行政院國家科學委員會專題研究計畫 成果報告

不同的檳榔子成份所誘導的細胞凋亡與自體吞噬之探討
研究成果報告(精簡版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 98-2314-B-041-002-
執 行 期 間 ：98 年 08 月 01 日至 99 年 07 月 31 日
執 行 單 位 ：嘉南藥理科技大學生物科技系（所）

計 畫 主 持 人：林美惠
共 同 主 持 人：劉永超
計畫參與人員：此計畫無其他參與人員

處 理 方 式 ：本計畫可公開查詢

中 華 民 國 99 年 10 月 26 日

行政院國家科學委員會補助專題研究計畫成果報告

Characterization of the apoptosis and autophagy induced by different areca nut ingredients

不同的檳榔子成份所誘導的細胞凋亡與自體吞噬之探討

計畫類別：■ 個別型計畫　　□ 整合型計畫
計畫編號：NSC 98-2314-B-041-002-
執行期間：　98　年　8　月　1　日至　99 年　7　月　31　日

計畫主持人：林美惠
共同主持人：劉永超
計畫參與人員：謝宛芳、鄭詠之、許凱程、林品妍

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：財團法人奇美醫院牙醫部

中　華　民　國　99　年　10　月　20　日

中文摘要

本計劃過去一年來的三項工作成果簡述如下：

1. **calmodulin-dependent protein kinase kinase β (CaMKKβ)/ AMP-activated protein kinase（AMPK）訊息途徑分別在檳榔鹼（arecoline）與 ANE 30-100K 所誘導的細胞凋亡與自體吞噬中之關聯性：**在 OECM-1, CE81T/VGH 與 Jurkat T 三種癌細胞中，Compound C 與 STO-609 只在 Jurkat T 細胞中能有效保護 ANE 30-100K 所誘導的自體吞噬之死亡，對 OECM-1 與 CE81T/VGH 的保護效果則不明顯。此外，利用 RNAi 的策略，亦發現在大部分 AMPK 被抑制表現的 OECM-1 純系細胞株中，對 ANE 30-100K 的耐受性與母株細胞類似。因此，AMPK 在由 ANE 30-100K 所誘導的自體吞噬上扮演的角色，可能在不同的細胞有所差異，未來尚有待確認。另一方面，由檳榔鹼在這三種細胞所誘導的細胞凋亡已證實有賴於 AMPK 受到抑制，並且此過程是透過胞內穀胱甘肽缺乏（glutathione depletion）所致。我們正在撰寫有關這些新發現的論文。
2. **在已知的壓力訊號中比較檳榔鹼與 ANE 30-100K 可能誘導的訊號：**目前在已知的壓力訊息中，已得知檳榔鹼與 ANE 30-100K 對 AMPK 有不同的調節作用；此外我們也採用蛋白二維電泳的方法，試圖找出新的作用分子。目前初步的結果顯示，經 ANE 30-100K 處理後的 OECM-1 其 endoplasmin precursor, thyroid hormone binding protein precursor, human protein disulfide isomerase (Chain A)與 glutathione-insulin transhydrogenase 等四種蛋白的表現量增加；而 CE81T/VGH 則有 elongation factor-1 α1 與 glyceraldehyde 3-phosphate dehydrogenase 的提升，值得繼續分析這些蛋白質在 ANE 30-100K 所誘導的自體吞噬中之角色。
3. **鑑定 ANE 30-100K 在細胞上（中）的接受器：**這部份的實驗並未有成果，但我們進一步分析了 ANE 30-100K 的單糖組成，有 GalNH2 (10.6%), GlcNH2 (14.0%), Galactose (21.4%), Glucose (36.3%) 與 Mannose (17.6%)等四種單糖，在中研院楊文斌博士的協助之下，未來擬繼續純化並分析造成 ANE 30-100K 誘導自體吞噬的多醣類之結構。

關鍵詞：檳榔子、凋亡、自體吞噬

Abstract

The progresses of three works in this project are briefly described below:

1. **The involvement of calmodulin-dependent protein kinase kinase β (CaMKKβ)/AMPK pathway in arecoline- and ANE 30-100K-mediated apoptosis and autophagy, respectively.** Among OECM-1, CE81T/VGH, and Jurkat T cells, Compound C and STO-609 significantly prevented ANE 30-100K-induced autophagic cell death only in Jurkat T cells, and the protection effect was not evident in OECM-1 and CE81T/VGH. Moreover, by using RNAi stategy, the tolerance of ANE 30-100K in most AMPK knocked down clones was found to be similar to the parental cells. Thus, the role of AMPK in ANE 30-100K-induced autophagy ,ay be varied among different cells, which needs further confirmation. On the other hand, the arecoline-induced apoptosis in these three cells were proven to rely on the inhibition of AMPK, which is mediated by the depletion of intracellular glutathione. We are preparing the manuscript for these findings.
2. **Comparison of the known stress signals which are possibly transmitted by arecoline and ANE 30-100K.** Among the known stress signals, AMPK are known to be contrarily regulated by arecoline and ANE 30-100K. In addition, by using protein 2D electrophoresis, our preliminary results revealed that protein levels of endoplasmin precursor, thyroid hormone binding protein precursor, human protein disulfide isomerase (Chain A), and glutathione-insulin transhydrogenase in OECM-1 and elongation factor-1 α1and glyceraldehyde 3-phosphate dehydrogenase in CE81T/VGH were elevated after ANE 30-100K treatment. It is worthy to keep on analyzing the roles of these proteins in ANE 30-100K-induced autophagy.
3. **Identification of the cellular receptor of ANE 30-100K.** No further result was achieved in this section; however, we analyzed the composition of monosaccharide of ANE 30-100K, and found that it is composed of 10.6% galactose-NH_2, 14.0% glucose-NH_2, 21.4% galactose, 36.3% glucose, and 17.6% mannose. With the help of

Dr. Wen-Bin Yang in Academia Sinica, we will try to further purify the active component responsible for autophagy induction and analyze its carbohydrate structure.

Keywords: areca nut, apoptosis, autophagy

前言與研究目的

Arecoline is the major alkaloid of areca nut (AN) capable of triggering apoptosis in a variety of oral cells. However, we previously demonstrated that AN extract (ANE) itself stimulate autophagy in a wide range of cell types. The molecular weight of autophagy-inducing AN ingredient (AIAI) was further traced to the 30-100 kDa fraction of ANE, designated as ANE 30-100K. This finding renewed the knowledge of AN mediated cytotoxicity and rendered the underlying mechanism worthy to be studied. Investigation of the three issues (1. the involvement of calmodulin-dependent protein kinase kinase β (CaMKKβ)/AMP-activated protein kinase (AMPK) pathway in arecoline- and ANE 30-100K-mediated apoptosis and autophagy, respectively; 2. Comparison of the known stress signals which are possibly transmitted by arecoline and ANE 30-100K; 3. Identification of the cellular receptor of ANE 30-100K) is the main goal of this project and was expected to provide some insights into the autophagic and apoptosis mechanisms respectively induced by ANE 30-100K and arecoline.

Introduction:

We have been studying the role of matrix metalloproteinases in the oral diseases in the past few years (1-5). Despite the increasing numbers of apoptosis-inducing areca nut (AN) components, such as arecoline and oligomeric procyanidins, having been identified, we recently found that AN extract (ANE) and the 30-100 kDa fraction of ANE (ANE 30-100K) induce autophagy-like responses in oral cancer OECM-1 cells (6, 7). With the support of this project, we further confirmed that both ANE and ANE 30-100K indeed trigger the execution of autophagic flux in both normal and malignant cells (8). This project was to analyze (i) possible implication of an autophagy mediator, AMP-activated protein kinase (AMPK) in ANE 30-100K- and arecoline-induced death signals; (ii) signals differentially regulated by ANE 30-100K and arecoline; (iii) cellular receptor of ANE 30-100K.

Although the activator of AMPK, AICAR, was originally reported to inhibit the execution of autophagy, AMPK was subsequently demonstrated to be required for this process by using its specific inhibitor Compound C and the expression of a dominant negative AMPK (9, 10). More recently, newly synthesized AMPK activators have been shown to enhance autophagy (11). It is therefore reasonable to speculate that this kinase may also play a role in the ANE 30-100K-induced autophagy. Indeed, our preliminary results showed that ANE 30-100K stimulated a profound elevation of AMPK phosphorylation. Furthermore, a specific inhibitor of calmodulin-dependent protein kinase kinase β (CaMKKβ), the upstream regulator of AMPK, STO-609 and Compound C attenuated ANE 30-100K-induced autophagic cell death of Jurkat T cells, suggesting a similar role of AMPK in this type of autophagy initiated by the autophagy-inducing AN ingredient (AIAI). However, changes of AMPK levels were not evident in both OECM-1 and CE81T/VGH cells after ANE 30-100K treatment. STO-609 and Compound C also failed to protect these two cells from the insult of ANE 30-100K. Collectively, it is suggested that although ANE 30-100K induces autophagy in a wide range of cell types, the dependence on AMPK may vary among different cells.

Interestingly, AMPK also regulates apoptosis; however, its role in regulating apoptosis remains controversial. Take more recent studies as examples, apoptosis induced by temozolomide, bioenergetic stress, and C6 ceramide was shown to depend on the activation of AMPK (12-14); however, AMPK was shown also to negatively regulate apoptosis in cisplatin-treated tumour cells and high glucose-treated glomerular epithelial cells (15, 16). It is suggested that this kinase protects cells from transient energy depletion, but prolong activation triggers apoptosis by transcriptional activation of the pro-apoptotic Bcl-2 family member, bim (17, 18). Our unpublished results indicated that arecoline inhibits AMPK within 3 h, and the inhibiting effect did not recover after 24 h in the three cells mentioned above. Compound C and RNAi interference further increased arecoline-induced apoptotic death; while AICAR attenuated this event. Therefore, inhibition of AMPK seems to be required for arecoline-induced apoptosis. Whether arecoline-mediated apoptosis goes through a Bim-independent pathway remains further study.

We have tried to purify and sequence cellular proteins bound to the ANE 30-100K-coated

plate separated by SDS-PAGE and transferred onto PVDF membrane. It is not clear why the protein signals were not reproducible. On the other hand, with the help of DR. Wen-Bin Yang in the Genomics Research Center of Academia Sinica, we preliminary characterize the monosugar composition of ANE 30-100K as listed in the abstract section. Further purification and characterization of ANE 30-100K is suggested by Dr. Yang, which is also included in our future works.

References:

1. Liu YC, Liu SY, Lin MH, Cheng PC, Chen LL, Chen TC, Cheng YC, Hsu KC, Chiang WF, Lee CH, Liu YC. Possible mechanism of betel quid extract (BQE)-induced MMP-2 expression. J Formos Med Assoc. 2009 Dec; accepted.
2. Liu SY, Liu YC, Huang WT, Huang GC, Su HJ, Lin MH. Requirement of MMP-3 in anchorage-independent growth of oral squamous cell carcinomas. J Oral Pathol Med. 2007 Aug;36(7):430-5.
3. Lin MH, Liu SY, Su HJ, Liu YC. Functional role of matrix metalloproteinase-28 in the oral squamous cell carcinoma. Oral Oncol. 2006 Oct;42(9):907-13. Epub 2006 May 26.
4. Liu SY, Lin MH, Yang SC, Huang GC, Chang L, Chang S, Yen CY, Chiang WF, Kuo YY, Chen LL, Lee CH, Liu YC. Increased expression of matrix metalloproteinase-2 in oral cells after short-term stimulation and long-term usage of areca quid. J Formos Med Assoc. 2005 Jun;104(6):390-7.
5. Liu SY, Lin MH, Yang SC, Huang GC, Chang L, Chang S, Yen CY, Chiang WF, Lee CH, Kuo YY, Liu YC. Areca quid chewing enhances the expression of salivary matrix metalloproteinase-9. J Formos Med Assoc. 2005 Feb;104(2):113-9.
6. Liu SY, Lin MH, Hsu YR, Shih YY, Chiang WF, Lee CH, Chou TH, Liu YC. Arecoline and the 30-100 kDa fraction of areca nut extract differentially regulate mTOR and respectively induce apoptosis and autophagy: a pilot study. J Biomed Sci. 2008 Nov;15(6):823-31.
7. Lin MH, Liu SY, Liu YC. Autophagy induction by a natural ingredient of areca nut. Autophagy. 2008 Oct 1;4(7):967-8. Epub 2008 Oct 20.
8. Lin MH, Hsieh WF, Chiang WF, Hong WZ, Hsu YR, Cheng YC, Chen TC, Hsu KC, Lin PY, Liu SY, Liu YC. Autophagy induction by the 30-100kDa fraction of areca nut in both normal and malignant cells through reactive oxygen species. Oral Oncol. 2010 Sep 30. [Epub ahead of print]
9. Samari HR, Seglen PO. Inhibition of hepatocytic autophagy by adenosine, aminoimidazole-4-carboxamide riboside, and N6-mercaptopurine riboside. Evidence for involvement of amp-activated protein kinase. J Biol Chem. 1998 Sep 11;273(37):23758-63.
10. Meley D, Bauvy C, Houben-Weerts JH, Dubbelhuis PF, Helmond MT, Codogno P, Meijer AJ. AMP-activated protein kinase and the regulation of autophagic proteolysis. J Biol Chem. 2006 Nov 17;281(46):34870-9.
11. Vingtdeux V, Chandakkar P, Zhao H, d'Abramo C, Davies P, Marambaud P. Novel synthetic small-molecule activators of AMPK as enhancers of autophagy and amyloid-{beta} peptide degradation. FASEB J. 2010 Sep 17. [Epub ahead of print]
12. Zhang WB, Wang Z, Su F, Jin YH, Liu HY, Wang QJ, Yang Y. Activation of AMP-activated

protein kinase by temozolomide contributes to apoptosis in glioblastoma cells via p53 activation and mTORC1 inhibition. J Biol Chem. 2010 Sep 29. [Epub ahead of print]
13. Kilbride SM, Farrelly AM, Bonner C, Ward MW, Nyhan KC, Concannon CG, Wollheim CB, Byrne MM, Prehn JH. AMP-activated protein kinase mediates apoptosis inresponse to bioenergetic stress through the activation of the pro-apoptotic BH3-only protein BMF. J Biol Chem. 2010 Sep 14. [Epub ahead of print]
14. Ji C, Yang B, Yang YL, He SH, Miao DS, He L, Bi ZG. Exogenous cell-permeable C6 ceramide sensitizes multiple cancer cell lines to Doxorubicin-induced apoptosis by promoting AMPK activation and mTORC1 inhibition. Oncogene. 2010 Aug 30. [Epub ahead of print]
15. Harhaji-Trajkovic L, Vilimanovich U, Kravic-Stevovic T, Bumbasirevic V, Trajkovic V. AMPK-mediated autophagy inhibits apoptosis in cisplatin-treated tumour cells. J Cell Mol Med. 2009 Sep;13(9B):3644-54.
16. Eid AA, Ford BM, Block K, Kasinath BS, Gorin Y, Ghosh Choudhury G, Barnes JL, Abboud HE. AMPK negatively regulates NOX4-dependent activation of p53 and epithelial cell apoptosis in diabetes. J Biol Chem. 2010 Sep 22. [Epub ahead of print]
17. Concannon CG, Tuffy LP, Weisová P, Bonner HP, Dávila D, Bonner C, Devocelle MC, Strasser A, Ward MW, Prehn JH. AMP kinase-mediated activation of the BH3-only protein Bim couples energy depletion to stress-induced apoptosis. J Cell Biol. 2010 Apr 5;189(1):83-94.
18. Weisová P, Dávila D, Tuffy LP, Ward MW, Concannon CG, Prehn JH. Role of AMP-activated protein kinase (AMPK) in cell survival and death responses in neurons. Antioxid Redox Signal. 2010 Aug 16. [Epub ahead of print]

The principle methods used to study this project are cell culture, Western blot analysis, RNAi interference, XTT assay, acidic vesicle staining, and protein 2D electrophoresis. These methods were well established in our laboratory and are described in the references. Protein identification and transmission electron microscopy are helped and analyzed by Cheng Kung University.

結果與討論（含結論與建議）

While executing this project in the past year, we observed that ANE 30-100K can induce autophagy in both normal and malignant oral cells, esophageal and bladder carcinomas, as well as peripheral blood lymphocytes and Jurkat T cells, the new findings published on Oral Oncology lately (8).

This interesting AN component may activate AMPK and lead to autophagy in Jurkat T cells; however, these events were not observed in OECM-1 and CE81T/VGH cells. More studies are required before obtaining a conclusive conclusion of the role of AMPK in ANE 30-100K-induced autophagy. On the other hand, arecoline significantly reduced the phosphorylation level of AMPK, and pharmacological activator and inhibitor of AMPK, as well as RNAi interference revealed that AMPK inhibition is required for arecoline-induced apoptosis. We are now preparing a manuscript describing the role of AMPK in the arecoline-induced apoptosis.

Possibly for a quantitative reason, no protein signals could be detected after the binding of cellular proteins from 10^9 cells to ANE 30-100K coated plate. It is also possible for the cellular receptor of ANE 30-100K to be carbohydrate, or mainly composed of carbohydrate. To solve this problem, we are to analyze the sugar composition of the bound materials described above.

Finally, characterization of the sugar structure of AIAI is also included in our future works.

計畫成果自評部份，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、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. In this project, we expected to address the role of AMPK in the ANE 30-100K- and arecoline-induced autophagy and apoptosis, respectively. Our preliminary results suggest that the dependence of ANE 30-100K-induced autophagy on AMPK may fluctuate among different cells. On the other hand, The arecoline-induced apoptosis is consistently rely on the inhibition of AMPK among different cells. Therefore, most of the proposed works are done in the past year.
2. We at least identified the differential regulation patterns on AMPK by ANE 30-100K and arecoline; and we also measured some potential proteins upregulated after ANE 30-100K treatment.
3. ANE 30-100K was firstly identified to be composed by five monosaccharide.

All these findings provide new insight into AN-associated pathology, worthy of continual study.

無衍生研發成果推廣資料

98 年度專題研究計畫研究成果彙整表

計畫主持人：林美惠	計畫編號：98-2314-B-041-002-
計畫名稱：不同的檳榔子成份所誘導的細胞凋亡與自體吞噬之探討	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	3	0	30%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	1	0	50%		
國外	論文著作	期刊論文	1	1	100%	篇	Autophagy induction by the 30-100kDa fraction of areca nut in both normal and malignant cells through reactive oxygen species. Lin MH, Hsieh WF, Chiang WF, Hong WZ, Hsu YR, Cheng YC, Chen TC, Hsu KC, Lina PY, Liu SY, Liu YC. Oral Oncol. 2010 Sep 30. [Epub ahead of print] PMID: 20920876
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	

	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)		無					

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

國科會補助專題研究計畫成果報告自評表

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

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

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)

我們進一步發現檳榔子萃取液（ANE）30-100K 透過 Reactive Oxygen Species 不同類型的細胞均可誘導自體吞噬的發生(Oral Oncology, 2010 Sep 30. [Epub ahead of print] PMID: 20920876)。此外我們也發現在 Jurkat T cells 中，ANE 30-100K 誘導 AMPK 的活化，進而導致自體吞噬的發生；而 Arecoline 則是抑制 AMPK，進而導致細胞凋亡的發生（撰寫中）。在檳榔相關的研究中有其創新之處，並且值得繼續探討其機轉。