科技部補助專題研究計畫成果報告
期末報告

探討甘草酸衍生物抑制不同鼻咽癌細胞株細胞增生及誘導細胞自噬作用及凋亡之機制

計 畫 類 別 ： 個別型計畫
計 畫 編 號 ： MOST 104-2314-B-040-018-
執 行 期 間 ： 104年08月01日至105年07月31日
執 行 單 位 ： 中山醫學大學醫學系

計 畫 主 持 人： 莊俊義
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計畫參與人員： 博士班研究生-兼任助理人員：何心妤

中 華 民 國 105 年 10 月 19 日

中 文 摘 要 ： 鼻咽癌是中國人特有的癌症。一般而言，經正規治療之結果，其病人五年之存活率約有60%。但若能更有效抑制癌細胞的生長或轉移，將可大幅減低癌症的死亡率。因此發展新穎天然藥物來當作癌症預防或是治療藥物是目前最重要的課題。甘草(Licorice)為豆顆甘草屬，是傳統中藥材之一。甘草中的黃酮類化合物主要為chalcones，而chalcones中的licochalcone A化合物在先前研究已經證實具有抗癌的特性。如licochacone A對於人類大腸癌、前列腺癌與口腔癌具有誘導細胞凋亡、抑制癌細胞增生的效果。然而licochalcone A化合物對於人類鼻咽癌細胞株的影響仍尚未釐清。我們利用licochalcone A化合物，處理人類三株鼻咽癌細胞 (HONE-1，NPC-39，NPC-BM)，分析其細胞存活率。結果發現licochalcone A會抑制三株鼻咽癌細胞的生長，我們也利用flow cytometer 分析方式發現licochalcone A會誘導HONE-1的細胞凋亡。此外，licochalcone A會經由活化caspase 3、8、9及裂解PARP來誘發鼻咽癌細胞凋亡。而我們也發現licochalcone A會增加了Erk1/2, JNK1/2及P38 MAPK磷酸化。而利用了JNK1/2及P38的專一性抑制劑也顯著地降低了licochalcone A所誘發的caspase -3,-8,-9的活化。綜合歸納後，我們的結果顯示licochalcone A是經由JNK1/2及P38路徑來誘發HONE-1細胞產生凋亡，這也暗示著licochalcone A適合作為另一種有效的鼻咽癌的抗癌輔助藥物。

中 文 關 鍵 詞 ： 鼻咽癌、甘草、細胞凋亡

英 文 摘 要 ： Nasopharygeal carcinoma, a disease that affects the population in South-East Asia, has high lymph-node metastastic ability. Numerous investigations demonstrated that licochalcone A can modulate many cancer targets, suppressing cancer cell growth and/or inducing apoptosis. However, the effect of licochalcone A on nasopharygeal carcinoma remains unclear. Here, the molecular mechanism by which licochalcone A -induced apoptosis effects in human nasopharygeal carcinoma cells was investigated. The results showed that licochalcone A significantly inhibited cell proliferation of three nasopharygeal carcinoma cell lines (HONE-1, NPC-39 and NPC-BM). Furthermore, licochalcone A induced apoptosis of HONE-1 cells through caspases-3, -8, and -9 activations and PARP cleavage in dose-dependent manner. Moreover, western blot analysis also showed that licochalcone A increase phosphorylation of Erk1/2, JNK1/2 and p38 MAPK in dose-dependent manner. Inhibition of JNK1/2 and p38 by specific inhibitors significantly abolished the licochalcone A-induced activation of the caspase-3, -8 and -9. Taken together, our results suggest that licochalcone A-induced HONE-1 cell apoptosis through JNK1/2 and p38 pathways and could serve as a potential additional chemotherapeutic agent for treating nasopharygeal carcinoma.

英文關鍵詞：Nasopharygeal carcinoma, licochalcone A, apoptosis

Licochalcone A mediates caspases activation and induces apoptosis through MAPK pathway in human nasopharyngeal carcinoma

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Competing Interests: The authors declare that no competing interests exist.

Short Title: Licochalcone A induces apoptosis in nasopharyngeal carcinoma

Abstract

Nasopharygeal carcinoma, a disease that affects the population in South-East Asia, has high lymph-node metastastic ability. Numerous investigations demonstrated that licochalcone A can modulate many cancer targets, suppressing cancer cell growth and/or inducing apoptosis. However, the effect of licochalcone A on nasopharygeal carcinoma remains unclear. Here, the molecular mechanism by which licochalcone A -induced apoptosis effects in human nasopharygeal carcinoma cells was investigated. The results showed that licochalcone A significantly inhibited cell proliferation of three nasopharygeal carcinoma cell lines (HONE-1, NPC-39 and NPC-BM). Furthermore, licochalcone A induced apoptosis of HONE-1 cells through caspases-3, -8, and -9 activations and PARP cleavage in dose-dependent manner. Moreover, western blot analysis also showed that licochalcone A increase phosphorylation of Erk1/2, JNK1/2 and p38 MAPK in dose-dependent manner. Inhibition of JNK1/2 and p38 by specific inhibitors significantly abolished the licochalcone A-induced activation of the caspase-3, -8 and -9. Taken together, our results suggest that licochalcone A-induced HONE-1 cell apoptosis through JNK1/2 and p38 pathways and could serve as a potential additional chemotherapeutic agent for treating nasopharygeal carcinoma.

Introduction

Nasopharyngeal carcinoma (NPC) is the most common primary malignancy occurred in nasopharynx, has a high incidence in South-East Asia region, especially Guangzhou and Taiwan [1]. The main causes of NPC include the difference in diet, such as salted or smoke fish, genetics, and Epstein-Barr virus (EBV) infection [2, 3]. Because of the location of nasopharyngeal tumor, NPC has the highest risk for regional lymph node metastasis in head and neck carcinoma [4]. NPC is a high radiosensitivity cancer compared with most squamous cell cancer, therefore radiotherapy becomes the standardize method [5]. The overall 5-year survival rate for NPC patients ranges from 60% to 80%. However, 20-30% patients finally progress with loco-regional recurrence and 5-8% with distant metastasis [6, 7].

The hallmarks of cancer cell include evading growth suppressor, sustaining proliferative signaling, resisting cell death, inducing angiogenesis, enabling replicative immortality, and activating invasion/ metastasis [8]. Licochalcone A (Figure 1A) is one of the flavonoids isolated from the root of Glycyrrhiza inflate [9, 10]. It has been shown to have anti-angiogenesis, anti-inflammatory and anti-cancer properties [11-18]. For example, Licochalcone A has been shown to induce intrinsic and extrinsic apoptosis via ERK1/2 and p38 phosphorylation-mediated TRAIL expression in head and neck squamous carcinoma [15]. However, there are no reports

concerning the anticancer effects of licochalcone A on human nasopharygeal carcinoma cells. In the present study, we investigated the cytotoxic effects of licochalcone A on three nasopharygeal carcinoma cell lines (HONE-1, NPC-39 and NPC-BM), and its underlying mechanisms in vitro.

Materials and Methods

Cell culture

The human nasopharyngeal carcinoma cell line HONE-1 was purchased from Food Industry Research and Development Institute (Hsinchu, Taiwan); NPC-39 and NPC-BM, established from Taiwanese patients with NPC [19], were gifts from Dr. M-K Chen, Department of Otolaryngology, Chang Hua Christian Hospital, Chang Hua, Taiwan. HONE-1, NPC-39, and NPC-BM cells were cultured in RPMI-1640 (GIBCO, Grand Island, NY, USA) supplemented with 10% FBS (GIBCO, Grand Island, NY, USA), 100 U/mL penicillin (Sigma Chemical Co, St. Louis, MO, USA), 100 μg/mL streptomycin (Sigma Chemical Co, St. Louis, MO, USA), 100 μg/mL sodium pyruvate (Sigma Chemical Co, St. Louis, MO, USA), sodium bicarbonate (Sigma Chemical Co, St. Louis, MO, USA), and 4-(2-hydroxyethyl)-1-piperazinee--thanesulfonic acid (HEPES, Sigma Chemical Co, St. Louis, MO, USA). Cultures

were maintained at 37 ℃ in a humidified atmosphere containing 5% CO_2.

Cell viability assay by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

HONE-1, NPC-39 or NPC-BM sells (7×10^4 cells per well) were seeded in 24-well plates, and subsequently incubated with indicated licochalcone A concentration. To examine cell viability, cells were incubated with medium containing 0.5 mg/ml (3-(4,5-Dimethylthiazol-2-yl)-2,5- di--phenyltetrazolium bromide (MTT, Sigma Chemical Co, St. Louis, MO, USA) for 4 h. The amount of MTT formazan product was dissolved in isopropanol and analyzed spectrophotometrically at a wavelength of 563 nm.

Flow Cytometric Analysis

HONE-1 cells (2×10^6/ml) were treated with 0.5% DMSO or licochalcone A (0, 20, 40 and 80 μM) for 24 h. At the end of incubation, cells were collected and fixed with 70% ethanol. Cells were stained with propidium iodide (PI) buffer (4 μg/ml PI, 1% Triton X-100, and 0.5 mg/ml RNase A in phosphate-buffered saline (PBS)) for 30 min in the dark at room temperature and then filtered through a 40-μm nylon filter (Falcon, San Jose, CA). The cell cycle distribution was analyzed for 10,000 collected cells by a

FACS Vantage flow cytometer that uses the Cellquest acquisition and analysis program (Becton-Dickinson FACS Calibur, San Jose, CA). Apoptotic cells with hypodiploid DNA content were detected in the sub-G_1 region. All results were obtained from three independent experiments.

Annexin-V/PI Staining Assay

Apoptosis-mediated cell death of tumor cells was examined using a double-staining method with an FITC-labeled Annexin-V/PI Apoptosis Detection kit (BD Biosciences, San Jose, CA). For PI and Annexin-V double-staining, cells were suspended in 100 µl of binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, and 2.5 mM $CaCl_2$ at pH 7.4) and stained with 5 µl of FITC-conjugated Annexin-V and 5 µl of PI (50 µg/ml) for 30 min at room temperature in the dark, and then 400 µl of binding buffer was added. Apoptotic cells were analyzed via flow cytometry, by a FACScan system flow cytometric analysis. Data acquisition and analysis were performed in a Becton-Dickinson FACS Calibur flow cytometer using Cell Quest software.

Western Blot Analysis

Cell lysates were prepared as previously described [20]. Equal amounts of

protein extracts (20 μg) were subjected to 10% or 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a polyvinylidene fluoride membrane (Millipore, Belford, MA). After blocking, the membrane was incubated with primary antibodies for caspases-3, -8, and -9, PARP, ERK1/2, p-ERK1/2, p38, JNK1/2, p-JNK1/2 and β-actin. Blots were then incubated with a horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody. Signals were detected via enhanced chemiluminescence using Immobilon Western HRP Substrate (Millipore, Billerica, MA).

Statistical Analysis

Values are shown as the mean ± SE. Statistical analyses were performed using the Statistical Package for Social Science software, vers. 16 (SPSS, Chicago, IL). Data comparisons were performed with Student's *t*-test when two groups were compared. A one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used when more than three groups were analyzed. Differences were considered significant at the 95% confidence level when $p < 0.05$.

Results

The cytotoxic effects of licochalcone A-treated nasopharyngeal carcinoma cell lines

To determine the cytotoxicity of licochalcone A on three nasopharyngeal carcinoma cell lines (HONE-1, NPC-39 and NPC-BM), cells were treated with different concentrations of licochalcone A (0-80 μM) for 24 h. After treatment for 24 h, licochalcone A significantly reduced the cell viability in a concentration-dependent manner for the three nasopharyngeal carcinoma cell lines (Figure 1B-1D).

Licochalcone A induces HONE-1 cell apoptosis

To determine whether the inhibitory effect of cell viability of licochalcone A is associated with induction of cell apoptosis, HONE-1 cells were treated with different concentrations (0-80 μM) of licochalcone A for 24 h. Cell cycle analysis by flow cytometry was showed a dose-dependent increased accumulation of cell population in sub-G1 phase after 24 h treatment with licochalcone A (Figure 2). We assessed the translocation of phosphatidylserine (PS) using Annexin-V and PI double-staining. Apoptotic cells (PI-negative/Annexin-V-positive and PI-positive/Annexin-V-positive) increased from 16.72% to 14.09%, 27.21%, 73.94% and 81.83% after respectively treating HONE-1 cells with 10, 20, 40 and 80 μM licochalcone A (Figure 3).

Licochalcone A induces expression of cleaved caspase-3, -8, -9 and PARP in HONE-1 cells

To further confirm the involvement of caspase activation in licochalcone A -induced apoptosis, activation of caspases-3, -8, and -9 and cleavage of PARP were detected. Figure 4A-4D shows that licochalcone A caused dose-dependent increased of the cleaved fragments of caspases-9, -8, -3 and PARP.

In a further investigation for the underlying molecular mechanisms, we determined whether MAPKs were activated in licochalcone A -treated HONE-1 cells by a Western blot analysis. Results showed that the phosphorylation of Erk 1/2, JNK1/2 and p38 MAPK were increased in cells treated with licochalcone A in a dose-dependent manner (Figure 5). Next, we further investigated relationships among licochalcone A induced activation of caspases-3, -8 and -9 and MAPKs. HONE-1 cells were pretreated with 1 μM JNK-IN-8 (a JNK inhibitor), or 10 μM SB203580 (a p38 inhibitor) for 1 h, treated with 40 μM licochalcone A for another 24 h, and then analyzed by Western blotting. As shown in Figure 6, treatment JNK-IN-8 and SB203580 significantly attenuated licochalcone A-induced caspase-3, -8 and -9 activation. These findings suggest that activation of JNK1/2 and p38 might play a critical upstream role in licochalcone A-mediated caspase activation in HONE-1 cells.

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Figure 1

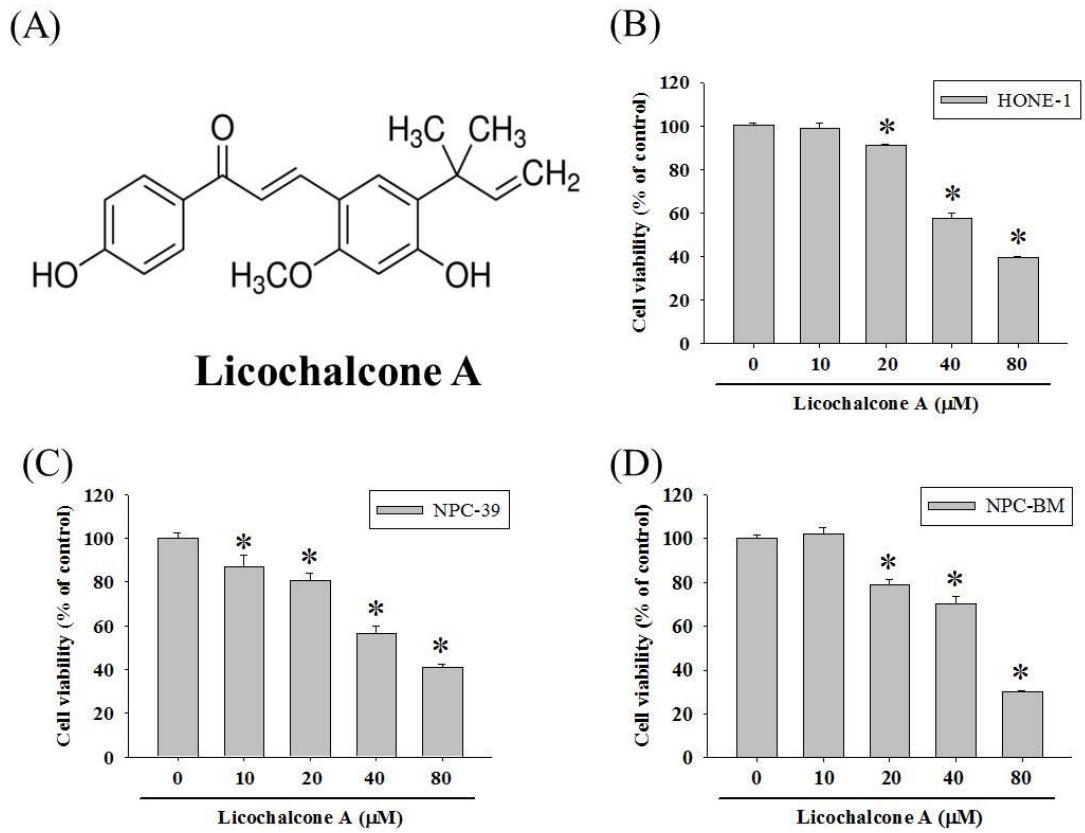

(A)
O
H3C CH3
CH2
HO
H3CO
OH
Licochalcone A
(B)
HONE-1
Cell viability (% of control)
120
100
80
60
40
20
0
0 10 20 40 80
Licochalcone A (μM)
(C)
NPC-39
Cell viability (% of control)
120
100
80
60
40
20
0
0 10 20 40 80
Licochalcone A (μM)
(D)
NPC-BM
Cell viability (% of control)
120
100
80
60
40
20
0
0 10 20 40 80
Licochalcone A (μM)

Figure 2

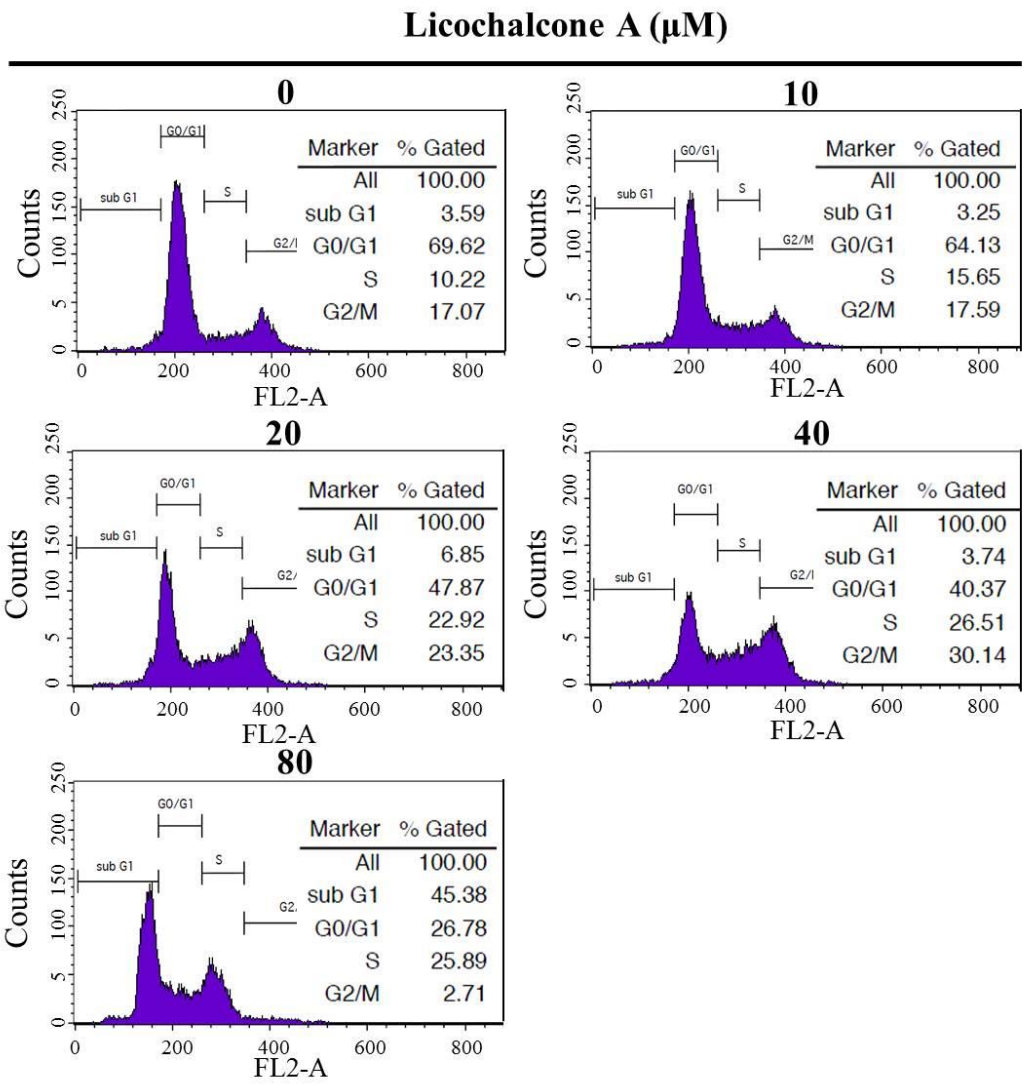

Licochalcone A (μM)
0
Marker % Gated
All 100.00
sub G1 3.59
G0/G1 69.62
S 10.22
G2/M 17.07
10
Marker % Gated
All 100.00
sub G1 3.25
G0/G1 64.13
S 15.65
G2/M 17.59
20
Marker % Gated
All 100.00
sub G1 6.85
G0/G1 47.87
S 22.92
G2/M 23.35
40
Marker % Gated
All 100.00
sub G1 3.74
G0/G1 40.37
S 26.51
G2/M 30.14
80
Marker % Gated
All 100.00
sub G1 45.38
G0/G1 26.78
S 25.89
G2/M 2.71
Counts
FL2-A
sub G1
G0/G1
S
G2/M

Figure 3

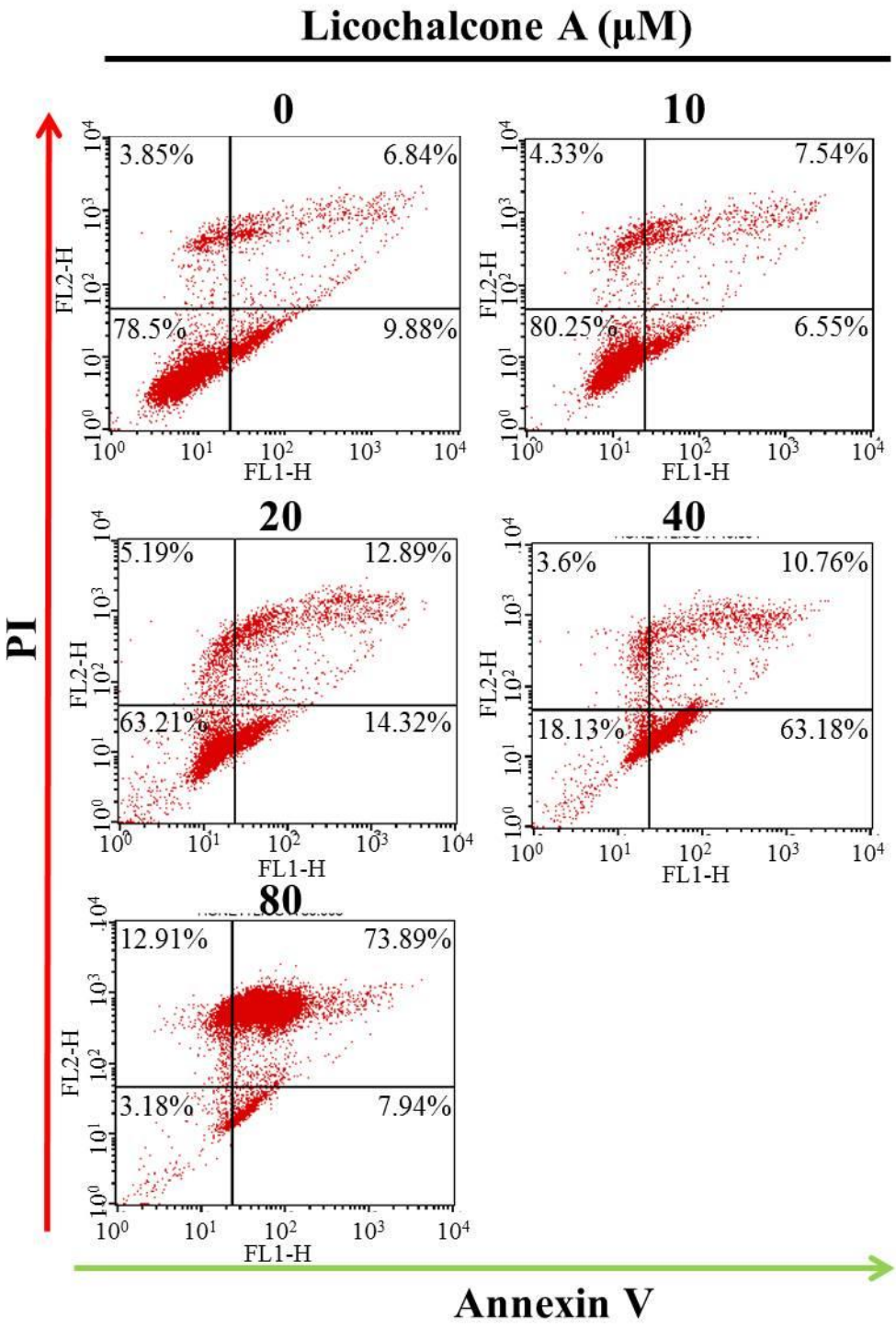

Licochalcone A (μM)
0
10
20
40
80
3.85%
6.84%
78.5%
9.88%
4.33%
7.54%
80.25%
6.55%
5.19%
12.89%
63.21%
14.32%
3.6%
10.76%
18.13%
63.18%
12.91%
73.89%
3.18%
7.94%
FL1-H
FL2-H
PI
Annexin V

Figure 4

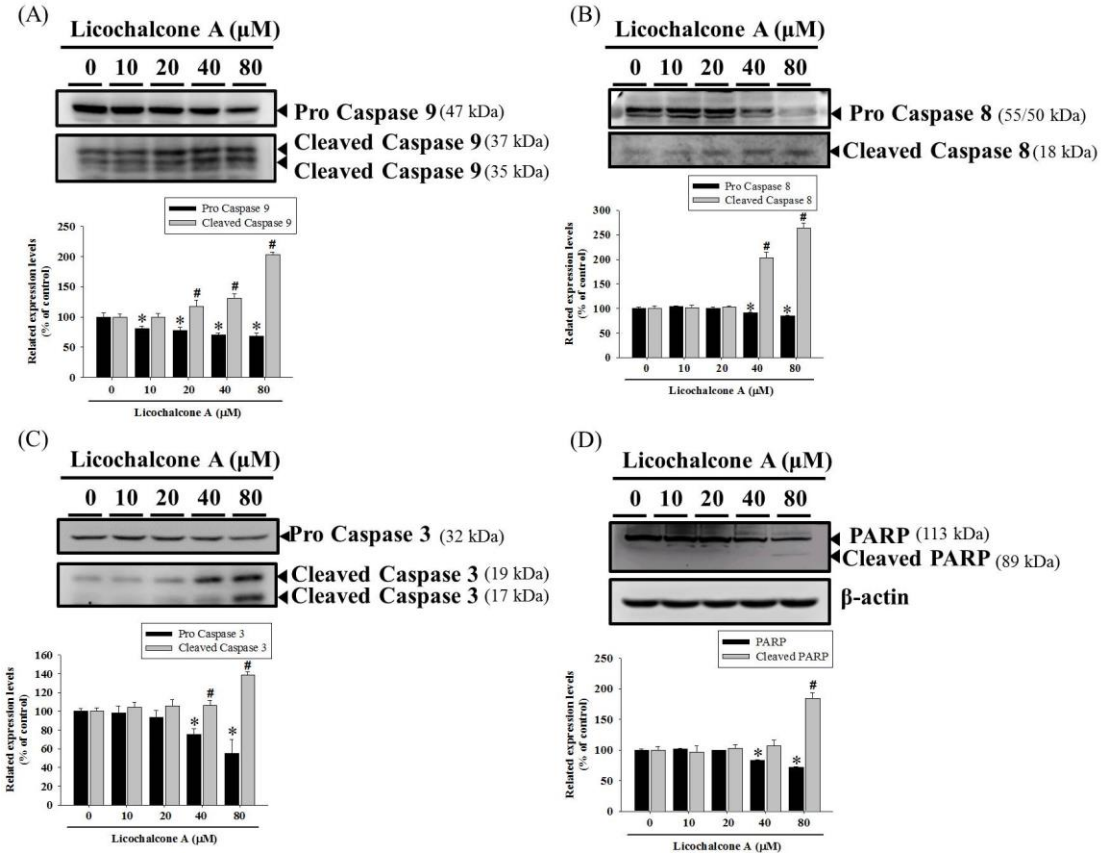

(A)
Licochalcone A (μM)
0 10 20 40 80
Pro Caspase 9 (47 kDa)
Cleaved Caspase 9 (37 kDa)
Cleaved Caspase 9 (35 kDa)
Pro Caspase 9
Cleaved Caspase 9
Related expression levels (% of control)
Licochalcone A (μM)
(B)
Licochalcone A (μM)
0 10 20 40 80
Pro Caspase 8 (55/50 kDa)
Cleaved Caspase 8 (18 kDa)
Pro Caspase 8
Cleaved Caspase 8
Related expression levels (% of control)
Licochalcone A (μM)
(C)
Licochalcone A (μM)
0 10 20 40 80
Pro Caspase 3 (32 kDa)
Cleaved Caspase 3 (19 kDa)
Cleaved Caspase 3 (17 kDa)
Pro Caspase 3
Cleaved Caspase 3
Related expression levels (% of control)
Licochalcone A (μM)
(D)
Licochalcone A (μM)
0 10 20 40 80
PARP (113 kDa)
Cleaved PARP (89 kDa)
β-actin
PARP
Cleaved PARP
Related expression levels (% of control)
Licochalcone A (μM)

Figure 5

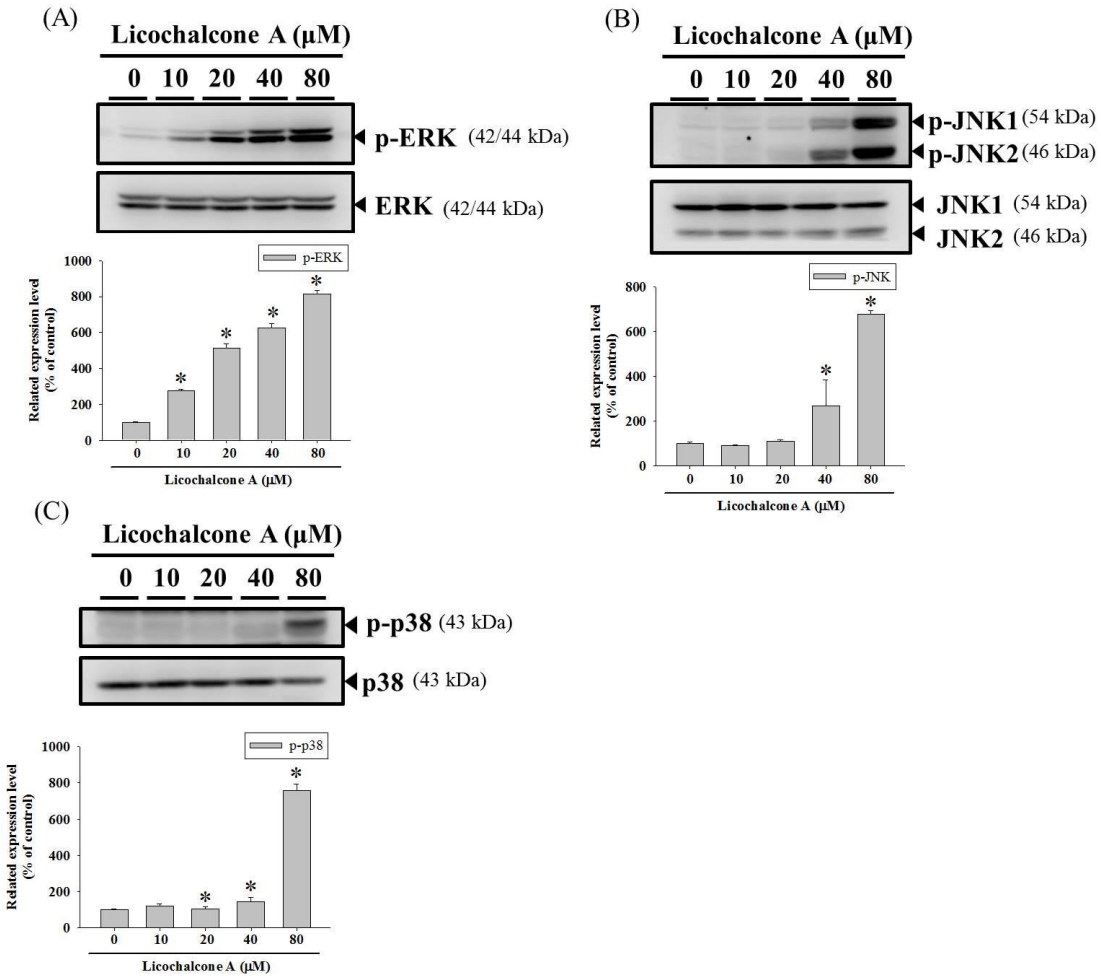
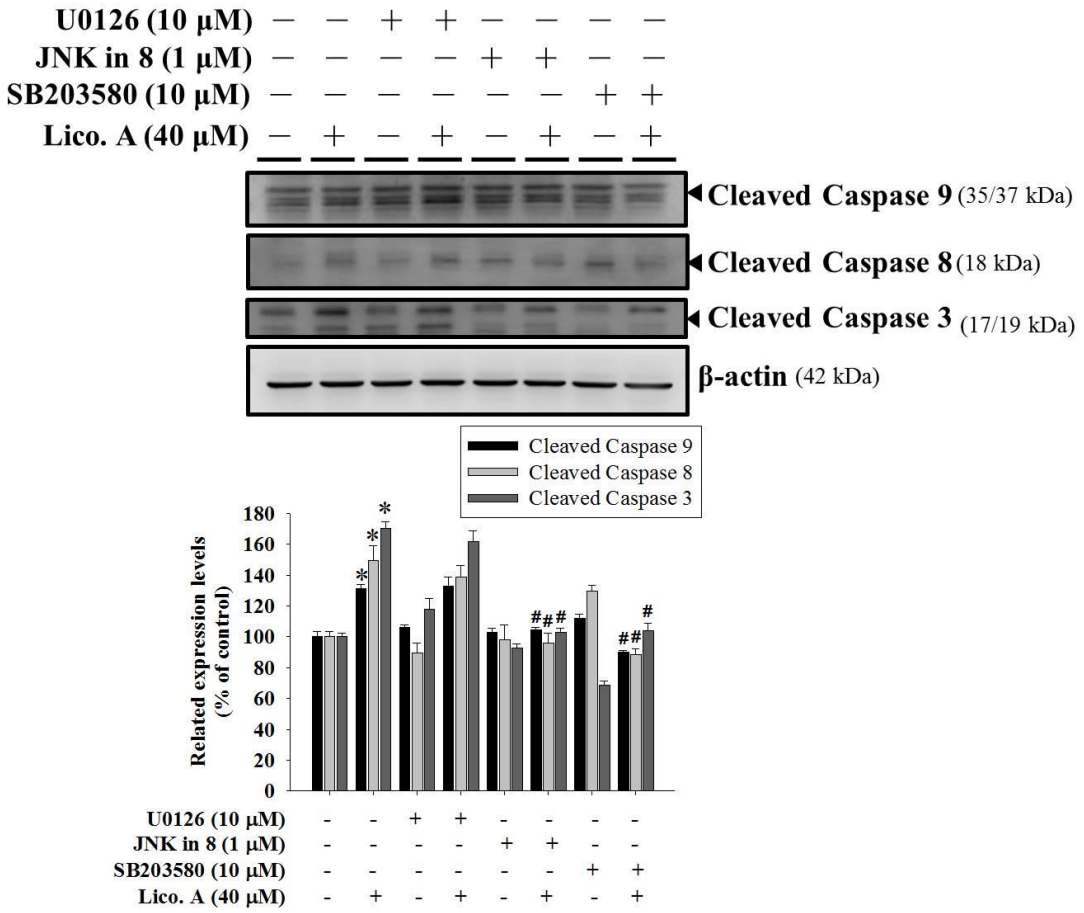

(A)
Licochalcone A (μM)
0 10 20 40 80
p-ERK (42/44 kDa)
ERK (42/44 kDa)
p-ERK
Related expression level (% of control)
Licochalcone A (μM)
(B)
Licochalcone A (μM)
0 10 20 40 80
p-JNK1 (54 kDa)
p-JNK2 (46 kDa)
JNK1 (54 kDa)
JNK2 (46 kDa)
p-JNK
Related expression level (% of control)
Licochalcone A (μM)
(C)
Licochalcone A (μM)
0 10 20 40 80
p-p38 (43 kDa)
p38 (43 kDa)
p-p38
Related expression level (% of control)
Licochalcone A (μM)

Figure 6


U0126 (10 μM) − − + + − − − −
JNK in 8 (1 μM) − − − − + + − −
SB203580 (10 μM) − − − − − − + +
Lico. A (40 μM) − + − + − + − +
Cleaved Caspase 9 (35/37 kDa)
Cleaved Caspase 8 (18 kDa)
Cleaved Caspase 3 (17/19 kDa)
β-actin (42 kDa)
Cleaved Caspase 9
Cleaved Caspase 8
Cleaved Caspase 3
Related expression levels (% of control)
0
20
40
60
80
100
120
140
160
180
U0126 (10 μM) - - + + - - - -
JNK in 8 (1 μM) - - - - + + - -
SB203580 (10 μM) - - - - - - + +
Lico. A (40 μM) - + - + - + - +

科技部補助計畫衍生研發成果推廣資料表

日期:2016/10/19

科技部補助計畫	計畫名稱: 探討甘草酸衍生物抑制不同鼻咽癌細胞株細胞增生及誘導細胞自噬作用及凋亡之機制
	計畫主持人: 莊俊義
	計畫編號: 104-2314-B-040-018- 學門領域: 耳鼻喉

無研發成果推廣資料

104年度專題研究計畫成果彙整表

計畫主持人：莊俊義	**計畫編號：**104-2314-B-040-018-
計畫名稱：探討甘草酸衍生物抑制不同鼻咽癌細胞株細胞增生及誘導細胞自噬作用及凋亡之機制	

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			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	
參與計畫人力	本國籍	大專生	0	人次	
		碩士生	0		
		博士生	1		
		博士後研究員	0		
		專任助理	0		
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	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。）					

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）
   鼻咽癌是中國人特有的癌症。一般而言，經正規治療之結果，其病人五年之存活率約有60%。但若能更有效抑制癌細胞的生長或轉移，將可大幅減低癌症的死亡率。因此發展新穎天然藥物來當作癌症預防或是治療藥物是目前最重要的課題。甘草(Licorice)為豆顆甘草屬，是傳統中藥材之一。甘草中的黃酮類化合物主要為chalcones，而chalcones中的licochalcone A化合物在先前研究已經證實具有抗癌的特性。然而licochalcone A化合物對於人類鼻咽癌細胞株的影響仍尚未釐清。我們利用licochalcone A化合物，處理人類三株鼻咽癌細胞(HONE-1，NPC-39，NPC-BM)，分析其細胞存活率。結果發現licochalcone A會抑制三株鼻咽癌細胞的生長，此外，licochalcone A會經由活化caspase 3、8、9及裂解PARP來誘發鼻咽癌細胞凋亡。綜合歸納後，我們的結果顯示licochalcone A是經由JNK1/2及P38路徑來誘發HONE-1細胞產生凋亡，這也暗示著licochalcone A適合作為另一種有效的鼻咽癌的抗癌輔助藥物。

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）