro-fibrogenic cytokines, such as transforming growth factor (TGF)-β [18-20]. In fact, activation of TGF-β signaling has been postulated as one of the main causative events for induction of myofibroblast transdifferentiation in OSF [18-20]. Also, TGF-β induces cells to undergo epithelial to mesenchymal transition (EMT), which is crucial in carcinogenesis and a possible origin of activated myofibroblasts. Previously, we have demonstrated that various EMT transcriptional factors have involved in dysregulation of myofibroblast in arecoline-induced OSF [21,22]. Since myofibroblasts are implicated in many fibrotic diseases, targeting molecules that regulate myofibroblast transdifferentiation may be a potential approach to understand pathogenesis of areca quid-associated OSF.

It is known that ~70–80% of the human genome is actively transcribed into RNA but only approximately 2% is translated into protein [23]. Around 98% of transcripts are noncoding RNAs (ncRNAs) and can be divided into small/short ncRNAs (such as microRNAs) and long non-coding RNAs (lncRNAs) according to their length of less or more than 200 nucleotides, respectively [24]. NcRNAs have been found to regulate gene expression that control diverse physiological and pathological processes, such as cell proliferation, apoptosis, migration and carcinogenesis. For example, lower level of microRNA-204 (miR-204) is observed in OSCC cancer stem cells and over-expression of miR-204 suppresses cancer stemness and tumor-growth by regulating the EMT mediators, Slug and Sox4 [25]. Several reports have revealed aberrant expression of ncRNA in various fibrotic diseases, such as liver [26,27], renal [28], pulmonary [29,30], cardiac [31] fibroses and ncRNAs serve as modulators of these fibroses. It has been shown that pro-fibrogenic miR-21 enhances TGF-β-induced myofibroblast differentiation [29,32]. And the level of miR-21 is associated with the activity of TGF-β in fibroblasts via targeting the negative regulators of myofibroblast, including Smad3, Smad7 [29] or programmed cell death 4 [32] genes. MiR-145 increases lung myofibroblast differentiation and pulmonary

fibrosis by acting on KLF4, a known negative regulator of α-SMA [30]. On the other hand, miR-29 acts as anti-fibrogenic miRNA by inhibiting ECM formation and interfering with the profibrogenic pathways including PDGF-B and PDGF-C signaling [33]. And miR-153 disturbs TGF-β1 signal transduction and reduces activities of pulmonary fibroblasts [34]. There have been significant advances over the past few years in the identification of ncRNAs, especially miRNA, as crucial components of myofibroblast biology and fibrosis. However, evidence for lncRNAs specifically influencing premalignant OSF pathogenesis is extremely limited.

LncRNAs include intergenic sequences, transcripts that overlap with other coding regions in either sense or antisense orientation, and enhancer RNAs, which activate transcription at distal promoters [35]. Functions of lncRNAs include sequestration of transcription factors, allosteric mechanisms and scaffolding for three-dimensional chromosomal structures. They can also cooperate with nucleic acids or proteins as activating or inhibiting signal molecules [36]. Emerging research findings suggest that lncRNAs are key contributors to fibrosis formation as well. One of the recent studies identified over 3600 lncRNAs that are expressed in human hepatic myofibroblasts and form networks with genes related to ECM components [37]. The interaction of lncRNAs and miRNAs has been examined in pulmonary fibrosis and several lncRNAs did correlate with the miRNAs expression. And they found lncRNA CD99P1 inhibited proliferation and α-SMA expression of lung fibroblasts [38]. A number of studies have shown that lncRNA H19 expression was significantly upregulated in TGF-β-induced renal and cardiac fibroses [28,39]. And H19 promotes fibroblast proliferation [39], while H19 inhibition attenuated fibroses [28]. In addition, several LncRNAs that have been established as tumor suppressors [40,41] also participate in fibrosis pathophysiology. For instance, lncRNA maternally expressed 3 (MEG3) has been found downregulated in liver fibrosis and is associated with anti-fibrogenic miR-29 [42]. It has been demonstrated that MEG3 regulated the TGF-β pathway genes [43] and overexpression of MEG3 in TGF-β1-treated hepatic stellate cells significantly decreased α-SMA mRNA and protein levels [44]. Although numerous lncRNAs involve in the fibrotic diseases, the functional roles of lncRNAs in the progression of OSF remain to be examined. Given the current lack of proper therapeutic options for oral submucous fibrosis, manipulation of non-coding RNAs may represent a novel and potent way to modulate myofibroblast phenotypes for effective treatment.

LncRNA growth arrest-specific 5 (GAS5) was first identified in 1988 in a search aimed to screen for novel tumor suppressors which were preferentially expressed during growth arrest. GAS5 is approximately 630 nt in length and localized at chromosome 1q25.1. GAS5 is a member of 5′-terminaloligopyrimidine (5′TOP) gene family and this motif is critical for the translational control. GAS5 introns encode 10 small nucleolar RNAs (snoRNAs) and exons are spliced to yield 2 mature lncRNA isoforms: GAS5a and GAS5b. At the 3' terminus of GAS5, there is partial overlap of 40 or so nucleotides with another non-protein coding gene, GAS5-antisense-1 (GAS5-AS1). Function of GAS5-AS1 is largely uncharacterized and the effect of its transcription on GAS5 expression remains unknown. GAS5, as its name suggests, is proven to be associated with the cell cycle progression and critical to normal growth arrest. GAS5 was reported to be down-expressed [45-47] and could inhibit cell proliferation, migration and invasion in various types of cancers [46-50]. A recent study revealed the expression of GAS5-AS1 was also downregulated in non-small cell lung cancer cells. They suggested that ectopic expression or downregulation of GAS5-AS1 influenced NSCLC cell migration and invasion [51]. Moreover, GAS5 represses liver fibrogenesis by competing with miR-222 [52], an indicative marker of fibrosis [53], and acts as a negative regulator of pro-fibrogenic miR-21 [49,54].

Materials and Methods

Reagents: Arecoline and collagen solution from bovine skin will be purchased from Sigma-Aldrich (St. Louis, MO, USA).

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 will be cultivated as previously described. Cell cultures between the third and eighth passages will be used in this study.

Quantitative real-time PCR (qRT-PCR): Total RNA will be prepared from cells using Trizol reagent according to the manufacturer's protocol (Invitrogen Life Technologies, Carlsbad, CA, USA). qRT–PCRs of mRNAs will be 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 will be performed on an ABI StepOne™ Real-Time PCR Systems (Applied Biosystems). The primer sequences are listed below:

Primer Name	Forward Primers	Reverse Primers
GAS5	CAGAGCGGTTGGCATTCATC	CTTTGCGAATGTTGCGGGTT
GAS5-AS1	TGCCTTAAACCAGTTGTGCC	TTCATAGGCCCCTGTGCTAA
SMA	AGCACATGGAAAAGATCTGGCACC	TTTTCTCCCGGTTGGCCTTG
COL1A1	GGGTGACCGTGGTGAGA	CCAGGAGAGCCAGAGGTCC
PAI-1	GAAATCCACAGCCCGGTAAC	GTCACACGGGCACAGAAAG
Vimentin	CAATGTTAAGATGGCCCTTG	GGGTATCAACCAGAGGGAGT
Fibronectin	CCCAGACTTATGGTGGCAATTC	AATTTCCGCCTCGAGTCTGA
GAPDH	CTCATGACCACAGTCCATGC	TTCAGCTCTGGGATGACCTT

Overexpression of GAS5 and GAS-AS1: GAS5 or GAS-AS1 cDNA will be cloned into pLV-EF1a-MCS-IRES-Puro (BioSettia, Cat. No: cDNA-pLV01; San Diego, CA, USA). Lentivirus production will be 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, Calsbad, CA, USA).

Lentiviral-mediated RNAi for silencing GAS5 and GAS-AS1: 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 human GAS5 and GAS-AS1 were synthesized and cloned into pLVRNAi to generate a lentiviral expression vector. The target sequences for GAS5 and GAS5-AS1 are listed as follows

Sh-GAS5-1	5'-AAAAGGAAGGATGAGAATAGCTATTGGATCCAATAGCTATTCTCATCCTTCC-3'
Sh-GAS5-2	5'-AAAAGCAGACCTGTTATCCTAAATTGGATCCAATTTAGGATAACAGGTCTGC-3'
Sh-GAS5-AS1-1	5'-AAAAGCCCAGAAACAGATTTGTATTGGATCCAATACAAATCTGTTTCTGGGC-3'
Sh-GAS5-AS1-2	5'-AAAAGCCTATGAAACCTGACAATTTGGATCCAAATTGTCAGGTTTCATAGGC-3'

Collagen gel contraction assay: Cells will be suspended in collagen gel solution (Sigma-Aldrich, St. Louis, MO, USA) and added into a 24-well-plate followed by incubation at 37°C for 2 hours. After polymerization, the gels will be further incubated within 0.5 ml medium for 48 h. The collagen gel size change (contraction index) will be quantified using ImageJ software (NIH, Bethesda, MD, USA).

Transwell migration assays: 1 x 10^5 cells in a medium with low serum will be added into the upper chamber of a transwell (Corning, Acton, MA) and medium supplemented with higher serum will be used as a chemoattractant in the lower chamber followed by 24 h incubation. Cells on the lower surface of the insert membrane will be stained with crystal violet. The number of migration cells in a total of five randomly selected fields will be measured.

Western blot analysis: All procedures in this assay will follow the previously described protocols. The primary antibodies against p-Smad2 and Smad2 will be applied.

Statistical analysis: Data are presented as mean ± SD. A Student's *t* test or analysis of variance (ANOVA) test will be used to compare the continuous variables among groups. $P < 0.05$ will be considered statistically significant.

Results

We have found lower expression level of GAS5/GAS5AS-1 in OSF tissues compared with normal buccal mucosa subjects **(Figure 1)**. Elevated expression of GAS5-AS1 represses the migration ability in arecoline-stimulated BMFs and fBMFs **(Figure 2)**. More importantly, overexpressed GAS5 significantly decreased the TGF-β1-induced collagen contractility in buccal mucosal fibroblasts **(Figure 3)**. GAS5 repressed TGF-β1-induced collagen contractility in BMF **(Figure 4)**. And there was a negative correlation between the expression of GAS5 and fibrotic and myofibroblasts marker expression **(Figure 5)**. Hence, it is attractive to elucidate whether GAS5/GAS5-AS1 involve in the progression of OSF and their clinical significance in the treatment of OSF.

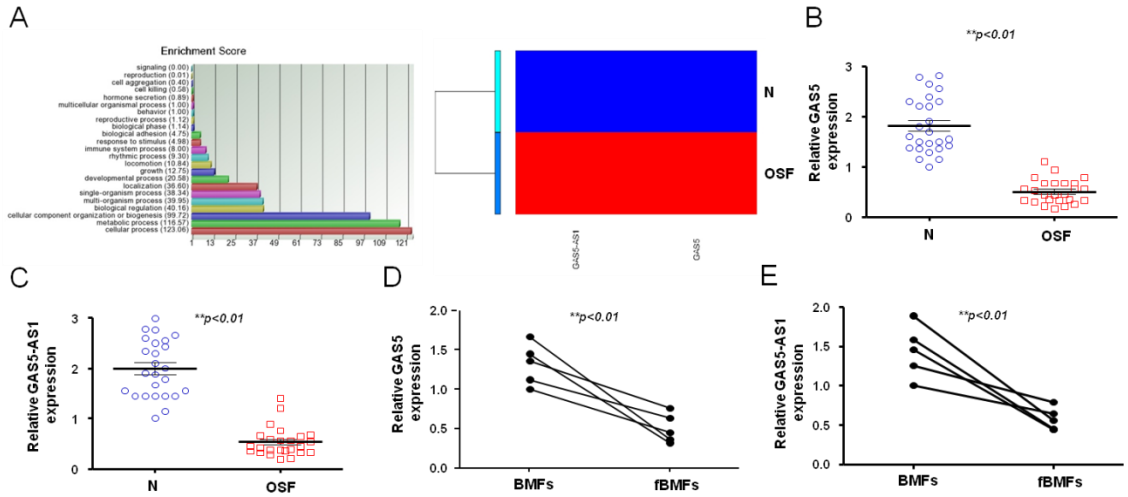

Figure 1. Downregulation of GAS5/GAS5-AS1 in OSF tissues and fBMFs. (A) In order to identify the association between lncRNAs and OSF pathogenesis, we used high-throughput RNA sequencing approach to recognize the putative targets, starting from total RNAs of normal buccal mucosa (N) and OSF tissues. The overlapped lncRNAs that were significantly increased (> 2 fold) or suppressed (<0.5 fold) in OSF specimens relative to their respective counterparts were considered for further analysis. To this end, we selected the downregulated lncRNAs (GAS5 and GAS5-AS1) as the promising candidates for the study; A significant decrease in GAS5 (B) and GAS5-AS1 (C) expression were found in OSF compared to N tissues by qRT-PCR analysis; The relative expression level of GAS5 (D) and GAS5-AS1 (E) were lower in human fibrotic buccal mucosal fibroblasts (fBMFs) relative to human buccal mucosal fibroblasts (BMFs).

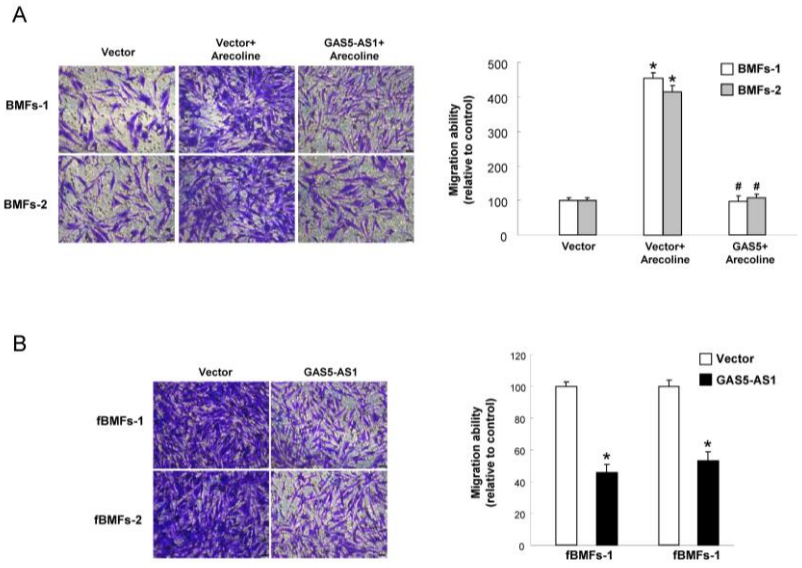

Figure 2.The effect of overexpressed GAS5-AS1 on cell migration

The transwell migration assay was performed in (A) GAS5-AS1 overexpressing BMFs with arecoline treatment and (B) GAS5-AS1 overexpressing fBMFs. The elevated expression of GAS5-AS1 suppressed the arecoline-induced migration of BMFs and also inhibited the migration capacity in fBMFs. The experiments were repeated three times and representative results were shown. *p < .05 compared to vector group; # p < .05 compared to vector+ arecoline group.

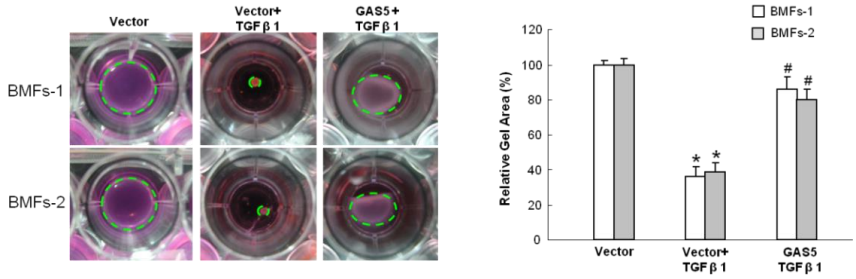

Figure 3. GAS5 repressed TGF-β1-induced collagen contractility in BMFs. BMFs treated with or without TGF-β1 were transduced with lentiviral vectors overexpressing GAS5 and embedded into collagen gels. After 48hr, contraction of the gels was photographed and measured using ImageJ software (NIH) to calculate their areas.

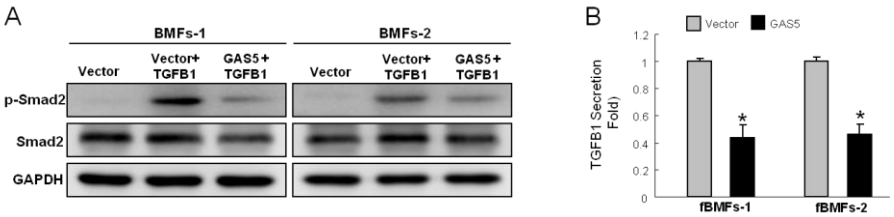

Figure 4. GAS5 repressed TGF-β1/Smad signaling (A) The protein expression levels of p-Smad2 and Smad in GAS5-transfected TGFＢ1-stimulated BMFs were analyzed by western blotting. (B) The secretion of TGFＢ1 in control and GAS5-overexpressing fBMFs by ELISA analysis.

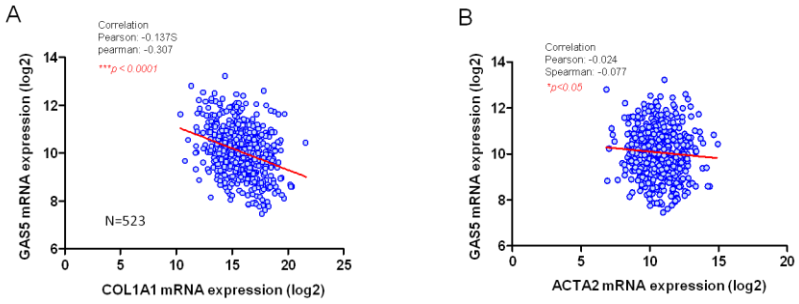

Figure 5. The negative correlation between the expression levels of GAS5 and myofibroblasts markers. Expression of GAS5 was inversely correlated with (A) COL1A1 and (B) ACTA2 (α-SMA) expressions in OC samples from the TCGA dataset using Pearson's correlation coefficient.

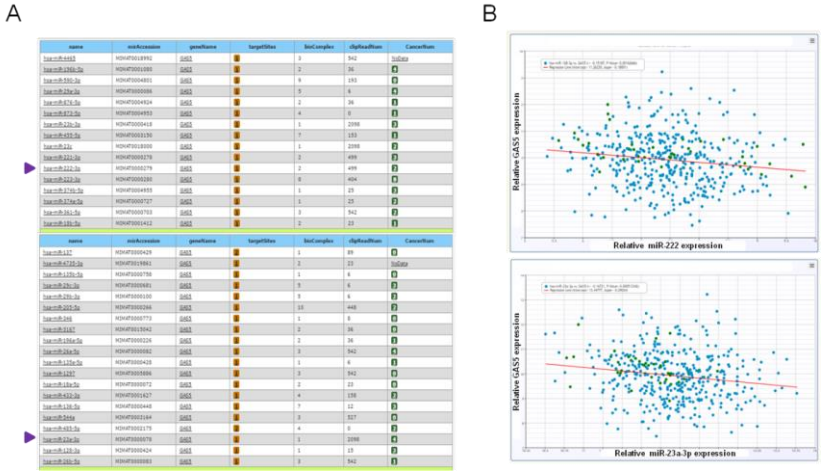

Figure 6. GAS5 might function as ceRNA for miR-222 or miR-23a-3p. (A) View the predicted miRNA-GAS5 interactions by scanning GSA5 sequences overlapping with CLIP-Seq peaks for potential microRNA targets (miRanda/mirSVR) and then output the detailed information. Purple arrows indicated miR-222 and miR-23a-3p. (B) Negative correlation between GAS5 and miR-222 (*upper*) or miR-23a-3p (*lower*) in oral cancer patients).

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106年度專題研究計畫成果彙整表

計畫主持人：余權航	**計畫編號：**106-2314-B-040-004-MY3
計畫名稱：GAS5/GAS5-AS1透過競爭性結合RNA功能化於口腔黏膜下纖維化症之機制剖析及治療意涵	

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	2	篇	1. J Formos Med Assoc. 2018 Aug;117(8):727-733. 2. J Formos Med Assoc. 2020 Apr;119(4):879-883.
		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	
參與計畫人力	本國籍	大專生	0	人次	
		碩士生	0		
		博士生	0		
		博士級研究人員	0		
		專任人員	1		培養一名碩士級研究助理蔡弼合操作分子生物學實驗能力
	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。）			由於口腔黏膜纖維化症多數患者其 GAS5及GAS5-AS1表現低下，當 GAS5及 GAS5-AS1參與口腔黏膜纖維化症進程分子機制及臨床意涵獲得驗證，可藉以開發口腔黏膜纖維化標靶藥物並減少病患及家屬負擔。		