科技部補助專題研究計畫成果報告
期末報告

長鏈非編碼RNA作為口腔癌前病變及口腔癌新穎診治標的之轉譯研究

計 畫 類 別 ： 整合型計畫
計 畫 編 號 ： MOST 106-2632-B-040-002-
執 行 期 間 ： 106年08月01日至107年07月31日
執 行 單 位 ： 中山醫學大學牙醫學系（所）

計畫主持人： 張育超
共同主持人： 柯俊良、楊順發、余承佳
計畫參與人員： 此計畫無其他參與人員

中　華　民　國　107　年　10　月　22　日

中 文 摘 要 ： 流行病學研究指出，臺灣地區口腔癌前病變及口腔癌發生率與嚼食檳榔有密切關係。根據衛生署癌症登記報告指出，口腔癌在臺灣男性十大癌症中死亡率與發生率皆位居第四位，口腔癌年增率也是臺灣地區年增率最高之癌症。其特性為容易復發及轉移，五年內存活率極低，因此尋找新穎生物標誌及有效療法將提供臨床很大的幫助。長鏈非編碼RNA (long non-coding RNA, LncRNAs)與發育、幹細胞維持、癌症及一些疾病相關，由於表現非常具有特異性，因此也是發展成疾病生物指標的熱門標的。然而，長鏈非編碼RNA於口腔癌前病變及口腔癌之角色仍不清楚。這個整合型轉譯醫學計畫，結合了臨床醫師、及基礎研究人員，以四個子計劃進行以下的研究成果發現：(一)探討長鏈非編碼RNA HOTAIR於口腔癌前病變致病角色；發現HOTAIR於口腔癌前病變組織高度表現，HOTAIR可調節纖維母細胞活性，檳榔素處理可增強正常頰黏膜纖維母細胞中HOTAIR表現，降低HOTAIR表現可抑制檳榔素所誘發肌纖維母細胞活性。(二)長鏈非編碼RNA調控癌幹性及驅使口腔癌惡化之功能性探討；發現MEG3於口腔癌低表達且可調控幹細胞化，鑑定MEG3具備ceRNA功能及預測MEG3 promoter可能結合轉錄因子上游關鍵因子，PrognoScan database鑑定出口腔癌病患高表現MEG3預後較佳，MEG3過度表現可降降低ALDH1活性及活體腫瘤生成力。(三)調節口腔癌細胞自噬與長鏈非編碼RNA之恆定增進5-Fluorouracil抗藥性敏感度及改善其不良副作用；發現真菌類免疫調節蛋白GMI能改善5-FU所誘發之粘膜炎。小鼠每隔4天以腹腔注射方式給予5-FU，進行兩循環。在5-FU注射前3天，小鼠將開始每天給予GMI或PBS直到第14天。以組織學分析，GMI可防止5-FU導致的腸粘膜和舌上皮損傷。GMI能增加5-FU對口腔癌細胞的細胞毒性，但在口腔正常細胞則無影響。總結，GMI能減輕5-FU誘導的損傷並減緩正常消化道組織中的細胞凋亡，期望GMI可能改善病患的生活品質。(四)探討長鏈非編碼RNA在天然化合物抗口腔癌轉移之作用機轉；發現丹酚酸A (salvianolic acid A)對口腔癌細胞SCC-9的細胞存活率並沒有顯著的影響，並可以藉由抑制MMP-2的分泌來降低癌細胞的轉移與侵襲。丹酚酸A對於p-c-Raf, p-MEK1/2, and p-ERK1/2都具有明顯的抑制效果。可以有效的增加lncRNA-Gas5的表現量，且利用Gas5的siRNA處理下，會反轉由丹酚酸A所抑制的細胞轉移現象。綜合以上結果，lncRNA-Gas5可能參與在丹酚酸A所抑制的口腔癌細胞轉移現象，lncRNA-Gas5在未來或許可以應用在預防口腔癌的轉移或輔助口腔癌的治療。

中 文 關 鍵 詞 ： 口腔癌前病變、口腔癌、長鏈非編碼核醣核酸、轉譯醫學、5-Fluorouracil、天然化合物

英 文 摘 要 ： Oral cancers can be preceded by clinically evident oral potentially malignant disorders such as leukoplakia, oral submucous fibrosis, and oral epithelial hyperplasia. To reach the goal, an integrated project is organized with four sub-projects that coordinates collaboration among basic researchers, clinicians, pathologists to investigate. The results of each part of project are shown as followings: (1) Functional investigation the roles of the lncRNA HOTAIR during oral potentially malignant disorders

pathogenesis: the upregulation of HOTAIR in oral submucous fibrosis tissues, modulation of HOTAIR in fibrotic buccal mucosal fibroblasts, arecoline enhanced HOTAIR in normal buccal mucosal fibroblasts, downregulation of HOTAIR inhibited arecoline induced gel construction. (2) Probing the mechanisms and clinical significance of lncRNAs on oral cancer stemness and mesenchymal transdifferentiation: lncRNA MEG3 lower expression in oral cancer specimens, the role of MEG3 as a promoter, better prognosis with MEG3 expression by PrognoScan database, over MEG3 overexpression reduced ALDH1activity and in vivo tumor size. (3) Balance of autophagy and long non-coding RNA regulation contribute to sensitization of 5-Fluorouracil resistance and alleviation of 5-Fluorouracil-induced adverse effects in oral cancer: GMI, one fungal immunomodulatory proteins from Ganoderma microsporum, prevented intestinal mucosa and epithelium of tongue from damaged by 5-FU. GMI enhanced the cytotoxicity of 5-FU in two oral cancer cells while GMI would not promote this effect in an oral normal cell. In conclusion, GMI alleviates 5-FU-induced damage and decelerates apoptosis in normal alimentary tract tissue. GMI may improve patients' life quality. (4) The mechanisms of long non-coding RNAs for anti-oral cancer effect on metastasis and apoptosis by natural compounds: Salvianolic acid A, a traditional Chinese medicine, has antithrombosis, antiplatelet, anti-inflammation, and antitumor activities. Our findings suggested that salvianolic acid A inhibits the invasion and migration of OSCC by inhibiting the c-Raf/MEK/ERK pathways that control MMP-2 expression. Our findings provide new insights into the molecular mechanisms that underlie the antimetastatic effect of salvianolic acid A and are thus valuable for the development of treatment strategies for metastatic OSCC.

英文關鍵詞： oral potentially malignant disorders、oral cancer、long non-coding RNAs、5-Fluorouracil、 natural compounds、translation research

科技部補助「私立大學校院發展研發特色專案計畫」成果報告

（□期中進度報告/■期末報告）

長鏈非編碼 RNA 作為口腔癌前病變及口腔癌新穎診治標的之轉譯研究

計畫類別：整合型計畫
計畫編號：MOST　106-2632-B-040-002
執行期間：106 年 8 月 1 日至 107 年 7 月 31 日

執行機構及系所：中山醫學大學牙醫學系（所）

計畫主持人：張育超教授
共同主持人：柯俊良教授、楊順發教授、余承佳教授
計畫參與人員：

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

中　華　民　國　107　年 10　月　15　日

成果報告內容

一、計畫方面：

（一）計畫目前執行進度及是否達到預定目標

（二）計畫執行迄今之成果(請具體明列成果項目，如：論文/專利/重大突破/或其他…)

（三）請說明計畫執行迄今經費運用狀況(含大型設備之購置狀況)及學校配合款支用情形。

（四）經費使用表：支用金額請統計至該年度計畫截止日

年　度	第一年			第二年			第三年		
項　目	科技部補助		學校配合款使用情形	科技部補助		學校配合款使用情形	科技部補助		學校配合款使用情形
	核定金額	使用金額		核定金額	使用金額		核定金額	使用金額	
金　額	3,600,000	3,600,000	900,000						

說　明：依科技部、學校會計總務採購規定辦理經費核銷。

二、團隊研究能力：總計畫與各子計畫之間的分工架構、整合性及工作協調為何？(請提出具體作法說明)。

本研究計畫係整合本校牙醫系所、口腔科學研究所、醫學研究所及本校附設醫院病理科、口腔外科進行臨床與基礎整合型計畫，針對口腔癌及期癌前病變以長鏈非編碼 RNA 作為新穎診治標的之轉譯研究之探討。每一位子計畫的主持人會依據各自的專長，分擔整合型計畫所需之工作內容。研究工作中所需之研究設備及技術，可由子計畫間共同使用及支援，且在人力配制上會以機動性的方式，協助研究團隊之成員， 總計畫主持人會定期召開研究會議，除了立即檢視各計畫之執行進度外，亦藉由會議召開之機會，增加此團隊之研究成果與經驗之交流，使各個子計畫的研發能力能完全發揮。每一位子計畫的主持人會依據各自的專長，分擔整合型計畫所需之工作內容。例如，本研究團隊固定開會討論基礎與臨床的整合細節。由臨床醫師提供檢體及病理科醫師加入討論從臨床重要性切入主題。而基礎老師也適時在臨床檢體分析實驗之外，提供更詳盡的實驗方法已深入了解詳細機轉。

三、學校方面：

（一）學校提供之具體配套措施(如：配合款；相關行政、空間、設備等之支援及協助；獎勵等)，請提出已配合之措施辦法或具體資料證明。

(二)學校對本計畫是否訂有考核機制？如有請列出，及考核結果為何？
(三)本計畫執行至今對於學校推展或提升研發環境有何具體成效？

學校依規定提供25%的相對配合款，來支應本計畫所需要的儀器及耗材費用。另學校亦依校內規定提供此計畫管理費的40%為經費獎勵。而在設備的支援及協助方面，學校亦提供此計畫使用付費的貴重儀器9折之優惠。

本計畫執行期間，目前發表1篇的SCI論文，其餘結果現正在整理中，準備申請專利或投稿期刊中，本計畫之執行對本校在推動特色研究(口腔醫學、癌症研究為本校五大特色研究)有極大的幫助，也提升了學校的研發動力。總主持人與子計畫主持人在執行計畫期間分別獲得「科技部彈性薪資」獎勵。

中文摘要

關鍵字: 口腔癌前病變、口腔癌、長鏈非編碼核醣核酸、轉譯醫學、5-Fluorouracil、天然化合物

流行病學研究指出，臺灣地區口腔癌前病變及口腔癌發生率與嚼食檳榔有密切關係。根據衛生署癌症登記報告指出，口腔癌在臺灣男性十大癌症中死亡率與發生率皆位居第四位，口腔癌年增率也是臺灣地區年增率最高之癌症。其特性為容易復發及轉移，五年內存活率極低，因此尋找新穎生物標誌及有效療法將提供臨床很大的幫助。長鏈非編碼 RNA (long non-coding RNA, LncRNAs)與發育、幹細胞維持、癌症及一些疾病相關，由於表現非常具有特異性，因此也是發展成疾病生物指標的熱門標的。然而，長鏈非編碼 RNA 於口腔癌前病變及口腔癌之角色仍不清楚。這個整合型轉譯醫學計畫，結合了臨床醫師、及基礎研究人員，以四個子計劃進行以下的研究成果發現：(一)探討長鏈非編碼 RNA HOTAIR 於口腔癌前病變致病角色；發現 HOTAIR 於口腔癌前病變組織高度表現，HOTAIR 可調節纖維母細胞活性，檳榔素處理可增強正常頰黏膜纖維母細胞中 HOTAIR 表現，降低 HOTAIR 表現可抑制檳榔素所誘發肌纖維母細胞活性。(二)長鏈非編碼 RNA 調控癌幹性及驅使口腔癌惡化之功能性探討；發現 MEG3 於口腔癌低表達且可調控幹細胞化，鑑定 MEG3 具備 ceRNA 功能及預測 MEG3 promoter 可能結合轉錄因子上游關鍵因子，PrognoScan database 鑑定出口腔癌病患高表現 MEG3 預後較佳，MEG3 過度表現可降降低 ALDH1 活性及活體腫瘤生成力。(三)調節口腔癌細胞自噬與長鏈非編碼 RNA 之恆定增進 5-Fluorouracil 抗藥性敏感度及改善其不良副作用；發現真菌類免疫調節蛋白 GMI 能改善 5-FU 所誘發之粘膜炎。小鼠每隔 4 天以腹腔注射方式給予 5-FU，進行兩循環。在 5-FU 注射前 3 天，小鼠將開始每天給予 GMI 或 PBS 直到第 14 天。以組織學分析，GMI 可防止 5-FU 導致的腸粘膜和舌上皮損傷。GMI 能增加 5-FU 對口腔癌細胞的細胞毒性，但在口腔正常細胞則無影響。總結，GMI 能減輕 5-FU 誘導的損傷並減緩正常消化道組織中的細胞凋亡，期望 GMI 可能改善病患的生活品質。(四)探討長鏈非編碼 RNA 在天然化合物抗口腔癌轉移之作用機轉；發現丹酚酸 A (salvianolic acid A)對口腔癌細胞 SCC-9 的細胞存活率並沒有顯著的影響，並可以藉由抑制 MMP-2 的分泌來降低癌細胞的轉移與侵襲。丹酚酸 A 對於 p-c-Raf, p-MEK1/2, and p-ERK1/2 都具有明顯的抑制效果。可以有效的增加 lncRNA-Gas5 的表現量，且利用 Gas5 的 siRNA 處理下，會反轉由丹酚酸 A 所抑制的細胞轉移現象。綜合以上結果，lncRNA-Gas5 可能參與在丹酚酸 A 所抑制的口腔癌細胞轉移現象，lncRNA-Gas5 在未來或許可以應用在預防口腔癌的轉移或輔助口腔癌的治療。

英文摘要

KEYWORDS: oral potentially malignant disorders、oral cancer、long non-coding RNAs、5-Fluorouracil、 natural compounds、translation research

Oral cancers can be preceded by clinically evident oral potentially malignant disorders such as leukoplakia, oral submucous fibrosis, and oral epithelial hyperplasia. In Taiwan, there are two million people who have the areca quid chewing habit, which has been suspected to elevate the incidence of oral potentially malignant disorders; about 80% of all oral cancer deaths are associated with this habit. Because of the higher incidence rate of oral cancer in Taiwan, to investigate the carcinogenesis of oral cancer is urgent and the molecular mechanisms will provide useful knowledge in prevention, diagnosis, and therapy. To reach the goal, an integrated project is organized with four sub-projects that coordinates collaboration among basic researchers, clinicians, pathologists to investigate. The specific aims of this proposal are: (1) Functional investigation the roles of the lncRNA HOTAIR during oral potentially malignant disorders pathogenesis; (2) Probing the mechanisms and clinical significance of lncRNAs on oral cancer stemness and mesenchymal transdifferentiation; (3) Balance of autophagy and long non-coding RNA regulation contribute to sensitization of 5-Fluorouracil resistance and alleviation of 5-Fluorouracil-induced adverse effects in oral cancer; (4) The mechanisms of long non-coding RNAs for anti-oral cancer effect on metastasis and apoptosis by natural compounds.

三、研究計畫內容

子計畫一: 功能性探討長鏈非編碼 RNA HOTAIR 於口腔癌前病變致病角色

研究背景及目的

1. HOTAIR（hox transcript antisense intergenic RNA）

HOTAIR是目前瞭解較為透徹的lncRNAs之一。Rinn等人於2007年發現這個能調節同源異型基因(Hox genes)的lncRNAs，並命名為HOTAIR[1]。其長度為2158個核苷酸，位於染色體12q13.13[2]。近年來不少研究已指出HOTAIR在許多癌症中有相當顯著地過度表現，包括像是乳癌、大腸直腸癌和喉癌等[3-5]。在乳癌細胞中已知HOTAIR過度表現會促使癌細胞轉移，因此其大量表現可做為癌細胞轉移或存活率不佳的有力預測因子[6]。同樣地在喉癌與肝癌，HOTAIR的過度表現也被認為是預後不佳的指標[7, 8]。HOTAIR雖然位於HOXC叢集（cluster）中，但它調節的方式並非直接影響HOXC叢集，而是透過甲基化抑制HOXD叢集上超過四萬個核苷酸的表現，包括像是HOXD8、HOXD9、HOXD10、HOXD11等許多非編碼核糖核酸[9]。作為指引因子時，HOTAIR有兩個主要的功能區位（functional domain）可與兩個染色質修飾複合體結合[10]。一個位在5’端能與polycomb repressive complex 2（PRC2）複合體結合。許多研究已指出HOTAIR藉由與PRC2複合體結合來徵募polycomb group（PcG）蛋白質到目標基因上[7]，並透過調節三甲基化組蛋白3賴胺酸27（H3K27me3）來影響一些抑制細胞轉移的基因[11]。另一個則位在3’端可與LSD1/CoREST1結合[11, 12]，藉由組蛋白3賴胺酸4（H3K4）去甲基化的機制抑制基因的表現[10, 13]。在大腸直腸癌組織中過度表現的HOTAIR被認為是透過與PRC2的交互作用來調控PcG蛋白質的基因表現，進而影響它所調控的染色質修飾作用[10]。HOTAIR也可以做為鷹架因子，組成 HOTAIR/ PRC2/ LSD1複合體，透過不同的機制來調節基因表現[12]。

此外，HOTAIR在肝癌組織中也是顯著地過度表現，而減弱HOTAIR的表現量可減少肝癌細胞株Bel7402的增生。而可能的機制被推測是因為抑制HOTAIR會使得基質金屬蛋白酶-9（matrix metalloproteinase-9）與血管內皮生長因子（VEGF）的表現量也跟著受到抑制[14]。而利用小干擾核糖核酸技術（siRNA）在肝癌細胞中降低HOTAIR的表現，被發現可以改變細胞的存活率與對TNF-α誘發細胞凋亡的敏感度。並提升對順鉑（cisplatin）與阿黴素（doxorubicin）的化學治療敏感度[14, 15]。同樣地，在口腔癌的組織中，HOTAIR也有過度表現的情形且和癌症轉移、腫瘤大小與不良預後相關[16, 17]。利用小干擾核糖核酸技術抑制HOTAIR則可以有效的降低細胞增生、侵入、轉移等特性並誘發癌細胞凋亡[16, 17]。其中一篇研究還發現HOTAIR與上皮間質轉換過程（epithelial-mesenchymal transition；簡稱EMT）的因子上皮鈣粘蛋白（E-cadherin）有負相關性。他們指出HOTAIR可藉由與EZH2、H3K27me3的結合來調節E-cadherin的表現量[16]。而EMT已被證實與口腔癌前病變[18]還有口腔癌[19]有著密切的關聯。此外，HOTAIR在病人或CCl_4誘發的肝纖維化病變中表現量皆升高。該研究指出HOTAIR能使PRC2複合體連結在另一具有抗纖維化功能的lncRNA MEG3啟動子（promoter）上，因而抑制MEG3/p53路徑[20]。雖然在口腔癌中HOTAIR的過度表現與癌症轉移等不良預後有關，其在口腔癌前病變的影響與相關機制目前仍未明。

2. 肌纖維母細胞與組織纖維化

口腔癌前病變之一的口腔黏膜下纖維化就跟其他纖維化疾病一樣，都是因為細胞外間質（extracellular matrix；簡稱 ECM）的合成與降解不平衡所造成[21]。目前已知肌纖維母細胞（myofibrolasts）是促成ECM 過度堆積的重要因子，且在眾多的纖維化疾病中都發現肌纖維母細胞的活化[22-24]。它的形態特徵是介於成纖維細胞（fibroblasts）與平滑肌細胞（smooth muscle cell）之間。除了會表現平滑肌肌動蛋白（α-smooth muscle actin；簡稱 α-SMA）[25]，其胞質內有大量的微絲束（microfilament bundles）[26]並具有收縮和遷移的能力。纖維母細胞一開始被認為是由在局部組織的成纖維細胞所轉化而來，但

許多文獻已經指出肌纖維母細胞可來自不同的前驅細胞。例如上皮細胞通過上皮間質轉換過程可以成為肌纖維母細胞的其中一個來源[27, 28]。在正常的傷口癒合情況下，肌纖維母細胞會短暫的出現促使傷口收縮與結締組織修復。但是在纖維化病變的過程中，肌纖維母細胞沒有在傷癒後及時的凋亡消失，而是在組織中持續的存在並分泌合成大量的 ECM 在組織中沉積。使正常組織結構發生病變，功能遭到破壞，最終導致纖維化。

肌纖維母細胞的分化是一個複雜的過程，受到多種訊息的調控。其中促纖維化細胞激素之一的 TGF-β 已經被廣泛地認作是關鍵的調控因子，不少文獻也指出 TGF-β 與口腔黏膜下纖維化的關聯[29-31]。檳榔素已被發現可活化 JNK/ATF2/Jun 路徑[31]，促使上皮細胞分泌 TGF-β[29]。而升高的 TGF-β 則會影響成纖維細胞的活化與調節 EMT 相關的基因[30]。當 TGF-β 被釋出後，會與 TGF-βⅡ型受體結合並磷酸化Ⅰ型受體。被活化的受體複合物則開始啟動細胞質的 Smad2/3，使之發生磷酸化。接著會與 Smad4 結合形成複合物進入細胞核內，與下游基因序列結合來引起或抑制相關基因的表現[32]。TGF-β 同時也與多條信號通路有著相互作用，包括像 focal adhesion kinase（FAK）[33, 34]、 MAPK[35]、Rho[36]、 INF-γ[37]等，這些訊號共同調控成肌纖維細胞分化為肌纖維母細胞的路徑。其中 INF-γ 在肝纖維化的實驗中已發現可抑制 TGF-β/Smad 路徑的活化並減少 α-SMA 與膠原蛋白的產生[38]。另外，在 TGF-β 誘使 EMT 發生的過程中，Rho 則被指出與 TGF-β 活化 α-SMA 啟動子的過程有有關[36]。

EMT是指上皮細胞失去原有特性，轉換成間質細胞的過程。此現象在胚胎發育過程、傷口癒合、組織再生、器官纖維化及癌症轉移中扮演重要角色，同時也是成熟細胞修復損傷的關鍵。如同上段所提及的，EMT已被證實與口腔癌前病變[18]還有口腔癌[19]有密切的關聯。TGF-β除了活化下游路徑，同時也促使細胞開始經歷EMT的過程[39]，進而有更多的肌纖維母細胞產生。我們先前的口腔黏膜下纖維化的實驗也發現許多EMT轉錄因子與檳榔素引起的肌纖維母細胞異常表現有關，例如ZEB1[40]或TWIST[41]。雖然已知HOTAIR與EMT相關，而TGF-β更是活化EMT的重要因子，目前仍缺乏針對TGF-β與HOTAIR的研究去闡明兩者之間在產生口腔黏膜下纖維化的關聯性。另外，若能了解調節HOTAIR是否能改變肌纖維母細胞活性，將可大大提升口腔癌前病變的治療。

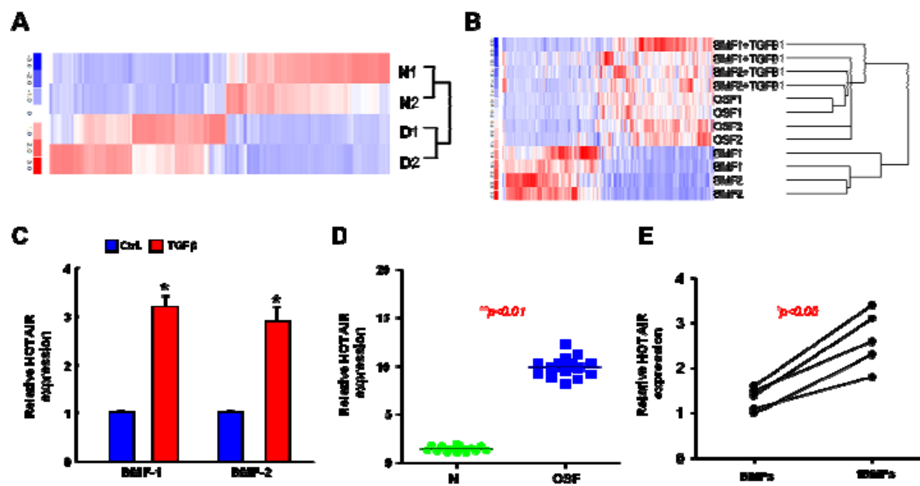
研究目的

我們的先導實驗已發現 HOTAIR 於口腔上皮異常增生（dysplasia）及口腔黏膜下纖維化（oral submucous fibrosis）的組織中具有高度表現。且 HOTAIR 已被發現可以用來做口腔癌轉移與預後的因子，它在口腔癌前病變中扮演甚麼角色便值得注目。若能提早利用 HOTAIR 作為口腔癌前病變的預測因子，勢必能減少病患惡化成口腔癌的比例。此外，我們也發現由 TGF-β1 處理過後的正常頰黏膜母細胞（buccal mucosal fibroblasts）會增加 HOTAIR 的表現。如同前言所提，檳榔素會增加 TGF-β1 的分泌因而促使肌纖維母細胞的分化。因此，增加 HOTAIR 的表現量將有很大的可能性與肌纖維母細胞的活化有關。若我們的假設為真，HOTAIR 在癌化轉變中的功能便有必要進一步闡明。本計畫欲探討 HOTAIR 於口腔癌前病變致病角色，希望利用此長鏈非編碼 RNA 為分子標靶對口腔癌前病變防治及癌化轉變及病理機轉了解有所助益。

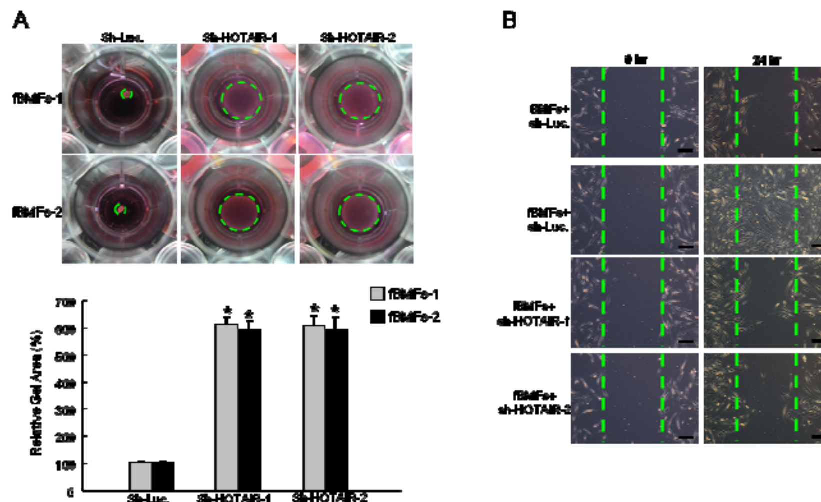
子計畫一研究結果

圖一: HOTAIR 於口腔癌前病變組織高度表現。(A)利用 lncRNA microarray 及生物資訊學鑑定出 HOTAIR 於上皮增生組織表現量（D1, D2)較正常口腔上皮組織(N1, N2)高;(B)利用 lncRNA microarray 及生物資訊學鑑定出 HOTAIR 於口腔黏膜下纖維化造纖維母細胞及 TGF-β 作用正常頰黏膜纖維母細胞（buccal mucosal fibroblasts, BMFs）高表現;(C) 用即時定量聚合酶連鎖反應驗證 TGF-β 作用於人類正常頰黏膜纖維母細胞會提昇 HOTAIR。(D) 用即時定量聚合酶連鎖反應 HOTAIR 於口腔黏膜下纖維化組織高表現。(E) 初代培養口腔纖維化頰黏膜母細胞(fibrotic buccal mucosal fibroblasts, fBMFs)HOTAIR

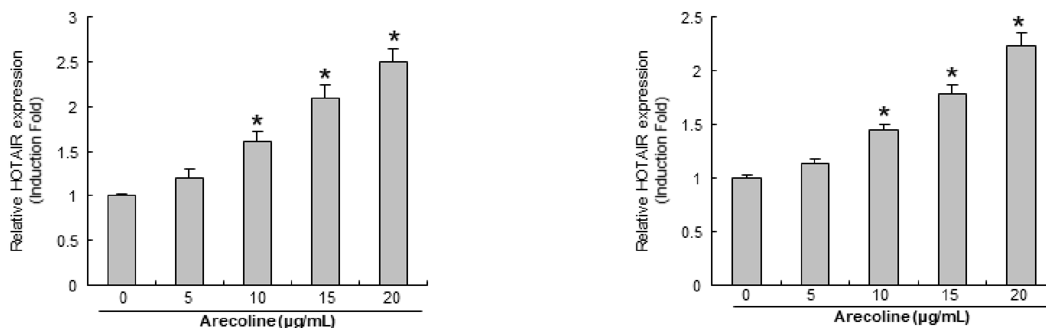
表現量較 BMFs 高。



圖二:HOTAIR可調節纖維母細胞活性。降低HOTAIR可抑制口腔纖維化頰黏膜母細胞（fibrotic buccal mucosal fibroblasts, fBMFs）膠體收縮力(A)及(B)傷口癒合(Wound healing)能力。

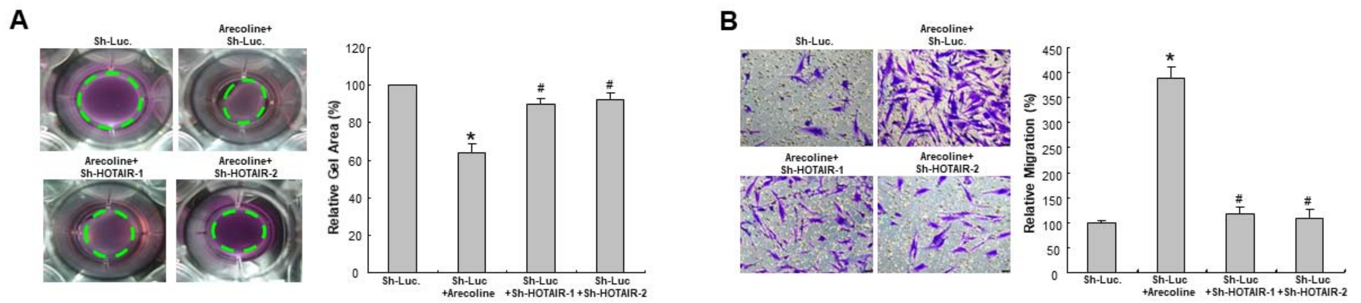


圖三:檳榔素處理可增強正常頰黏膜纖維母細胞中HOTAIR表現。



圖四:降低HOTAIR表現可抑制檳榔素所誘發肌纖維母細胞活性

降低HOTAIR表現可抑制檳榔素所誘發膠體收縮力(A)及細胞移動能力(B)。



研究方法

口腔癌前病變標本之收集及臨床資料庫之建立

經過人體試驗委員會(IRB)核准後開始蒐集檢體樣本,由本院口腔顎面外科協助收集。組織以RNA later（QIAGEN）保存，所有樣本皆在取得後48小時內完成RNA萃取步驟並置放於-80℃保存。以即時定量聚合酶連鎖反應分析HOTAIR表現量。

細胞總量 RNA 萃取

細胞以胰蛋白酶 (trypsin) 處理使細胞懸浮後，以 1000 rpm離心 5 分鐘並吸去上清液，加入 1ml TRIzol reagent (Invitrogen Life Technologies)，混合後靜置 5分鐘，之後加入 BCP (bromochloropropane) 100µl，上下搖晃使其混和均勻，靜置室溫 5 分鐘後以 12000 rpm (Eppendorf, F45-30-11) 在 4˚C 離心 15 分鐘，吸取上層液體到新的離心小管，再加入異丙酮 (isopropanol)混和均勻靜置 5 分鐘，於 4˚C 12000 rpm (Eppendorf, F45-30-11) 離心 10 分鐘，去除上清液，並且以 75D% 酒精 500µl 清洗沈澱於管底的 RNA，再於室溫 12000 rpm 離心 5 分鐘，倒掉酒精且於抽風櫃中風乾 RNA，RNA 沉澱物乾後加入 20 µl diethylpyrocarbonate (DEPC)處理過的水回溶，測量其260 nm吸光值並計算RNA濃度。

即時定量反轉錄聚合酶連鎖反應 (quantitative real-time transcriptional PCR, qRT-PCR) 分析

將 1μg 之RNA 反轉錄為互補去氧核醣核酸 (cDNA)，然後於 StepOnePlus™ Real-Time PCR System (美國Applied Biosystems 公司)進行定量即時聚合酶鏈反應 (quantitative real-time transcriptional PCR) 實驗。

Primer Name	**Forward Primers**	**Reverse Primers**
HOTAIR	GGTAGAAAAAGCAACCACGAAGC	ACATAAACCTCTGTCTGTGAGTGCC
GAPDH	CTCATGACCACAGTCCATGC	TTCAGCTCTGGGATGACCTT

統計分析

利用Sigma plot統計軟體分析結果，以Student's paired t test分析，當$p<0.05$則表示具有統計意義。實驗結果以mean+-SD表示。

利用病毒建立穩定表現抑制的HOTAIR shRNA

慢病毒載體由 biosettia 生技公司購得。將 pLV-hU6-EF1a-green-shHOTAIR 及 lentivector plus helper plasmid (VSVG 及 Gag-Pol) 混和後利用 Lipofectamine 2000 (LF2000, Invitrogen, Calsbad) 共同轉染至 293T 細胞，48 小時後收集上清液得到慢病毒 (lentivirus)。將欲感染的細胞加入 8 µg/ml polybrene (Sigma-Aldrich)並利用 lentivirus 感染，因為此載體帶有綠色螢光蛋白 (green fluorescent protein, GFP)，可利用流式細胞儀將帶有 GFP 的細胞分選，GFP 作為細胞是否有被感染成功並表現 shRNAi

的依據。HOTAIR shRNA 序列如下：Sh-HOTAIR-1: 5'-AAAAGGAGTACAGAGAGAATAATTTGGATCCAAATTATTCTCTCTGTACTCC-3';Sh-HOTAIR-2:5' -AAAAGCTTCCTTGCTCTTCTTATTTGGATCCAAATAAGAAGAGCAAGGAAGC-3'。

膠原蛋白膠體收縮實驗(Collagen contraction assay)

首先將細胞繼代收下後，以細胞量 1~2×10^5 加入 1.5 ml 微量離心管並以 300 g 離心 5 分鐘後移除上清液，並加入 500 μl 的 collagen solution(配置表如下)混合均勻後，注入 24 孔盤並置入 37°C 培養箱 2 小時等待凝固。接著準備合適且滅菌過的刮勺，以利將凝固的膠體從 24 孔盤壁面剝離，並額外加入無血清(serum-free)培養液，再放入 37°C 培養箱靜置 1~5 天觀察膠體收縮情形，拍照並使用 ImageJ 軟體分析膠體面積大小。

Collagen Solution(假設配置 4 ml):

2× basal medium	2 ml
Collagen (6mg / ml)	1.33 ml
滅菌 ddH_2O	0.67 ml

細胞移行試驗（Cell migration assay）

將每樣品取 2×10^4 細胞與不含血清的培養液 250 μl 加入轉移盤（Transwell®, 8μm pore size, polycarbonate membrane, Corning ® Costar）之上層盤中，並於下層盤加入 750 μl 含有10 % FBS 的培養液，放入細胞培養箱培養，誘使細胞移行。24 小時後，將轉移盤取出，並以 PBS 沾濕的棉花棒將上層盤內未移行的細胞清除，以 4 % paraformaldehyde 固定 10 分鐘，用PBS清洗 2 次，以 0.1% 結晶紫染細胞後，再用 PBS 清洗，將上層盤的膜小心切下放於載玻片，以顯微鏡 100 X 視野觀察，每個膜取5個視野，平均值為細胞相對移行能力，每次實驗各組皆做 3 重複。

統計分析

利用Sigma plot統計軟體分析結果，以Student's paired t test分析，當$p<0.05$則表示具有統計意義。實驗結果以mean+-SD表示。

子計畫二: 闡明長鏈非編碼 RNA 調節口腔癌幹細胞化及間質轉化之分子機制及臨床意義

研究計畫之背景及目的

1. MEG3（maternally expressed 3）

MEG3為一種母系銘記基因（maternally expressed imprinted gene），位於染色體14q32.2的位置，在2000年由Miyoshi等人發現[42]。MEG3是可以修飾染色質的一種lncRNAs。就像HOTAIR一樣，MEG3也會與PRC2複合體連結來調節不同的基因，包含像是TGF-β訊號路徑[43]。過表現MEG3被證實可以減少TGF-β路徑中的TGFB2、TGFBR1、SMAD2等基因。利用小干擾核糖核酸技術（siRNA）去降低MEG3的表現則能活化下游的ACTC1、CNN1與COL5A1 [43]。MEG3已知可以來抑制肝臟星狀細胞（hepatic stellate cells）的活化與肝纖維化。該研究指出，TGF-β會降低MEG3的表現量，而過表現MEG3則會使暴露於TGF-β1的肝臟星狀細胞降低產生α-SMA與Col1A1並開始細胞凋亡[44]。這些都顯示出MEG3與TGF-β路徑所引起的纖維化之關聯。MEG3普遍被認為是一種抑癌因子[45]，它的表現量在眾多癌症組織中皆降低，且與癌症預後和腫瘤大小有關[46]。另外降低MEG3的表現也被發現會增加細胞的侵入性[43]。但近期一篇研究卻指出MEG3的表現量在肺癌細胞經歷EMT的過程中會增加。讓MEG3基因減量（knockdown）會抑制由TGF-β誘發的EMT特徵與部分相關基因表現，過表現MEG則會加強TGF-β所造成的效果[47]。EMT的過程會讓細胞出現間質細胞的特性，開始能夠侵入附近的組織並脫離原先的群塊[48]。因此，EMT被認為是產生癌幹細胞的一個重要來源[49]，並且與癌症的侵入性與轉移性有密切關聯[50]。而與EMT相關的MEG3會如何影響癌症轉移，尤其在口腔癌方面的影響，便成為值得研究的議題。

作為抑癌因子的相關機制方面，MEG3已被發現可與p53的DNA結合區（DNA-binding domain）做結合而調節p53下游基因[51]，並且被證實可以減少非小細胞肺癌[46]與肝癌細胞[51]的增生。低表現量的MEG3與肺癌細胞對順鉑（cisplatin）產生抗藥性相關。目前已知可能是藉由調控p53/Bcl-xl [52]或活化Wnt/β-catenin路徑[53]。而MEG3也能誘發細胞週期停滯與促進癌細胞凋亡來增加化療的敏感度[52, 53]。最近也有研究利用MEG3的抑癌效果來檢測一種抗心律不整藥物adenosine對肝癌細胞的抗癌成效。他們發現adenosine可以藉由抑制DNA甲基化來增加MEG3的表現量，導致p53、caspase-3的增加與MDM2、cyclin D1減少。顯示由adenosine增加的MEG3同樣會促進癌細胞凋亡與細胞週期停滯而有抗癌效果[54]。與其他癌症的結果一致，在舌癌的病人檢體中，MEG3的表現量顯著減少。而在舌癌細胞中過度表現MEG3則可抑制細胞增生與促進細胞凋亡[55]。在檳榔素造成口腔鱗狀細胞癌的致癌機制中，目前已知檳榔素會降低MEG3的表現量，同時也活化Wnt-7b/GSK-3β/β-catenin路徑[56]。雖然該研究指出MEG3在口腔鱗狀細胞癌中是藉由甲基化機制被調節，MEG3的缺失是否會導致癌幹細胞的增加目前仍未明。

2. 癌幹細胞

癌幹細胞（cancer stem cells）又稱為腫瘤起始細胞（tumor initiating cells）。在 1997 年，Bonnet 與 Dick 從急性骨髓性白血病患（acute myeloid leukemia）身上獲取 $CD34^{+}CD38^{-}$細胞並從尾靜脈注射進免疫缺陷鼠，發現這群獨特的細胞會在小鼠身上誘發血癌的症狀[57]，成為第一篇證實癌幹細胞存在的有力證據。爾後，不同癌症陸續被發現有癌幹細胞。顧名思義，該種細胞對癌症的產生、復發、轉移，甚至抗藥性都有很大的關聯。腫瘤本身包含癌幹細胞與其他細胞，現存的抗癌治療僅能消除或減少非癌幹細胞。因此只要癌幹細胞存在，便可能不斷復發。癌幹細胞僅占腫瘤一小部分並且會表現幹細胞標記，像是 CD44 [58]、CD133 [59]和 ALDH [60]等。癌幹細胞不僅具有形成腫瘤的能力，同時也像其他幹細胞一樣，具有多向分化（multi-lineage differentiation）與自我再生（self-renewal）的潛能[61]。因此調控這些特性的基因若發生異常，便可能與癌幹細胞的產生有關[62]。例如像是 Bmi-1 就被發現對正常幹細胞與癌幹細胞中的自我再生能力相當重要[63, 64]，並且可能與 MEG3 的表現量有關。先前在肺幹細胞自我再生能力的相關研究中已指出，包含 MEG3 在內的許多銘記基因都受到 Bmi 的調節，顯示出這些銘記基因與幹細胞自我修復能力的關聯[65]。Wnt/β-catenin 路徑也被認為與正常幹細胞[66]和癌幹細胞自我再生能力有關[67]。不少研究已發現 MEG3 是經由 Wnt/β-catenin 路徑來調節許多生物特性。除了上段提及檳榔素可能會降低 MEG3 表現量而活化 Wnt/β-catenin 路徑並造成口腔鱗狀細胞癌之外[56]，低表現量的 MEG3 所引起的抗藥性也是經由活化 Wnt/β-catenin 路徑而來[53]。因此，低度表現的 MEG3 是否會藉由此路徑來產生癌幹細胞特性需要進一步的研究驗證。此外，不少實驗也指出 MEG3 對幹細胞分化能力有所影響。例如 MEG3 對骨髓幹細胞在成骨分化（osteogenic differentiation）時非常重要[68]；胚胎幹細胞若有 MEG3 缺失也會影響其神經分化的能力[69]。這些證據都指出 MEG3 與癌幹細胞特性的可能關聯，包含像是自我再生、分化能力、EMT 或抗藥性等。然而去證實 MEG3 的異常表現與癌幹細胞化的文獻目前仍相當缺乏，更遑論 MEG3 表現缺失在口腔癌幹細胞的影響。值得一提的是，我們的先導實驗已經發現在 OSF 的組織裡 HOTAIR 有高度表現，而在口腔癌的組織裡 MEG3 則是低度表現。HOTAIR 已被證實能夠增加癌幹細胞的腫瘤生成能力[70]，且在子實驗一我們提及已有文獻指出 HOTAIR 能抑制 MEG3/p53 路徑[20]，因此 HOTAIR 的變化是否會影響 MEG3 進而造成癌幹細胞的增加便成為我們所關心的主題之一。

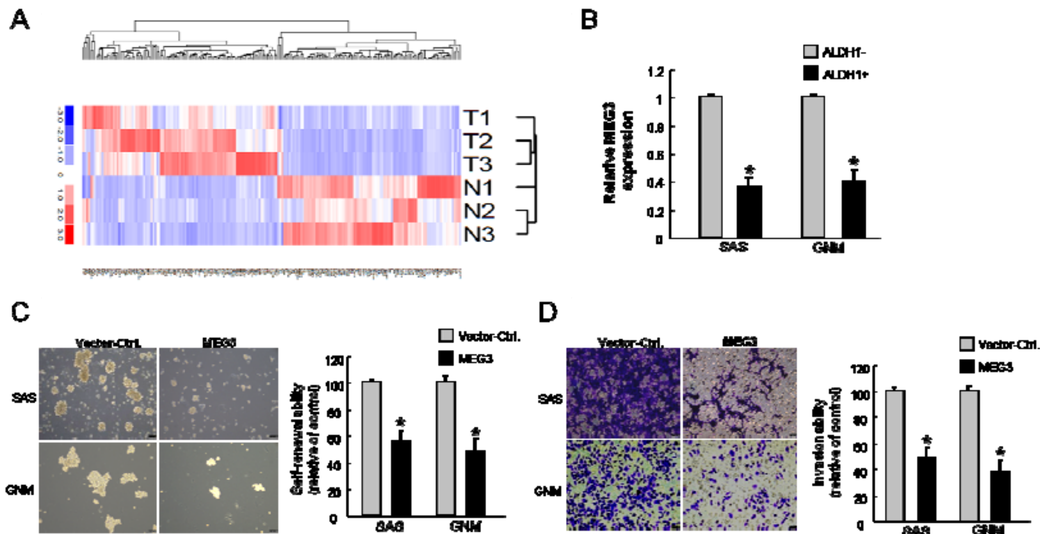
研究目的

本研究團隊先前研究發現，癌幹性存在對於傳統化療及放射性療法具備阻抗性及高度轉移力，癌幹細胞應是導致口腔癌病患病灶復發致死的主因。因此，尋找癌幹細胞新穎生物標誌及有效標靶療法將有助於口腔癌臨床上診斷及及輔佐治療。上皮間質轉換過程被認為是癌症產生與癌症轉移的一個重要步驟，而作為抑癌因子的MEG3在肺癌細胞進行此過程中有異常表現。因此，我們便產生想探討MEG3在口腔癌腫瘤轉移上的關聯性。我們的先導研究成果利用新世代定序技術及即時定量聚合酶連鎖反應分

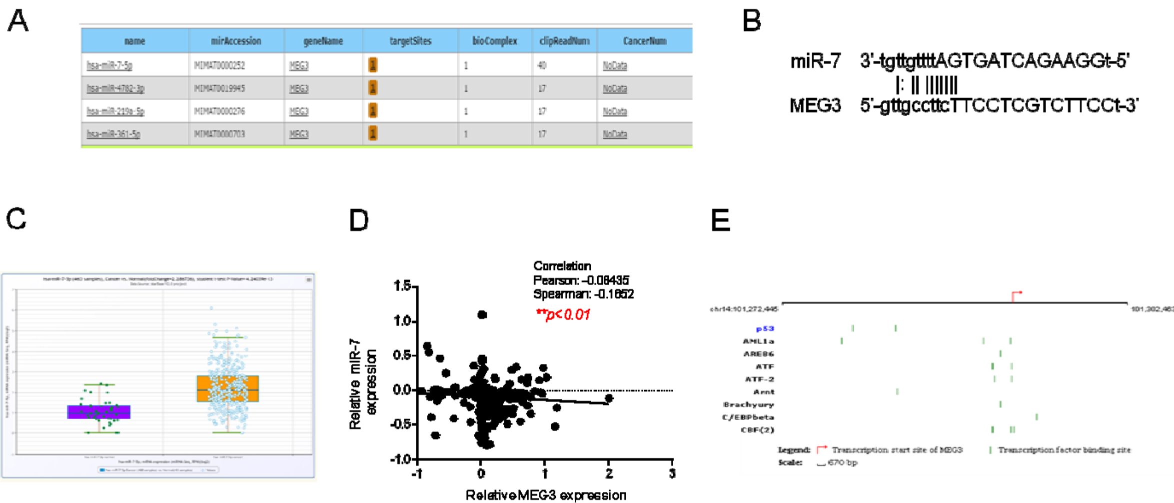
析鑑定出lncRNA MEG3於口腔癌檢體及口腔癌幹細胞中皆為低度表現。且不少文獻已經指出MEG3與癌幹細胞的許多特性有關，因此MEG3低度表現是否會造成癌幹細胞化的增加便需要進一步的去研究與確認。

子計畫二研究成果

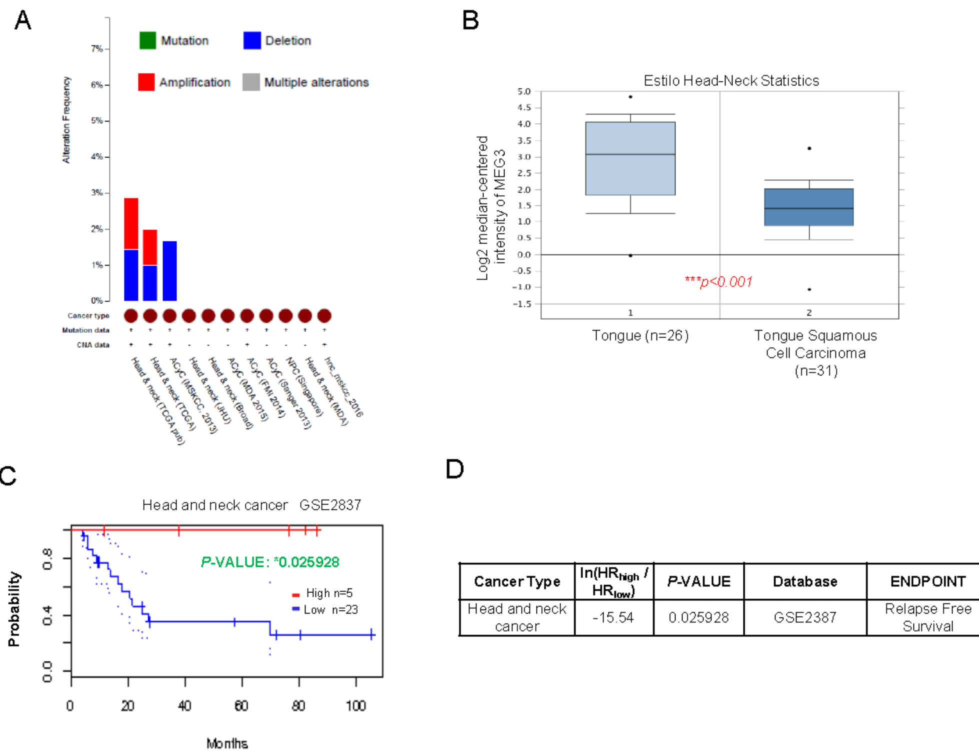
圖一: MEG3於口腔癌低表達且可調控幹細胞化。(A) 利用新世代定序技術鑑定出lncRNAs於口腔癌腫瘤組織中有差異性表現，MEG3是其中明顯降的lncRNA; (B) MEG3於ALDH1+口腔癌幹細胞表現低下; 過度表現MEG3可降低幹細胞球體形成力(C)及細胞侵襲性(D)。



圖二: 鑑定MEG3具備ceRNA功能及上游關鍵因子預測。(A) 利用生物資訊軟體starBase v2.0 database預測口腔癌中MEG3可結合之miRNAs; (B) miR-7與MEG3結合配對序列; (C) miR-7 在口腔癌檢體為高度表現(D) 美國癌症基因體圖譜計畫資料庫TCGA分析出口腔癌檢體中MEG3與miR-7表現高度負相關; (E)預測MEG3 promoter可能結合轉錄因子

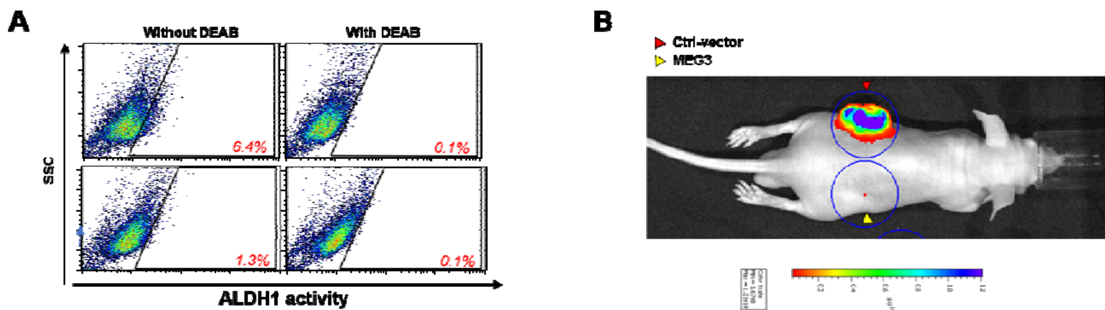


圖三: MEG3於口腔癌臨床病理意義。(A)於cBioportal for cancer genomics 資料庫中MEG3於頭頸癌檢體多為表現缺失; (B) Oncomine database鑑定出MEG3 於舌癌檢體為低表現; (C)(D) PrognoScan database鑑定出口腔癌病患高表現MEG3預後較佳



Cancer Type	$ln(HR_{high} / HR_{low})$	P-VALUE	Database	ENDPOINT
Head and neck cancer	-15.54	0.025928	GSE2387	Relapse Free Survival

圖四: MEG3過度表現可降降低ALDH1活性(A)及活體腫瘤生成力(B)



研究方法

研究方法

ALDH1+癌幹細胞分選

口腔癌細胞株(SAS及GNM)用以ALDEFLUOR 試劑(Stem cell Technologies)利用Fluorescence-activated cell sorter (BD FACS Aria)方式偵測細胞內癌幹細胞標記Aldehyde dehydrogenase 活性並分選ALDH1高表現(ALDH1+)及低表現(ALDH1-)的細胞。

癌幹細胞球體培養

利用無血清幹細胞生長因子培養液自口腔癌細胞篩選並且培養瘤源幹細胞。無血清幹細胞生長因子培養液配方如下：DMEM/F-12 (Gibco)、bFGF 20 ng/ml、EGF 20 ng/ml、N2-supplement (R&D System, Minneapolis, MN。先種 1 x 10^4 細胞於直徑 10 公分的低貼附細胞培養皿，隔天將培養液換成選擇性培養液，持續觀察 一 週，細胞由原本貼附在培養皿的狀態，漸漸聚集成許多細胞所構成的球體。

細胞爬行能力

詳細實驗步驟如同子計畫一。

活體腫瘤生成及轉移試驗

由國家實驗動物中心購入5~6 週之BALB/cAnN.Cg-Foxn1nu/CrlNarl 雌性裸鼠。將基因轉殖成功口腔癌幹細胞經皮下或尾靜脈注入，為了進一步觀察癌細胞在小鼠體內轉移的情形，將利用 IVIS Lumina

Imaging System 活體影像系統來監測 小鼠腫瘤轉移與生長情形。六週後犧牲老鼠，取出腫瘤及肺臟以觀察其腫瘤大小、重量及 轉移至肺部的能力。動物存活率分析以Kaplan Meier 方式分析。

統計分析

利用Sigma plot統計軟體分析結果，以Student's paired t test分析，當$p<0.05$則表示具有統計意義。實驗結果以mean+-SD表示。

子計畫三: 調節口腔癌細胞自噬與長鏈非編碼 RNA 之恆定增進 5-Fluorouracil 抗藥性敏感度及改善其不良副作用

中文摘要:

5-FU 是頭頸癌治療用藥，然而其副作用如粘膜炎與胃口不佳，常導致化療中止。本研究的目的為評估真菌類免疫調節蛋白 GMI 是否能改善 5-FU 所誘發之粘膜炎。小鼠每隔 4 天以腹腔注射方式給予 5-FU，進行兩循環。在 5-FU 注射前 3 天，小鼠將開始每天給予 GMI 或 PBS 直到第 14 天。以組織學分析，GMI 可防止 5-FU 導致的腸粘膜和舌上皮損傷。GMI 能增加 5-FU 對口腔癌細胞的細胞毒性，但在口腔正常細胞則無影響。總結，GMI 能減輕 5-FU 誘導的損傷並減緩正常消化道組織中的細胞凋亡，期望 GMI 可能改善病患的生活品質。

實驗步驟

Preparation for 5-fluorouracil, GMI

5-FU was purchased from NANG KUANG PHARMACEUTICAL CO., LTD. (Tainan, Taiwan). The structure of 5-FU is shown in **Fig. 1A**. The original concentration of 5-FU was 50 mg/ml and was diluted to 10 mg/ml with PBS. For animal experiments, Reishimmune-S were used which were purchased from Mycomagic Biotechnology Co., LTD. (New Taipei City, Taiwan) and GMI was the main ingredient of the edible mushroom nutritional supplement in Reishimmune-S. Reishimmune-S is a dietary ingredient approved by FDA in 2015. The protein structure of GMI was demonstrated in **Fig. 1B**. A slice of Reishimmune-S had 72 mg and was dissolved in 800 µl PBS. For cell experiments, GMI, manufactured by Mycomagic Biotechnology Co., Ltd. (Taipei, Taiwan), was generated and ameliorated from G. microsporum. The detailed expression methods of GMI were described in our previous study[16].

Animal experiments

All animal experimentation procedures were conducted according to the Affidavit of Approval of Animal Use Protocol, Chung Shan Medical University Experimental Animal Center, Taichung, Taiwan (Approval No: 1375). Five-week-old male BALB/c mice (body weight 18-20 g) were purchased from the National Laboratory Breeding Research Center in Taiwan. Mice were housed under pathogen-free conditions with a 12 h light/12 h dark cycle, and fed an autoclaved diet with ad libitum access to standard rodent chow (LabDiet, 5001) during the study. After one week, animals were randomly divided into three groups consisting of five animals each: (1) Control group: from day -3, 200µl PBS were administered by gavage twice daily; (2) 5-FU group: 5-FU were dissolved in PBS and administered by a single intraperitoneal (i.p.) injection (50 mg/kg/day) to induce oral and intestinal mucositis on days 1 to 4 and 8 to 11. The accumulated dose of 5-FU was similar to those reported in previous studies[17]. (3) 5-FU+GMI group: Reishimmune-S (GMI) 72 mg was dissolved in 800 µl PBS and administered by gavage at 18 mg per mouse twice for 3 days before 5-FU injection. The schematics of the treatment regimen is shown in **Fig. 1C**. All animals were sacrificed on day 14. Blood samples were obtained with some of the blood rinsed EDTA to prevent coagulation and used to determine complete blood count (CBC) on a Hemavet automated cell counter (Sysmex KX-21). The remaining blood

was centrifuged at 4 °C and the plasma was frozen at−80 °C until analysis. A 10-cm section of the proximal jejunum was collected and gently flushed with saline. The contents of the jejunum and tongue were immediately removed were removed and fixed in 10% buffered formalin for 24 h for histological analysis and scoring.

Histological analysis

For the assessment of pathological changes in jejunum and tongue, these organs were fixed in 10% formaldehyde solution and embedded in paraffin. Five micrometer sections were stained with haematoxylin and eosin (H & E). The method for producing histological samples was followed by our previous study[12].

Cell line

SCC9, a human tongue squamous cell carcinoma cell line, SAS, a human oral squamous cell carcinoma were cultured in Dulbecco's modified Eagle's medium supplemented with an equal volume of a nutrient mixture, F-12 Ham's medium (Life Technologies, Grand Island, NY, USA), 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA), 2 mM glutamine, 100 U/ml penicillin, 100 μg/mL streptomycin. SG, immortalized human gingival keratinocytes was cultured in Dulbecco's modified Eagle's medium supplement with compounds mentioned above without F-12 Ham's medium. The medium for SCC-9 also contains 400 ng/ml hydrocortisone and 1% non-essential amino acids (NEAA). All cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Cell viability

2×10^4 tongue squamous carcinoma cells, SAS and SCC9 were seeded onto 96-well plates containing 100 μl of culture medium. After 24 h incubation, the medium was carefully removed and 100 μl of fresh medium with different concentration of 5-FU (0, 1, 10, 100 μM) and different concentration of GMI (0, 0.3, 0.6, 1.2 μM) was added. GMI was pretreated for one hour before 5-FU was added. Cells were treated with 5-FU and GMI for 24 and 48 h. At the end of this process, the medium was carefully removed and 100 μl of fresh medium containing 0.5 mg/ml MTT (Thiazolyl Blue Tetrazolium Bromide) (Sigma, M 2128) were added to the wells. The intensity is measured colorimetrically at a wavelength of 570 nm. Absorbance values are presented as the mean ± SE of three replicates for each treatment. Cells in controls and compound controls were included. Absorbance of untreated cells was considered 100%.

Western blot analysis

Cells were lysed with RIPA buffer containing protease and phosphatase inhibitor cocktail (Roche, 04 693 159 001) and protein concentration was assayed with Bio-Rad Protein Assay
Kit (Bio-Rad, 500-0006). Equal amounts of proteins from each sample were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (PVDF) (Amersham, RPN303F). Membrane was blocked for 1 h in TBS containing 5% nonfat milk and 0.2% Tween 20. For the detection of Bax, Bcl-2, PARP, caspase 7, cleaved caspase 7 and β-actin, monoclonal anti-Bax (Cell Signaling, 2772), monoclonal-Bcl-2 (Cell Signaling, 4223), monoclonal anti-PARP (Cell Signaling, 9542), monoclonal caspase 7 (Novus Biologicals, NB100-56529), monoclonal cleaved caspase 7 (Cell signaling, 9491) and monoclonal anti-β-actin (Sigma, AC-40) were incubated with membranes at 4°C overnight. Membranes were subsequently washed for 3–5 min in 0.2% TBS-Tween 20, incubated in HRP-conjugated secondary antibody for 1 h, washed again and visualized with enhanced luminol reagent for chemiluminescence (PerkinElmer, NEL104).

Flow cytometry

SAS and SCC9 cells (8×10^5 cells/60 mm dish) were first pretreated with GMI (0.3 or 0.6 μM) for 1 h, and then co-treated with GMI and 5-FU (10 μM) for 48 h. After that, cells were washed twice with pre-cooled

PBS, trypsinized, and incubated with a binding buffer containing Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (BioVision, K101). Flow cytometry analysis was performed using FACScalibur Flow Cytometer (BD Biosciences). A minimum of 10,000 cells were analyzed per sample and illustrated as dots plot using CellQuest Pro software.

Statistical analysis

All data are presented as mean ± standard deviation (SD). In our animal study, statistical comparisons of the different treatment groups were carried out by Student's t test or Tukey post-hoc test in ANOVA. $p < 0.05$ was considered statistically significant.

實驗結果:

Body weight loss and anorexia are common adverse effects after treatment with 5-FU. To figure out whether these adverse effects are associated with mucositis, the weight, diet, and water intake were first measured daily and the results of all groups were compared. Those mice in the 5-FU group had higher body weight (BW) loss than those in the control group and there was significant loss on day 9 to 11(**Fig. 2**). However, the BW loss of the mice in the 5-FU+GMI group were significantly less than those in the 5-FU group on the second week. In addition, all groups which were injected 5-FU had worse food and water intake after 5-FU injection in first week (Data were not shown). And then, two to three days after the injection in the first-week, the group of 5-FU, 5-FU+GMI could have a better diet. Food intake of the 5-FU+GMI group could even recover closely to the control group. However, because we didn't use the metabolic cage and all mice in the same group were fed in one cage, the standard deviation of diet and water consumption could not be measured.

Effects of GMI on Complete Blood counts (CBCs)

Chemotherapy, including 5-FU usually leads to severe leukopenia. To figure out whether GMI could prevent mice from 5-FU-induced leukopenia, we analyzed the complete blood counts. The data is shown in **Table 1**. When comparing to the control group, we observed significant decreases in white blood cell WBC counts in 5-FU-treated mice ($p < 0.01$). However, GMI administration could only slightly increase the WBC counts but there was no significant difference between the 5-FU alone group and the 5-FU+GMI group. In addition, mean corpuscular hemoglobin content (MCH) and mean corpuscular hemoglobin concentration (MCHC) significantly rose in the 5-FU+GMI group compared to the control group ($p < 0.05$).

Effects of GMI on oral and intestinal histology in 5-FU treated mice

When mucositis happens after treatment with 5-FU, leukocytes infiltration is an important process of inflammation and helps healing of tissue. To know whether GMI can prevent damage of intestinal mucosa induced by 5-FU and whether GMI can alleviate leukocytes infiltration, the histology of jejunum samples were observed with H&E staining. Repeated administration of 5-FU (50 mg/kg) caused substantial changes in the intestinal mucosal layer including flattened epithelial layer, shortened villi and thinning lamina propria with inflammatory cells infiltration (**Fig. 3A**). The mucosa in 5-FU group underwent nercrosis and the villi were almost unable to be determined. Intestinal villus length and crypt of Lieberkühn depth was determined on NIS-Elements D 3.2 imaging system. Mice treated with GMI prior to 5-FU showed significant reductions in structural damage to the mucosal layer and shortening of intestinal villus length compared to mice treated with 5-FU alone (**Fig. 3B** and **3C**).

As for tongue tissue, we can see that 5-FU administration led to a significantly thinner mucosa when comparing to the control group (**Fig. 3E**). In addition, 5-FU also damaged the filiform papilla on the mucosal layer and made them fewer compared to the control group. However, GMI administration could protect the tongue epithelium from damage. After GMI treatment, the thickness of tongue mucosa could recover to a

degree that is similar to the control group (**Fig. 3F**) and the filiform papilla could be observed more frequently than the 5-FU group.

GMI protects intestine from apoptosis after 5-FU treatment

5-FU might inhibit proliferation of mice intestine crypt and increase the number of cleaved caspase 3 and caspase 8-positive cells[18]. This indicates 5-FU induced apoptosis of intestine mucosa. Therefore, to figure out whether GMI can attenuate apoptosis of enterocytes in small intestine caused by 5-FU, proteins of mice intestine tissue were extracted and the expression of some apoptosis markers was analyzed by western blot. The data is shown in **Fig. 3D**. In 5-FU+PBS group, the expression of anti-apoptosis protein, Bcl-2 was decreased while the expression of Bax and cleaved caspase 7 were increased. At the same time, the 5-FU+GMI group showed reversed expression of Bcl-2 and Bax. However, there were no expression difference of cleaved caspase 7 between the 5-FU+PBS and the 5-FU+GMI group.

GMI enhances the cytotoxic effects of 5-FU on oral cancer cells

To confirm whether GMI may interfere with the therapeutic effect of 5-FU on head and neck cancer, we assessed the cell viability of two oral cancer cell line and one oral keratinocyte cell line. Cells were first pretreated GMI with three doses (0.3, 0.6, and 1.2 μM) for one hour and then treated 5-FU with different doses (1, 10, 100 μM) for 24 and 48 hours. Finally, MTT assay were used to analyze the cell viability. The results showed 5-FU induced oral cancer cell death in a concentration-dependent manner. Compared to the untreated cells, cell death of 38% and 6% at 24 h (**Fig. 4A** and **4C**) and 53% and 17% at 48 h (**Fig. 4B** and **4D**) were observed for SAS and SCC9 cells treated with 5-FU at 100 μM. All data have significance except SCC9 at 24 h. For treatment of lower dose of GMI (0.3 μM), GMI only could significantly enhance the cell death of SAS cells on the treatment of highest dose of 5-FU (100 μM) at 48 h while the highest dose of GMI (1.2 μM) could induce higher cell death when cells were co-treated with different doses of 5-FU. The combination effect was more obvious in SCC9. However, there was little influence on cell death when SCC9 cells were co-treated with different doses of GMI. As for the results of SG, the oral keratinocyte, significant cell death was only observed when cells were treated 100 μM 5-FU at 48 h while there were no enhancing effects observed in co-treatment of 5-FU and GMI (**Fig. 4E** and **4F**). For two cell models, we didn’t observe a higher cell viability when cells were pretreated with GMI, which indicated GMI would not interfere with the effect of 5-FU on oral cancer but may enhance the cytotoxicity. However, this enhancing effect only appeared in oral cancer cells but not normal cells.

GMI enhances apoptosis of oral cancer cells induced by 5-FU

With the cell viability result, we further investigate whether GMI could enhance the 5-FU-induced apoptosis on oral cancer. 0.3 or 0.6 μM of GMI were pretreated for one hour and then 10μM of 5-FU were co-treated for 48 hours. Western blot and flow cytometry were used to analyze the apoptosis. For the western blot results, the level of cleaved caspase 7 was increased in all treatment group in SAS cells (**Fig. 5A**), especially the 0.6 μM GMI alone and the 5-FU alone group. The cleaved form of PARP was expressed most obviously in the 5-FU plus 0.3 μM GMI group. For SCC9 cells, the highest level of cleaved caspase 7 were observed in the GMI alone groups and the 5-FU plus 0.3 μM GMI group (**Fig. 5B**). Also, there were an increasing expression of cleaved PARP appeared in the 5-FU alone group. Furthermore, Annexin-V and PI staining were applied to confirm apoptotic changes in 5-FU and GMI treated with SAS and SCC9 cells (**Fig. 5C** and **5D**). The results revealed that both cells treated 5-FU for 48 h exhibited late apoptosis. There were 17.25 and 8.61% for SAS and SCC9 cells in the late apoptosis stage respectively. SAS cells showed a dose-dependent apoptosis after treated GMI while obvious apoptosis was observed in SCC9 treated the middle dose of GMI. For combined treatment, both cells exhibited a good response of either apoptosis or necrosis. More cells in necrosis appeared

in SAS cells while more cells in late apoptosis appeared in SCC9 cells. Both cells demonstrated a dose-dependent response after co-treatment of 5-FU and GMI for 48 h.

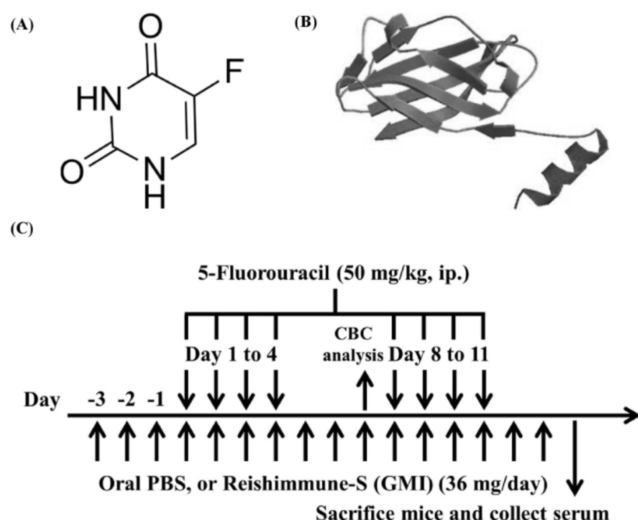


Fig. 1
The schematics of experimental procedure and the chemical structure of 5-FU and GMI. (A) The structure of 5-FU (B)The protein structure of GMI. (C) Six week-old mice were divided into three groups and treated as follows: (1) PBS as control, (2) 5-Fluorouracil plus PBS, (3)5-Fluorouracil plus GMI. The 5-FU-treated mice were pretreated with Reishimmune-S (GMI) (36 mg/day) for 3 days before 5-FU injection. 5-FU was injected on day 1 to 4 and day 8 to 11. At day 7, blood was collected to do CBC analysis. At day 14 after the final injection of 5-FU, mice were sacrifice and the blood serum were collected.

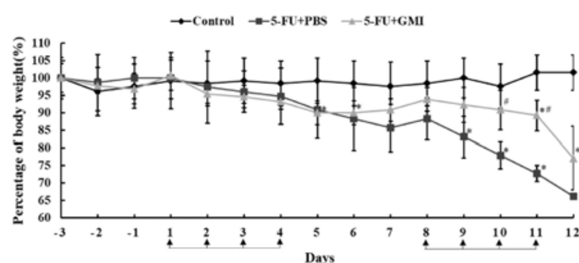


Fig. 2
The effects of GMI on mice body weight. Body weights of mice were measured every day before GMI was fed. The percentage of body weight on every mice were calculated when it was compared to the control group at day -3. Means ± SD were showed. *, $p < 0.05$ when it was compared to the control group. #, $p < 0.05$ when it was compared to the 5-FU + PBS group.

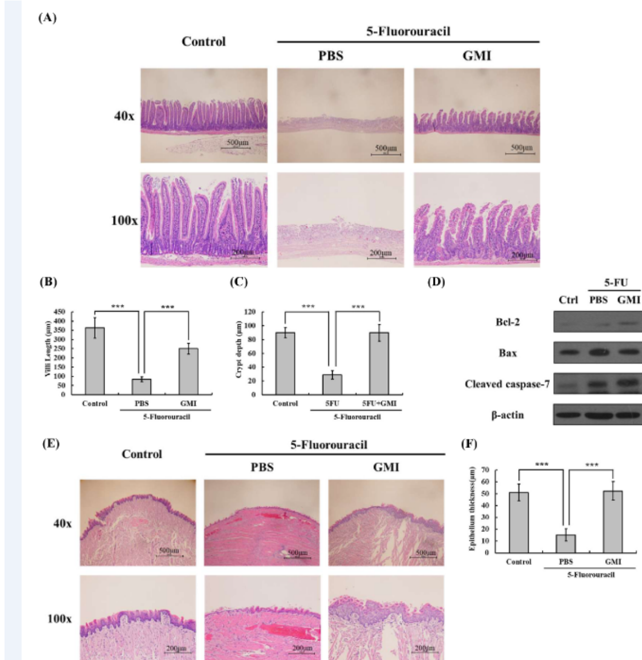


Fig. 3

Histological changes in the intestinal villi and tongue were determined using H&E staining. (A) The upper three pictures showed the longitudinal section of jejunum on 40x field while the lower three pictures showed the structure on 100x field. (B)(C) The villi length and crypt depth of intestinal villi were randomly measured in different parts of jejunum on the same group of samples. ***, $p < 0.001$. (D) Cell lysates of intestinal samples were extracted and the level of Bcl-2, Bax, and cleaved caspase 7 were analyzed by western blot. β-actin was used as an internal control. (E) The upper three pictures showed the cross section of tongue on 40x field while the lower three pictures showed the structure on 100x field. (F) The epithelial thickness of the tip tongue was measured. ***, $p < 0.001$. Data presented are means ± SD.

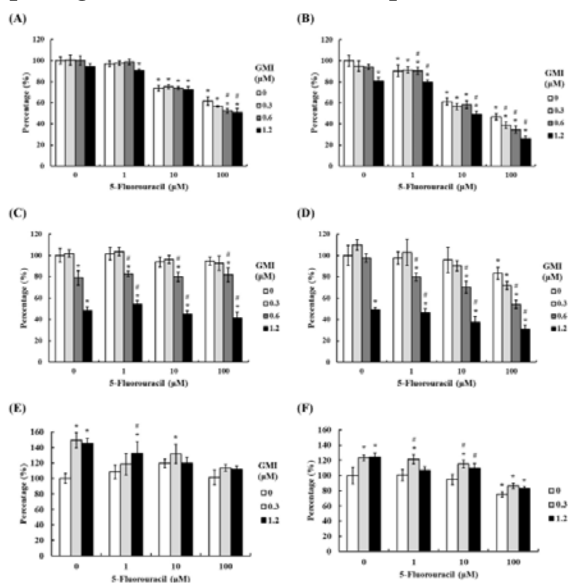


Fig. 4

The effects of 5-FU and GMI on cell viability of SAS and SCC9 cells. (A)(B) SAS, (C)(D) SCC9 cells, and (E)(F) SG cells (1 x 104 cells/well of 96-well plate) were treated with various concentrations of GMI (0, 0.3, 0.6 and 1.2 μM) and various concentration of 5-FU (0, 1, 10, 100μM) for 24 and 48 h. Cell viability was analyzed by the MTT assay. The untreated group was presented 100 % and the percentages of other groups were calculated versus the untreated group. Means ± SD were showed. *, $p < 0.05$ versus the untreated group while #, $p < 0.05$ versus the same concentration of 5-FU but non-treatment of GMI group.

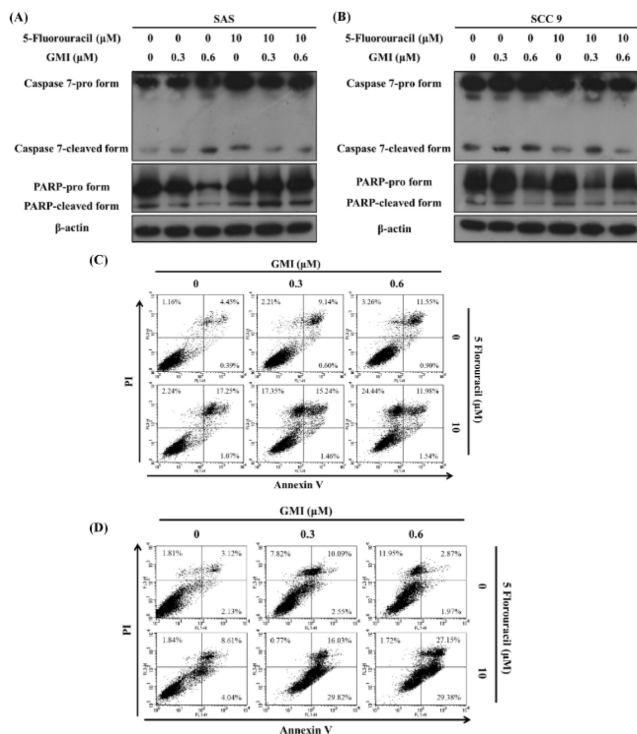


Fig. 5
The effects of 5-FU and GMI on the apoptosis of SAS and SCC9 cells. Apoptosis-related proteins were detected by western blot after (A) SAS and (B) SCC9 cells (8 x 105 cells/60 mm dish) were treated with various concentrations of GMI and 5-FU for 48 h. GMI was pretreated for 1 h before 5-FU. β-actin was used as an internal control. (C) SAS and (D) SCC9 cells (8 x 105 cells/60 mm dish) were treated the same way as (A) and (B). The number of apoptotic cells were assessed by Annexin-V and PI staining through flow cytometry.

Table 1 The effect of 5-FU and GMI on complete blood counts.

Group	WBC ($\times10^3$/μl)	RBC ($\times10^6$/μl)	HGB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	PLT ($\times10^3$/μl)
Control	9.53±1.066	10.27±1.21	15.45±2.03	51.78±7.51	50.3±1.67	15.05±0.98	29.93±1.70	942.5±252.6
5-FU	3.42±1.00**	9.03±0.51	14.58±0.68	45±2.70	49.82±0.36	16.16±0.61	32.44±1.24	416.6±54.2
5-FU+GMI	3.7±0.53**	9.48±2.20	15.675±2.53	47.05±11.46	49.6±0.88	16.6±2.04*	33.5±4.88*	379.5±138.1

*, $p < 0.01$, **, $p < 0.01$ versus the control group. Data presented are means ± SD.

<u>子計畫四：探討長鏈非編碼 RNA 在天然化合物抗口腔癌轉移之作用機轉</u>

Abstract

The metastasis of oral squamous cell carcinoma (OSCC) is one of the most important causes of cancer-related deaths. Thus, various therapeutic strategies have been developed to prevent the metastasis of OSCC. Salvianolic acid A, a traditional Chinese medicine, has antithrombosis, antiplatelet, anti-inflammation, and antitumor activities. Here, we provide molecular evidence indicating that salvianolic acid A exerts its antimetastatic effects by markedly inhibiting the invasion and migration of oral squamous SCC-9 cells. SCC-9 cells were treated with various concentrations of salvianolic acid A to further investigate the precise involvement of salvianolic acid A in cancer metastasis. The results of zymography, and Western blotting indicated that salvianolic acid A treatment may decrease matrix metallopoteinase-2 (MMP-2) expression. Salvianolic acid A also inhibited p-c-Raf, p-MEK1/2, and p-ERK1/2 protein expression. In addition, salvianolic acid A also increased the long non-coding RNA (lncRNA) Gas5 expression, while treating Gas5 siRNA reversed SCC-9 cell migration. Our findings suggested that salvianolic acid A inhibits the invasion and migration of OSCC by inhibiting the c-Raf/MEK/ERK pathways that control MMP-2 expression. Our findings provide new insights into the molecular mechanisms that underlie the antimetastatic effect of

salvianolic acid A and are thus valuable for the development of treatment strategies for metastatic OSCC.

本子計畫首先利用MTT assay偵測丹酚酸A (salvianolic acid A)在不同濃度下對口腔癌細胞SCC-9存活率的影響 (0、12.5、25、50 μM)，結果顯示丹酚酸A對SCC-9的細胞存活率並沒有顯著的影響，並可以藉由抑制MMP-2的分泌來降低癌細胞的轉移與侵襲。另外，在訊息傳遞路徑部分也發現丹酚酸A對於p-c-Raf, p-MEK1/2, and p-ERK1/2都具有明顯的抑制效果。我們進一步利用Real-time PCR偵測與轉移有關的長鏈非編碼RNA (long non-coding RNA; lncRNA)，結果發現丹酚酸A可以有效的增加lncRNA-Gas5的表現量，且利用Gas5的siRNA處理下，會反轉由丹酚酸A所抑制的細胞轉移現象。綜合以上結果，lncRNA-Gas5可能參與在丹酚酸A所抑制的口腔癌細胞轉移現象，lncRNA-Gas5在未來或許可以應用在預防口腔癌的轉移或輔助口腔癌的治療。

RESULTS

Salvianolic acid A has no cytotoxic effects on SCC-9 Cells

In this study, the cytotoxicity of salvianolic acid A was determined by treating SCC-9 cells with various concentrations of salvianolic acid A for 24 h. Cell viability was then quantified through MTT assay. Relative to that of the controls, the cell viability of SCC-9 cells treated with salvianolic acid A was not significantly altered (Figure 1). Thus, at concentrations of 0 to 50 μM, salvianolic acid A exerted no cytotoxic effects on SCC-9 cells. Therefore, salvianolic acid A was applied at this concentration range in the following experiments.

Salvianolic acid A inhibits the migration and invasion of SCC-9 cells

The inhibitory effect of salvianolic acid A on the migration and invasion of SCC-9 cells were examined to screen for potential preventive effectors for oral cancer metastasis. A wound healing migration assay was performed to analyze whether salvianolic acid A affects cell migration. SCC-9 incubated with 0.5% FBS exhibited markedly promoted cell migration in the wound area for 0-48 h after wounding, whereas those treated with salvianolic acid A showed significant delays in wound healing under the same conditions. These results showed that salvianolic acid A significantly inhibited the migration of SCC-9 (Figure 2A) cells ($p < 0.05$). The inhibitory effect of salvianolic acid A on the invasion and migration of SCC-9 cells was examined with a Boyden chamber assay. Salvianolic acid A significantly reduced the migration (cells seeded on an uncoated filter) and invasion (cells seeded on Matrigel-coated filter) of SCC-9 cells (Figure 2B).

Salvianolic acid A has inhibitory effects on MMP-2 enzyme activity, protein expression, and mRNA expression

To clarify the involvement of MMP-2 in the inhibitory effects of salvianolic acid A on cell invasion, the effects of salvianolic acid A on MMP-2 activity was investigated through gelatin zymography assay. Salvianolic acid A significantly suppressed MMP-2 activity in SCC-9 cells (Figure 3A). Western blotting was then performed to assess the effects of salvianolic acid A on MMP-2 protein expression. Treatment with salvianolic acid A also inhibited MMP-2 protein expression in SCC-9 cells (Figure 3B). Semi-quantitative RT-PCR and real time PCR analysis were performed to further evaluate if the significant regulatory effect of salvianolic acid A on MMP-2 occurred through transcriptional regulation. Salvianolic acid A treatment significantly decreased MMP-2 mRNA levels (Figure 3C). The results of these analyses collectively demonstrated that salvianolic acid A reduces MMP-2 activity by inhibiting MMP-2 expression at the mRNA and protein levels.

Salvianolic acid A inhibits the phosphorylation of c-Raf, MEK1/2, and ERK1/2 in SCC-9 cells

The mechanisms that underlie the inhibitory effect of salvianolic acid A on the invasion ability of and MMP-2 activity in SCC-9 were further investigated by studying its effects on MEK/MAPK and FAK pathways. As shown in Figure 4, salvianolic acid A significantly inhibited the phosphorylation of c-Raf, MEK1/2, Src, and FAK in SCC-9 cells. Moreover, salvianolic acid A significantly inhibited the phosphorylation of ERK1/2 but did not affect that of p38, and JNK1/2 (Figure 5).

Salvianolic acid A inhibits MMP-2 expression and cell invasion by inactivating MEK /ERK1/2 signaling pathways

To further determine whether the inhibitory effects of salvianolic acid A on MMP-2 expression and cell migration are mainly mediated through the inhibition of the MEK/ERK1/2 signaling pathway, SCC-9 cells were pretreated with a MEK inhibitor (U0126; 10 μM) for 30 min and then incubated with or without salvianolic acid A for 24 h. The results of Gelatin zymography analysis demonstrated that combined treatment of salvianolic acid A and U0126 significantly suppressed MMP-2 activity in SCC-9 cells (Figure 6A). The results of Boyden chamber assays showed that combined treatment with 25 μM salvianolic acid A and 10 μM U0126 could further reduce cell migration by 59% (Figure 6B). These results indicated that salvianiolic acid inhibits MMP-2 expression and migration in SCC-9 cells partly by inactivating MEK/ERK1/2.

Salvianolic acid A Induces Expression of lncRNA Gas5, Causing reduced cell migration of SCC-9 cells

To examine whether lncRNAs participate in salvianolic acid A-mediated cell migration, real-time PCR was performed to assess lncRNA expression status in SCC-9 cells treated with salvianolic acid A. As Shown in Figure 7, expression of lncRNA, Gas5, was significantly increased after salvianolic acid A treatment, while lncRNA RAB11B-AS1, H19 and HOTAIR were no changed. Moreover, treatment with Gas5 siRNA significantly inhibited Gas5 expression (Figure 8A), and salvianolic acid A-mediated cell migration effects were reversed when Gas5 was silenced during Gas5 siRNA treatment (Figure 8B). These findings indicate that the anti-metastasis mechanisms of salvianolic acid A are due to regulation by lncRNA Gas5.

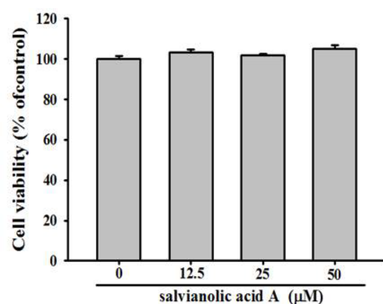


Figure 1. Effect of salvianolic acid A on the viability of SCC-9 cells. Cell viability of SCC-9 cells treated with 0, 12.5, 25, or 50 μM of salvianolic acid A for 24 h was determined through MTT assay. Data represent the mean ± SD of three independent experiments.

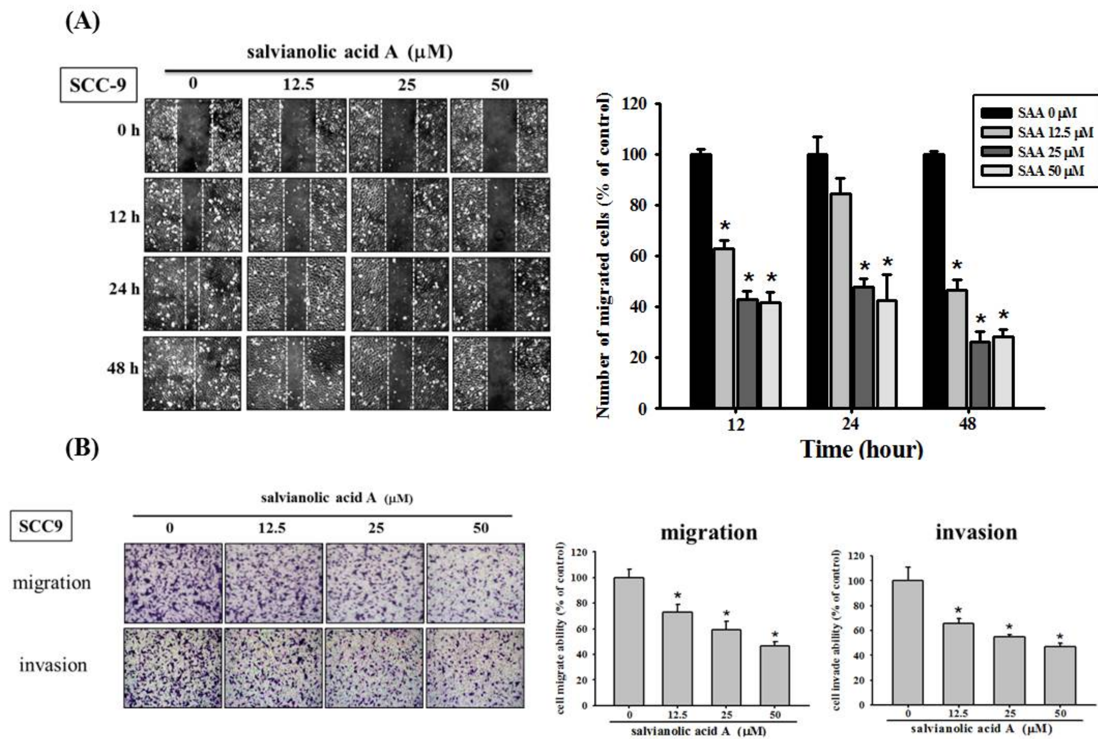


Figure 2 Effects of salvianolic acid A on the migration and invasion abilities of SCC-9 cells. (A) Photographs show the wound closure ability of SCC-9 cells treated with the salvianolic acid A (0, 12.5, 25, or 50 μM). Wound healing assay was then performed. (B) SCC-9 cells were treated with salvianolic acid A at a concentration of 0, 12.5, 25, or 50 μM for 24 h and then subjected to migration and invasion assays. **p*<0.05 as compared with the control.

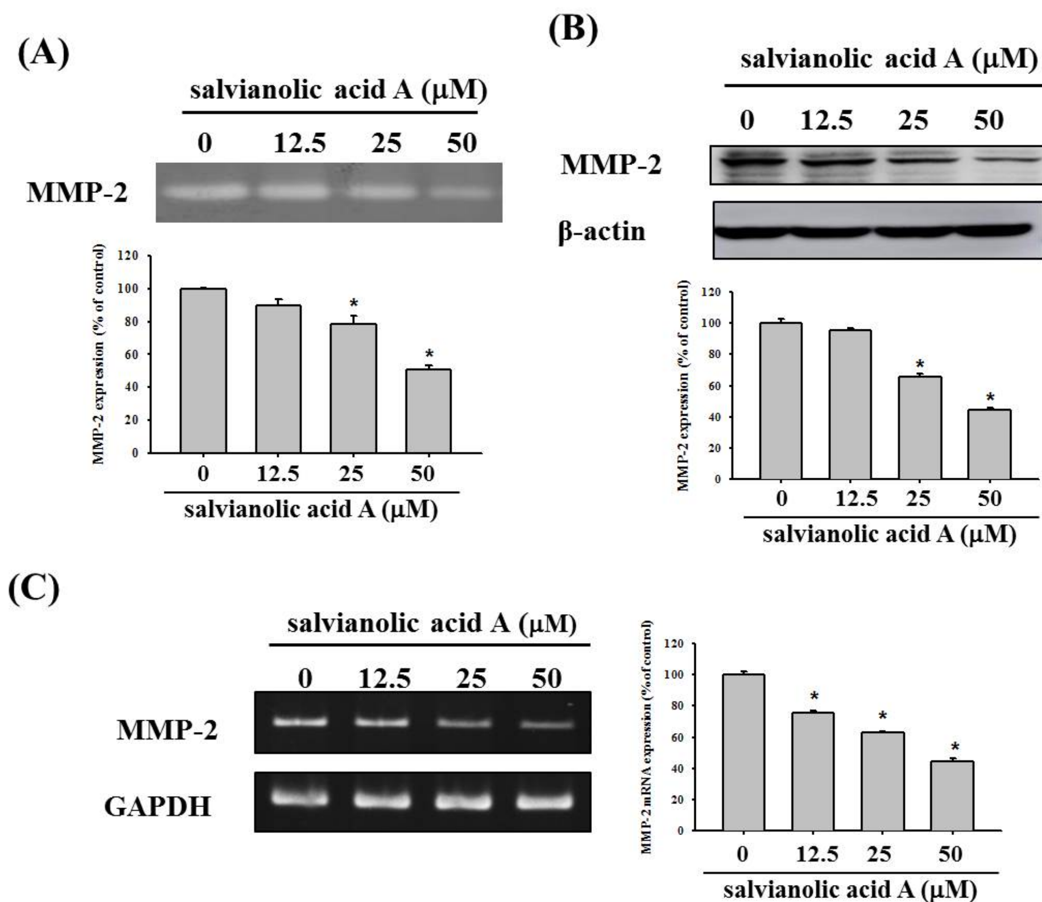


Figure 3 Effects of salvianolic acid A on MMP-2 activity, protein expression, and mRNA levels in SCC-9 cells. (A) Gelatin zymography was performed to measure MMP-2 activity in SCC-9 cells treated with different concentrations of salvianolic acid A for 24 h. (B) Western blot analysis was performed with β-actin as the internal control to measure MMP-2 protein expression levels in SCC-9 cells after salvianolic acid A treatments. (C) For the quantification of MMP-2 mRNA levels, total RNA was extracted from SCC-9 cells and subjected to semi-quantitative RT-PCR and real time PCR. *$p<0.05$ as compared with the control.

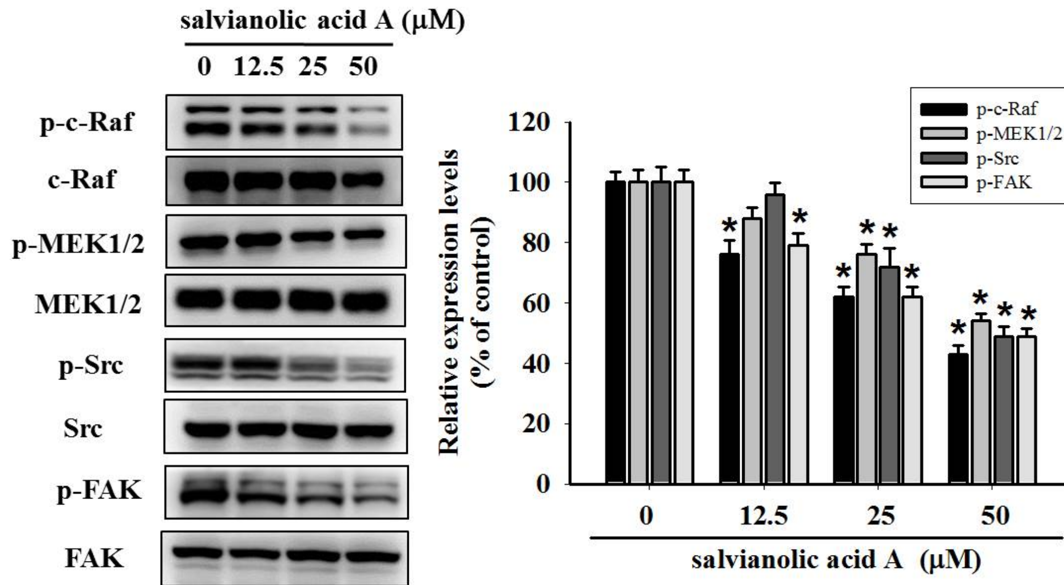


Figure 4 Inhibitory effect of salvianolic acid A on the phosphorylation of c-Raf, MEK1/2, Src and FAK. SCC-9 cells were treated with an indicated dose of salvianolic acid A (0, 12.5, 25, or 50 μM) for 24 h, and cell lysates were then subjected to SDS-PAGE followed by Western blotting. *$p<0.05$ as compared with the control.

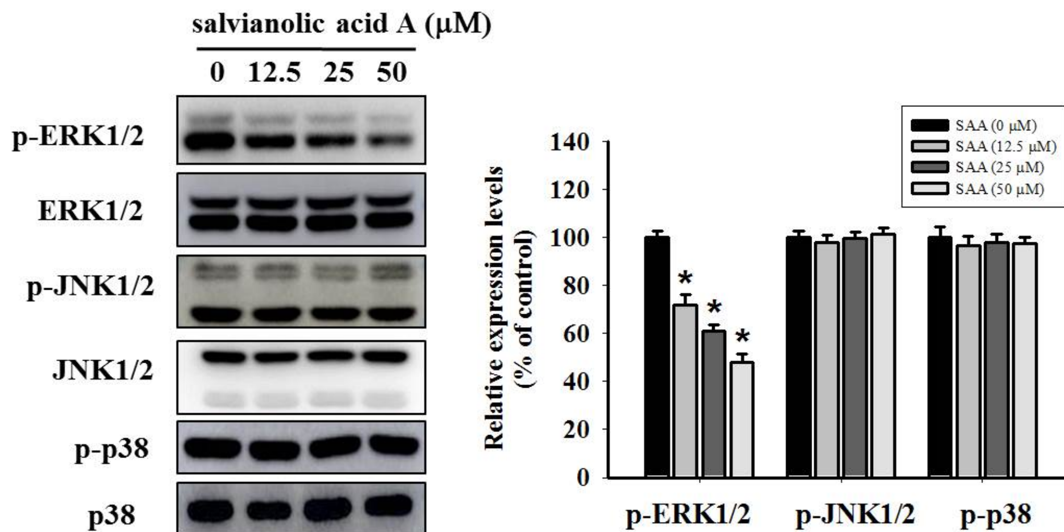


Figure 5 Inhibitory effect of salvianolic acid A on the phosphorylation of MAPK pathway. SCC-9 cells were treated with an indicated dose of salvianolic acid A (0, 12.5, 25, or 50 μM) for 24 h, and cell lysates were then subjected to SDS-PAGE followed by Western blotting. *$p<0.05$ as compared with the control.

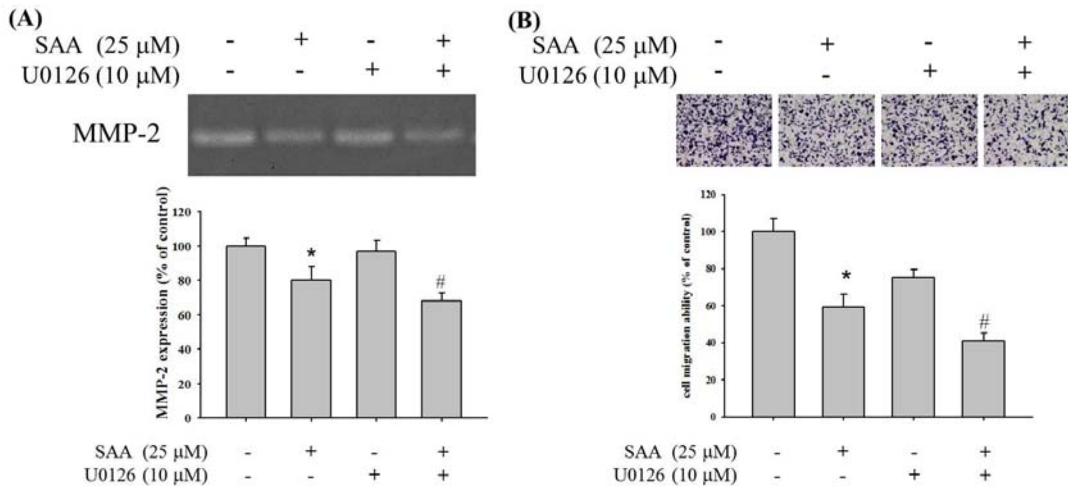


Figure 6 Effects of MEK inhibitor (U0126) and salvianolic acid A (SAA) on MMP-2 activity and cell migration. (A) SCC-9 cells were plated on 24-well plates, pretreated with U0126 (10 μM) for 30 min, and then incubated with or without salvianolic acid A (SAA) (25 μM) for 24 h. Culture media were subjected to gelatin zymography for the analysis of MMP-2 activity. (B) SCC-9 cells were pretreated with U0126 (10 μM) for 60 min and then incubated with or without salvianolic acid A (SAA) (25 μM) for 24 h. The cells were then subjected to Boyden chamber migration assay. Data represent the mean ± SD of at least three independent experiments. **p*<0.05 as compared with the control. #Significantly different, *p*<0.05, when compared with salvianolic acid A-treated group.

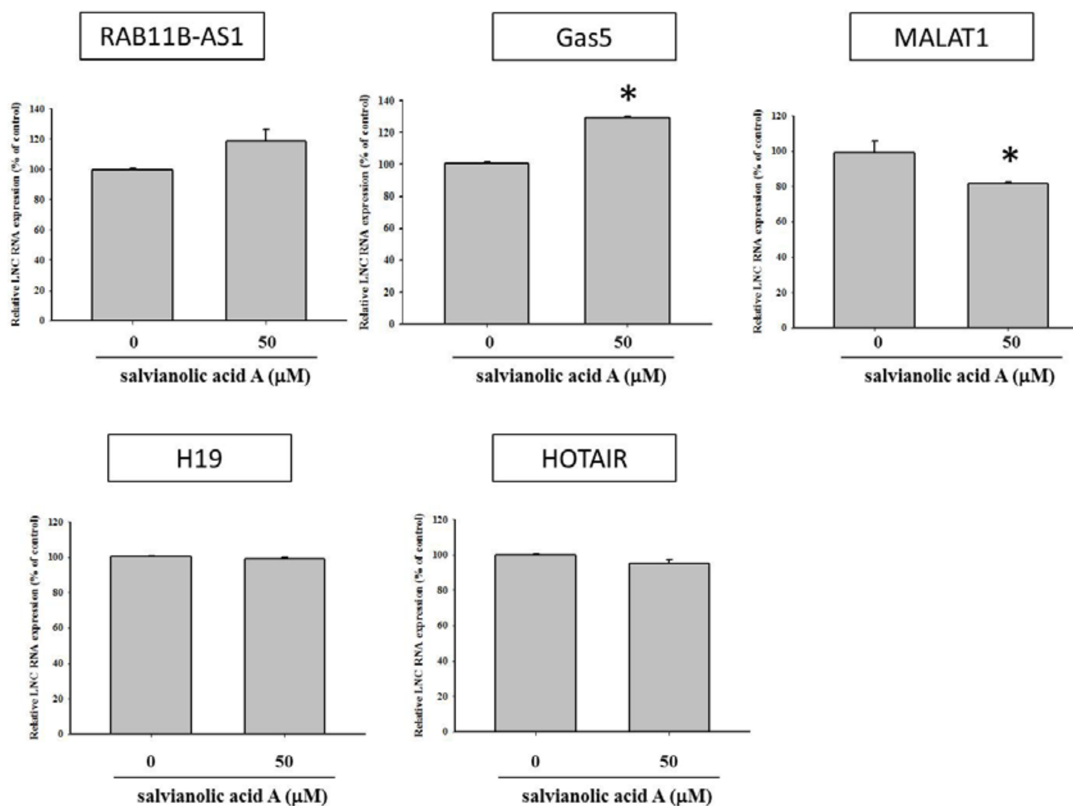


Figure 7. Effect of salvianolic acid A on the lncRNAs expression of SCC-9 cells. LncRNAs expression of SCC-9 cells treated with 0 or 50 μM of salvianolic acid A for 24 h was determined through real-time PCR assay. Data represent the mean ± SD of three independent experiments. **p*<0.05 as compared with the control.

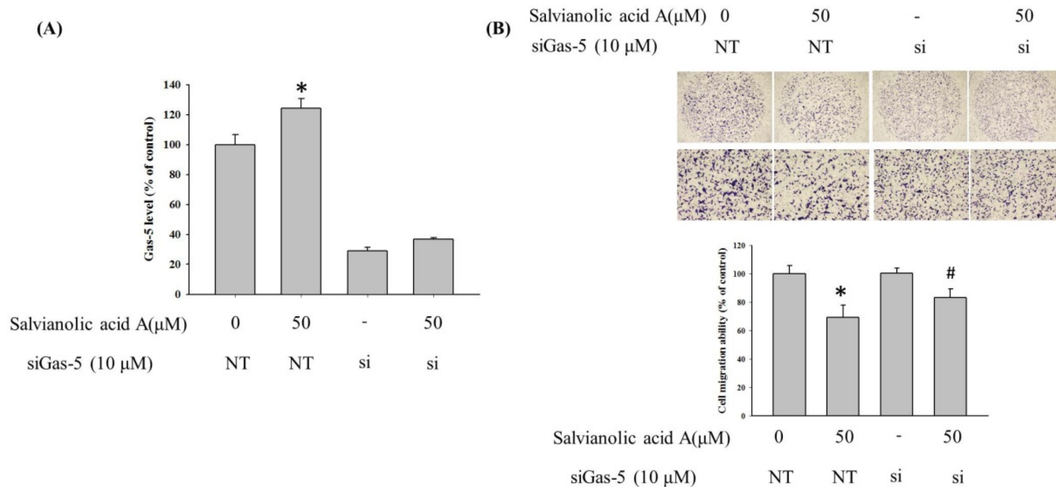


Figure 8. Effects of Gas5 siRNA and salvianolic acid A on Gas5 expression and cell migration. (A) SCC-9 cells were plated on 24-well plates, pretreated with siGas5 (10 μM), and then incubated with or without salvianolic acid A (50 μM) for 24 h. Real-time PCR assay was performed for the analysis of Gas5 expression. (B) SCC-9 cells were pretreated with siGas5 (10 μM) and then incubated with or without salvianolic acid A (50 μM) for 24 h. The cells were then subjected to Boyden chamber migration assay. Data represent the mean ± SD of at least three independent experiments. *$p<0.05$ as compared with the control. #Significantly different, $p<0.05$, when compared with salvianolic acid A-treated group.

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：**106-2632-B-040-002-
計畫名稱：長鏈非編碼RNA作為口腔癌前病變及口腔癌新穎診治標的之轉譯研究	

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	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
國外	學術性論文	期刊論文			1	篇	Antimetastatic potentials of salvianolic acid A on oral squamous cell carcinoma by targeting MMP-2 and the c-Raf/MEK/ERK pathway. Environ Toxicol. 2018 May;33(5):545-554.
		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	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
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<tr><td>博士後研究員</td><td>0</td><td></td></tr>
<tr><td>專任助理</td><td>0</td><td></td></tr>
<tr><td rowspan="5">非本國籍</td><td>大專生</td><td>0</td><td></td></tr>
<tr><td>碩士生</td><td>0</td><td></td></tr>
<tr><td>博士生</td><td>0</td><td></td></tr>
<tr><td>博士後研究員</td><td>0</td><td></td></tr>
<tr><td>專任助理</td><td>0</td><td></td></tr>
<tr><td colspan="3">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。）</td><td colspan="3"></td></tr>
</table>

科技部補助專題研究計畫成果自評表

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（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）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（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   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200字為限）

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）
   本整合型計畫發現, 長鏈非編碼RNA可成為口腔癌前病變及口腔癌新穎生物標誌及分子標靶，HOTAIR於口腔癌前病變組織高度表現, MEG3於口腔癌低表達且可調控幹細胞化, 真菌類免疫調節蛋白GMI能改善5-FU所誘發之粘膜炎, lncRNA-Gas5可能參與在丹酚酸A所抑制的口腔癌細胞轉移現象，lncRNA-Gas5在未來或許可以應用在預防口腔癌的轉移或輔助口腔癌的治療。 預期本計畫結果可利用長鏈非編碼RNA研發口腔癌之防治藥物, 目前資料彙整準備投稿。

4. 主要發現
   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
   說明：（以150字為限）