行政院國家科學委員會專題研究計畫 成果報告

槲黃素及其相關衍生物之抗癌活性及對癌細胞組蛋白乙醯化與其所調控基因表現之影響研究(第 3 年)
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行政院國家科學委員會補助專題研究計畫 v 成 果 報 告 □期中進度報告

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目　錄

中文摘要

組蛋白乙醯化可以調控基因的表現，許多癌症產生的原因可能涉及了由於組蛋白去乙醯化作用所產生抑制抑癌基因的轉錄，先前本計劃已於期中報告中指出類黃酮衍生物 槲黃素具有誘發血癌細胞走向凋亡之作用且此作用與誘發組蛋白乙醯化調控 Fas ligand 表現有相關聯,另外我們也發現另一類黃酮衍生物芹菜素具有抑制乳癌細胞增生之作用,而此作用與誘發組蛋白乙醯化進而調控 p21 表現有關,最後本年度以另一類黃酮衍生物木樨草素進行比較探討.木樨草素(Luteolin, 3',4',5,7-tetrahydroxyflavone)是一種普遍存在於各種植物的類黃酮,屬類黃酮當中的黃酮，在傳統醫學裡被應用於治療各種疾病，包括高血壓、發炎和癌症。在本實驗中發現木樨草素(35 μM)可以誘導 HL-60 細胞走向細胞凋亡，為了證實其中的分子機制，在此利用西方點墨法、DNA 電泳、聚合酶鏈鎖反應、反轉錄聚合酶鏈鎖反應、染色質免疫沉澱法等技術觀察，結果我們發現，木樨草素可以經由活化 JNK MAPK pathway 促使轉錄因子 c-jun 往核內移動，同時增加組蛋白 H3 之 *FasL* 基因操縱子區域高度乙醯化,改變染色質結構而增加 *FasL* 轉錄活性，促使 FasL 新合成而活化 Caspase 8、Caspase 3，並進一步造成 PARP 斷裂導致 HL-60 細胞走向細胞凋亡；另一方面，我們也發現，木樨草素也能造成 *Fas* 基因操縱子區域的高度乙醯化而增加其轉錄活性，共同引發外源途徑的細胞凋亡。綜合以上結果，我們知道，木樨草素誘導 HL-60 血癌細胞細胞凋亡是透過活化訊息傳遞 JNK/c-Jun signaling pathway 與組蛋白的修飾作用。另外在乳癌細胞研究上也發現其與芹菜素類似也可透過促進組蛋白 H3 乙醯化而正調控 p21 表現之作用.

關鍵詞: 木樨草素　細胞凋亡　組蛋白乙醯化

英文摘要

Histone acetylation appears to play an important role in transcriptional regulation of cancer. Inactivation of chromatin by histone deacetylation is involved in the transcriptional repression of several tumor suppressor genes. Previously, our study showed qurecetin increased acetylation of histone H3 which modulated the expression of FasLin HL-60 cells. In addition, another flavonoid, apigenin, increased histone H3 acetylation medialted p21expression resulting in cell cycle arrest in MDA-MB-231 cells. The following study will investigate the effect of luteolin, 3',4',5,7-tetrahydroxyflavone, in anticancer potential involving histone acetylation. Luteolin, , is a common flavonoid that exists in many types of plants including fruits, vegetables, and medicinal herbs. Plants rich in luteolin have been used in Chinese traditional medicine for treating various diseases such as hypertension, inflammatory disorders, and cancer. Here, we found that luteolin was able to induce HL-60 cells apoptosis. For further understanding of this molecular mechanism we applied western blot, DNA electrophoresis, PCR, RT-PCR and ChIP assay. As a result we discovered that luteolin activated JNK MAPK pathway facilitating c-jun transcription factor translocation from cytosol to nuclear. In addition, luteolin induced hyperacetylation of FasL promoter and change chromatin structure which increased transcriptional activity of FasL. This de novo FasL induced caspase 8 and caspase 3 activation and PARP cleavage in regulating the luteolin-induced apoptosis. Taken together, luteolin induced HL-60 cells apoptosis through JNK/c-Jun signaling pathway and histone modification. Overall, luteolim presents antitumor activity involving mediating histone H3 acetylation.

Keywords: luteolin, apoptosis, histone acetylation.

前　言

1.基因調控與癌症 (Epigentics and Cancer)

遺傳學上，癌症可以被視為是一種由於基因缺陷所產生的疾病， 一旦基因的遺傳序列發生突變、刪除或是染色體異常便會造成腫瘤抑制基因(tumor-supressor genes, TSGs)喪失功能或是增加致癌基因(oncognes)的表現，進而導致癌症的發展與形成。而近年來對於基因表現的研究逐漸證實，透過遺傳修飾(epigenetic changes)可以造成染色質重組(chromatin remodeling)致使基因表現的改變。DNA 經由組蛋白組裝纏繞成為染色質，其中組蛋白的 N 端會受到一些官能基的修飾，例如：乙醯化 (acetylation)、甲基化 (methylation)、磷酸化 (phosphorylation)，泛素化 (ubiquitinylation)，羰基化(carbonylation)，醣化(glycosylation)以及 SUMOylation，而這些修飾作用除了針對組蛋白外，對於其他的蛋白質，例如：轉錄因子也可能產生類似的修飾作用，而去影響基因表現.

近年來在所有的基因修飾上，屬乙醯化最為被廣泛研究，乙醯化的作用是讓透過組蛋白乙醯轉移酶(histone acetyltransferases, HATs)將乙醯輔酶 A(acetyl-coenzyme A)上的乙醯基轉移到組蛋白上的離胺酸，使得離胺酸上的 NH^{3+}被轉換成乙醯基，正電荷受到中和，而降低兩者之間的親和力，讓帶負電荷的 DNA 不再與組蛋白緊密結合，染色質結構因此鬆開，相反地，組蛋白去乙醯酶(histone deacetyltransferases, HDACs)能夠將組蛋白上的乙醯基移除，而讓染色質能夠再常染色質與異染色質間轉換調控基因轉錄的能力.

組蛋白乙醯化主要是由 HATs 與 HDACs 兩種酵素間的平衡所調控，在人類中已有將近 20 種以上的 HDAC 被發現，根據與酵母菌同源的 HDACs 做分類，可以將其分為三大類：Class Ⅰ(1,2,3,8)、Class Ⅱ(Ⅱa：4, 5, 7, 9、Ⅱb：6, 10)以及 Class Ⅲ(SIRT1-7)，第一類的 HDACs 同源於酵母菌的 RPD3 蛋白，此類 HDACs 大都位於細胞核中，且廣泛地表現在各種人類細胞株和組織；第二類 HDACs 則是同源於酵母菌的 Hda1，他們可以改變在細胞中的位置，在細胞核與細胞質中轉換，而 Class Ⅱb 則多位於細胞質，並且在結構上具有兩個去乙醯基的功能區；第三類的 HDACs 則是同源於 Sir2，必須要有 NAD^{+}作為輔因子，方能作用，主要是在因應細胞內氧化還原狀態所改變的基因表現，研究發現，SIRT1 可以對 p53 進行去乙醯化，抑制 p53 的轉錄活性；除了上述的 HDACs，HDAC11 被歸類在 Class Ⅳ HDACs，其結構類似 Class Ⅰ、Ⅱ但相似度卻不足以被歸類進去。

2. 類黃酮 (Flavonoids)

類黃酮屬於一種天然的抗氧化劑，許多蔬果中都含有這些多酚類化合物，能幫助清除體內的自由基。而類黃酮的基本結構包含 15 個碳原子排列在 3 個環上，而類黃酮其主體結構由 A、B、C 三個環組成，主要的分別在於碳環上的取代基，A 環及 B 環大多含有取代基，以氫氧基最多，甲氧基次之，也有許多是以糖基的形式取代，根據取代基及飽和的位置，又可將類黃酮分成五大類：黃酮類(Flavones)、黃酮醇類(Flavonols)、異黃酮類(Isoflavones)、黃烷醇類(Flavanols)、黃烷酮類(Flavanones)、花青素(Anthocyanins)，這些類黃酮的成分被發現具有各種生物活性，包括抗動脈粥狀硬化、抗發炎抗血栓形成以及抗癌活性。

木樨草素(Luteolin, 3',4',5,7-tetrahydroxyflavone)屬於黃酮類的一種，普遍存在於各種植物當中，例如：金銀花、菊花、荊芥，而其他像是百里香、甘藍、花菜、甜菜、花椰菜和胡蘿蔔等日常食用的蔬菜當中也有木樨草素，目前已經知道木樨草素本身就具有抗氧化、抗發炎和抗過敏等多種生物活性，且相較於其他類黃酮具有更低的作用濃度(Seelinger, Merfort, & Schempp, 2008)，除此之外，目前也已有研究證實了木樨草素具有的抗癌功能，這些研究指出，木樨草素對多種實體腫瘤都有顯著的抑制作用，例如能藉由誘導腫瘤壞死因子 α (Tumor Necrosis Factor-α, TNF-α)或是抑制拓蹼樸異構酶 I、II (Topoisomerase I、II)來誘導細胞凋亡；透過細胞實驗也證實木樨草素也能抑制由 VEGF 所誘導的血管新生，透過動物模式亦得到相同的結果(Kim et al., 2005)，另外，木樨草素亦能通過抑制 MAPK/ERKs 與 PI3K/AKT 兩條路徑來抑制由肝細胞生長因子所誘導的肝癌細胞侵犯作用(Lee et al., 2006)；也有報告指出，木樨草素具有抑制癌細胞增生的作用；由此可知，木樨草素在抗癌作

研 究 目 的

近年來由於醫療的進步，許多疾病得以被治療，然而，癌症高居國人十大死因之首，卻仍然未有有效的治療方法，因此，癌症的預防與治療仍是目前醫療科技研究的首要目標。癌症的產生，可以被視為一種由於基因表達異常所造成的疾病，而染色體結構在調控基因的表現上扮演非常重要的角色，其中，透過組蛋白去乙醯基酶抑制劑(HDAC inhibitor, HDACi)可以增加抑癌基因的表現，而抑制腫瘤細胞生長或導致癌細胞程式性死亡，同時許多研究也發現，這些組蛋白去乙醯基酶抑制劑對於細胞是具有選擇性毒性的，因此，組蛋白去乙醯基酶抑制劑便成為當前具有發展潛力作為治療癌症的藥物。然而，目前臨床上用來治療癌症的各種療法，包括傳統化療、放射療法或是外科手術都有其一定的風險和副作用，因此發展具有治療潛力與低副作用的藥物，一直是當前科學界努力研究的目標。而目前已有許多研究發現，一些存在於天然植物中的化學物質，例如：維生素、多酚和類黃酮這些存在於日常食用蔬果的物質，可以呈現出許多不同機制的抗癌活性，而其中的類黃酮，屬於一種多酚類的化合物，其結構包含兩個芳香環(A, B)透過一個異環(C)連結，並且廣泛存在於蔬菜水果中,本研究想了解類黃酮衍生物如槲黃素木樨草素等是否能夠經由調控組蛋白的乙醯化，改變染色質結構，進而影響基因表現，來達到腫瘤抑制的作用.

文 獻 探 討

類黃酮衍生物是一大群植物二次代謝產物,含有 diphenylpropane (C6-C3-C6) 之結構,廣泛分布於蔬果中,文獻指出這一類植物成份具有抗菌.抗發炎.抗氧化及抗癌活性, 類黃酮衍生物被認為是具有防癌及治癌潛力的一群天然物,其作用機制包括: 影響 ROS 的含量.抑制 topoisomerase I and II, 降低 NFkB and AP-1 活性,穩定 p53,抑制 PI3K, STAT3, IGF1R and HER2,對於調控組蛋白的乙醯化相關機制則有待了解.

研 究 方 法

(一)、細胞培養 (cell culture)

人類血癌細胞株HL-60 (from *The NHRI Cell Bank*) 源自急性前骨髓性白血病(acute promyelocytic leukemia)患者，培養於含有10% fetal bovine serum (FBS : Gibco BRL)、1% L-Glutamine (Gln：Gibco)、1% Penicillin-Streptomycin (PS : Gibco)的RPMI 1640 medium(RPMI-1640 : Gibco)，培養箱設定5% CO_2，37℃恆溫環境，細胞密度維持在 $2x10^5$～$1x10^6$，每週更換培養基 2～3次。

人類血癌細胞株U937 (from *The NHRI Cell Bank*) 源自急性前骨髓性白血病(acute promyelocytic leukemia)患者，培養於含有10% fetal bovine serum (FBS : Gibco BRL)、1% L-Glutamine (Gln：Gibco)、1% Penicillin-Streptomycin (PS : Gibco)的RPMI 1640 medium(RPMI-1640 : Gibco)，培養箱設定5% CO_2，37℃恆溫環境，細胞密度維持在 $2x10^5$～$1x10^6$，每週更換培養基 2～3次。

人類乳癌細胞株MDA-MB-231(from *The NHRI Cell Bank*)， 培養於含有10% fetal bovine serum (FBS : Gibco BRL)、1% L-Glutamine (Gln：Gibco)、1% Penicillin-Streptomycin (PS : Gibco)的Dulbecco's Modified eagle medium (DMEM : Gibco)，培養箱設定5% CO_2，37℃恆溫環境，細胞密度維持在 $2x10^5$～$1x10^6$，每週更換培養基 2～3次。

(二)、細胞活性分析 (MTT assay)

利用活細胞能經由粒腺體去氫酶(dehydrogenase)的作用，將(3-[4 , 5-Dimethylthiazol-2-yl]-2 , 5-diphenyl-tetrazolium bromide) [MTT]代謝還原成紫色的 formazan crystal，並於波長 570 nm 有最大吸光值，再以比色法量化分析細胞的存活及增殖。將細胞培養後，以培養基將細胞濃度調整至 $1x10^5$ cells / ml，各取 0.5 ml 種於 24-well 培養皿後，培養於 5 % CO_2，37℃恆溫環境下。待 24 小時後，加入含有各種不同濃度的各種類黃酮各 0.5 ml，處理 12 小時。將各 well 之細胞液轉換至 eppendorf，離心去除舊培養基，更換為每 ml 含有 100 µl MTT (5mg/ml)之新培養基，再度放回培養箱反應 4 小時候，離心除去培養基，最後以 1 ml 的 DMSO 將紫色的 formazan 結晶溶解，並於波長 570 nm 下測定吸光值(OD_{570})。

(三)、西方點墨法 (Western blotting)

將細胞培養後，以trypsin-EDTA將細胞由培養皿打下，PBS沖洗後，以1000 rpm離心5分鐘，取沉澱細胞，加培養基調整細胞濃度至$2x10^5$ cells/ml，將細胞培養於10 cm-dish 24小時，以實驗所需的luteolin濃度預先處理1小時，作用指定的時間後，將細胞以trypsin-EDTA由培養皿打下，加入RIPA buffer（150mM NaCl, 1% NP-40, 0.5% Deoxycholic acid, 0.1% SDS, 50mM Tris-base, pH7.5），內含1mM sodium orthovanadate, 100µg PMSF, 170 µg/ml leupeptin，於冰上震盪30分鐘，在4℃下以13000 rpm離心10分鐘，取上層液定量蛋白，將定量後之蛋白取30 µg，調整至相同體積，再加入等量的5X Sample loading dye （2ml 0.5M Tris-HCl pH6.8, 1.6ml Glycerol, 3.2ml 10% SDS, 0.8ml 2-β-mercaptoethanol, 0.4ml 0.5% bromophenol blue）以95℃加熱5分鐘，迅速置入冰中冷卻10分鐘，以離心機將Sample spin down後再loading至每個well中，上層膠以70伏特，下層以130伏特跑電泳，

待電泳結束後將蛋白質的轉漬至Nitrocellular paper上，以5%脫脂牛奶於室溫下進行blocking 1小時，以washing buffer（PBS with 0.5% tween-20）沖洗3次，將NC paper置於4℃冰箱中與一級抗體反應overnight，以washing buffer沖洗3次，再以Horseradish peroxidase conjugated的二級抗體反應1小時，以washing buffer沖洗3次，最後加入Western Blot Chemiluminescence Reagent Plus反應1分鐘後，以冷光螢光數位元影像分析儀（LAS-1000 plus system）觀察並定量。

(四)、去氧核醣核酸斷裂 (DNA Fragmentation)

細胞培養後，以培養基將細胞調整為 2×10^6 cells/ml，取 1 ml 種入 10 cm 培養皿，24 小時後加藥處理；將細胞沖下置入 15 ml 離心管，4℃下以 1200 rpm 離心 5 分鐘並去除上清液，再以 PBS 沖洗兩次並轉置於 eppendorf; 加入 lysis buffer [20 mM Tris、10 mM EDTA、0.2% Triton X-100 pH8.0) 0.5 ml/enpendrof，再加入 12.5 µl proteinase K (40mg/ml)]於 50℃水浴作用 6 小時，接著加入 5 µl RNAse (10mg/ml)於 37℃水浴作用 1 小時，加入等體積的飽和 phenol (pH=8.0)，輕輕搖晃均勻，之後在 4℃下以 12000 rpm 離心 10 分鐘，取上清液至新 eppendorf 後，加入等體積的 phenol/chloroform/isoamylalchol [25:24:1]，輕輕搖晃均勻(25-50 次)，接著在 4℃下以 12000 rpm 離心 10 分鐘，取上清液至新 eppendorf，加入等體積 isopropanol 及 4ul Glycogen (5mg/ml)，於-20℃靜置 overnight；接著在 4℃下以 12000 rpm 離心 10 分鐘，以 75% ehtanl 沖洗 pallete 後，4℃、12000 rpm 離心 10 分鐘，去除上清液後風乾 10~15 分鐘，加入適量 TE buffer (10mM Tris，1mM EDTA) (約 30λ) 37℃回溶 DNA。最後用 50V 跑 1.5%的洋菜膠電泳，再以 EtBr 染色後用 KODAK 數位元影像系統 EDAS290 定量。

(五)、反轉錄聚合酶鏈鎖反應 (RT-PCR)

細胞培養後，調整細胞濃度為2×10^6 cells/ml，取1 ml種入10cm dish(2×10^6 cells/10-cm dish)，隔天加藥處理，到達時間點後將細胞收入15 ml離心管，再以PBS wash兩次，最後將細胞轉入eppendorf，於4℃下、2000 rpm離心5分鐘後去除上清液，加入1 ml Trisolution Reagent/eppendorf並pipetting直至細胞溶解後置於室溫下反應5分鐘，接著加入Chloroform 0.2 ml/eppendorf，搖晃均勻後靜置於室溫下反應2~3分鐘，4℃、12000 rpm離心15分鐘，小心將上清液取至新eppendorf，加入isopropanol 0.5 ml/eppendorf搖晃均勻後靜置於室溫下10分鐘以沉澱RNA，4℃、12000 rpm離心15分鐘可以看到白色RNA沉澱，去除上清液再以75% Alcohol l ml/eppendorf wash，4℃、12000 rpm離心15分鐘，去除上清液後靜置風乾，再加入30µl DEPC water回溶，並測定RNA濃度(A_{260}/A_{280})。取1~5 µg RNA、oligo$(dT)_{18}$ primer (0.5 µg/µl) 1µl，並補DEPC water至12µl，70℃反應5分鐘，再加入5X reaction buffer 4µl、Ribonuclease inhibitor (20u/µl) 1µl和10mM dNTP mix 2 µl，37℃下反應5分鐘，再加入ReverstAidTM M-MuLV Reverse Transcriptase (200u/µl) 1µl作反轉錄(所有Reagent來自Fermentas, RevertAid^{TM} First Strand cDNA Synthesis Kit, #K1629)。取2.5 µl cDNA作PCR。

(六)、染色質免疫沉澱 (Chromatin Immunoprecipitation, ChIP)

細胞培養後，將細胞調整成2×10^7 cells/ml後，取1 ml種於15-cm dish，處理藥物後，均勻加入甲醛113.5µl後於室溫下反應10分鐘，在加入2.5M Glycine 0.75 ml繼續在室溫下反應5分鐘，將細胞

沖下置入15 ml離心管，離心4℃、5分鐘、1500 rpm，再以PBS沖洗三次，換至eppendorf，再加入FA Lysis Buffer 750 µl/ eppendorf；之後利用超音波細胞粉碎機以6 wart強度、10秒七次之頻率將DNA震成500~1000 bp.的片段大小，再以4℃、8000 g離心30秒，取上清液至新的eppendorf並各取50µl作為INPUT；加入5µl Proteinase K (20 mg/ml)於65℃下反應4~5小時，加入phenol:chloroform萃取DNA，取上清液至新eppendorf，再以含有10µl glycogen(5 mg/ml)5µl的99.8% ethanol沉澱DNA，最後以100µl二次水回溶；取25 µg DNA並以RIPA buffer作1:10稀釋後IP，加入一級抗體1~10 µg/ 25 µg DNA，再加入20 µl protein A/G beads於4℃之3D shaker下反應overnight；離心2000 g、4℃、1分鐘並去除上清液，再以Wash Buffer 1 ml沖洗beads三次，以Final Wash Buffer 1 ml沖洗一次後去除上清液；加入120 µl Elution Buffer於30℃下反應15分鐘，以2000 g離心1分鐘，將上清液轉至新eppendorf，加入280 µl phenol:chloroform抽取DNA，取上清液至新eppendorf，再以含有10µl glycogen(5 mg/ml)的75% ethanol沉澱DNA，最後以100µl二次水回溶，最後進行PCR，並以50 V作4%洋菜膠電泳，以數位元化影像處理系統拍攝分析。引子設計如下(Z. Huang et al., 2008; Jia et al., 2008)：

Fas promoter	Forward: 5’-GCAGAGCTTGGTGGACGATG-3’
	Reverse: 5’-TCACTATTGCTTTGGAACGGTAGA-3’
FasL	Forward: 5'-CTGTAAATTATGGTGATCGG-3'
promoter	Reverse: 5'-AACTCTAACAAAAATTGTTCAG-3'

(七)、 免疫沈澱法（Immunoprecipitation）

免疫沈澱法其原理在於利用抗體和抗原的專一性原理，將預觀察的蛋白從細胞內的所有蛋白中沈澱出來，將細胞培養並加藥處理後，將細胞收入15 ml離心管，PBS沖洗後，以1000rpm離心5分鐘，取沉澱細胞，加入RIPA buffer取得total cell lysate，定量後取1-0.5 mg/500 µl的蛋白質，加入欲觀察蛋白質的一級抗體10 µl（2 µg），於4℃下在3D shaker上反應overnight，接著加入對一級抗體具有結合力的Protein agarose A（取2.5 mg Protein agarose A加入500µl RIPA buffer）中，在3D shake上搖2~4小時。反應時間結束後，以4℃、 7000 rpm離心5分鐘。離心之後再以RIPA buffer清洗三次，最後離心沉澱物加入30 µl protein loading buffer，於95℃下反應5分鐘，之後置於冰上冷卻，即完成蛋白樣品的準備，接著依照西方點墨法的步驟跑SDS-PAGE。

結 果 與 討 論

一、 Luteolin 誘導 HL-60 與 U-937 血癌細胞死亡

利用 MTT assay 比色定量分析各種 Flavonoids (Apigenin、Luteolin、Genestine、Quercetin、Narnigenin)對 HL-60 血癌細胞生長之影響，以 Flavonoids 0~100 µM 處理 12 小時，結果發現，隨著處理劑量的增加，會促使 HL-60 血癌細胞死亡(Fig. 1)，以相同方式處理 U-937 血癌細胞株(Fig. 2)，在濃度 35 µM 就能殺死半數癌細胞。

二、 Luteolin 透過誘導細胞凋亡造成血癌細胞死亡

由前述實驗可知，luteolin 具有殺死血癌細胞的活性，因此我們進一步分析 luteolin 造成的細胞死亡現象是否與細胞的程式性死亡有關。利用細胞凋亡時所產生的 DNA 斷裂透可以觀察到，HL-60 血癌細胞在分別處理 luteolin 0、5、15、25、35、45 μM，12 小時後可以觀察到處理 35 μM、12 小時出現了 DNA 斷裂的現象 (Fig. 3),除此之外，細胞凋亡發生時所產生的 Caspase 3 和 PARP (Poly-ADP Ribose Polymerase)斷裂透過 Immunoblotting 觀察其表現，可以看到在處理 luteolin 35 μM 後的 6 小時就可以看到明顯的 PARP cleavage，而上游的 Caspase 3 也有明顯被活化的現象(Fig. 5)，證實 luteolin 造成的細胞死亡來自於細胞凋亡。 細胞凋亡主要通過兩條路徑：1.) Extrinsic pathway 2.) Intrinsic pathway 分別活化 Caspase 8 與 Caspase 9 造成，透過 Immunoblotting 分析發現，在處理不同濃度 (Fig. 4)與時間點 (Fig. 5)的 luteolin 後都可以看到 Caspase 8 有被活化的現象，因此，由此可知 luteolin 所誘導的細胞凋亡，會通過活化 Extrinsic pathway 所造成的。

三、 Luteolin 誘導活化 Fas 與 FasL 蛋白質表現

由於前面的實驗我們發現 HL-60 血癌細胞再處理 luteolin 後可以增加細胞凋亡的表現，因此，我們繼續觀察 luteolin 是透過何種路徑來誘導細胞凋亡。因此我們將 HL-60 細胞分別處理 luteolin 35 μM 6 與 12 個小時後以 western blot 作觀察，發現 Fas 的表現在 luteolin 6 小時後就有些微的增加，而 FasL 則是在處理 luteolin 12 小時後有明顯的表現 (Fig. 6)，顯示，luteolin 可以經由增加 Fas 與 FasL 的表現來誘導細胞凋亡的現象。

四、 Luteolin 誘導活化 ERK 與 JNK 訊息傳遞路徑

ERK1/2、JNK、p38 MAPK pathway 參與了細胞內的訊息傳遞，當細胞接受到不同的刺激，便會活化、傳遞訊息，調控細胞的增生、轉移、存活、凋亡等各種現象，因此為了證實 luteolin 誘導細胞凋亡的過程中，是否會通過活化 ERK1/2、JNK、p38 signaling pathway，於是利用 Westeren blot 觀察，結果可以看到，在處理 35 μM Luteolin 後，ERK 與 JNK 就迅速被磷酸化，並且隨著時間增加而增加，但是 p38 卻不受 luteolin 所影響 (Fig. 7)，由此可知，luteolin 能夠活化 HL-60 細胞內 ERK 與 JNK 兩條訊息傳遞路徑。

五、 Luteolin 誘導 c-jun 轉錄因子由細胞質移動至細胞核中

由於 luteolin 作用下可以誘導 Fas 與 FasL 蛋白質表現量的增加，而過去的文獻指出，c-jun 為 Fas 與 FasL 的轉錄因子(Kavurma & Khachigian, 2003)，而 c-jun 的表現又由 JNK pathway 所調控(Weston & Davis, 2002)，因此為了確認 luteolin 是否能藉由活化 JNK pathway 增加 c-jun 作為轉錄因子的能力，我們以 35μM luteolin 分別處理 HL-60 細胞 1、2、4、6、8、10 小時，之後抽取核蛋白並以 wesatern blot 觀察，結果發現，隨著 luteolin 處理時間的增加 c-jun 在細胞核內的表現也跟著增加，並在 4 小時的時候達到最高 (Fig. 8)，顯示，luteolin 的作用下，會增加 c-jun 在細胞核內的表現，並且有可能是作為凋亡基因的轉錄因子增加其轉錄活性。

六、 Luteolin 誘導 CD95 (APO-1/Fas)與 CD95L (APO-1L/FasL) mRNA 合成

當 Fas ligand 於其受器 Fas 結合活化 FADD，FADD 再活化下游的 Pro-Caspase 8，使得 Caspase 8 活化，因而引起下游的細胞凋亡路徑，因此，我們進一步分析，luteolin 的作用下，是否是透過誘導 Fas 和 FasL 生合成造成 Extrinsic pathway 的細胞凋亡。HL-60 血癌細胞分別處理 luteolin 35 μM、0、0.5、1、2、3、4、6、8、10 小時，經由 RT-PCR 分析，結果可以發現，隨著處理藥物時間的增加，Fas 與 FasL mRNA 的表現量逐步上升 (Fig. 9)，顯示，luteolin 透過活化 Fas 與 FasL mRNA 合成而造成 Extrinsic pathway 活化產生細胞凋亡現象。

七、 Luteolin 調控 FasL 操縱子區域和轉錄因子 c-jun 之關聯

隨著處理 luteolin 的時間增加會促使轉錄因子 c-jun 由細胞質移動至細胞核(Fig. 8)，同時促進轉錄因子 c-jun 與 FasL 操縱子結合(Fig. 10)，顯示 luteolin 促使 FasL 轉錄活化，可能是透過增加 c-jun 與 FasL 操縱子結合。然而 Fas 部份則無此作用.

八、 Luteolin 引起組蛋白乙醯化修飾作用

近年來的研究發現，組蛋白的乙醯化可以促使染色體結構打開，促進轉錄因子結合至操縱子區域，進而活化下游基因表現，有趣的是，我們又發現 luteolin 在處理 HL-60 細胞會誘導 FasL 與 Fas 的 mRNA 合成(Fig. 9)，因此，我們懷疑是否 luteolin 除了能經由活化 ERK1/2 與 JNK 兩條路徑來誘導細胞凋亡，還能直接透過調控基因表現的方式促使細胞凋亡？結果發現，以不同濃度的 luteolin 處理 12 小時後 (Fig. 11)，可以觀察到，隨著藥物的濃度增加，組蛋白 H3 (Histone H3)的乙醯化有逐漸增加的現象，但卻不會對組蛋白 H4 (Histone H4)造成乙醯化；而處理 35 μM luteolin 4 小時後也可以看到組蛋白 H3 有乙醯化的現象並隨時間增加而增加 (Fig. 12)，由此可知，luteolin 所誘導的細胞凋亡，有可能是透過改變染色質的結構來調控凋亡基因的表現。

九、 Luteolin 調控 Fas 與 FasL 操縱子區域乙醯化

由先前的實驗可以證實，luteolin 能夠造成組蛋白 H3 乙醯化 (Fig. 11、12、13)，同時也能促使 Fas 與 FasL mRNA 合成 (Fig. 10)，因此，為了證實 luteolin 是否能通過改變染色質結構造成目標基因的轉錄活性增加，我們利用染色質免疫沉澱法 (Chromatin Immunoprecipitation)觀察，結果發現，HL-60 細胞在受到 luteolin 刺激後，會在 4-8 小時後在 FasL 操縱子區域產生乙醯化；而同樣的，Fas 的操縱子區域也有乙醯化的現象產生，(Fig. 13)，因此，我們推斷 luteolin 有可能是藉由造成 Fas 與 FasL 操縱子區域的乙醯化進而改變染色質結構，增加轉錄活性，使得 Fas 與 FasL mRNA 合成增加。

十、 Luteolin 誘導 MDA-MB-231 乳癌細胞死亡

由先前的實驗我們知道 luteolin 作用在血癌細胞 HL-60 上具有相當的抗癌活性，可以

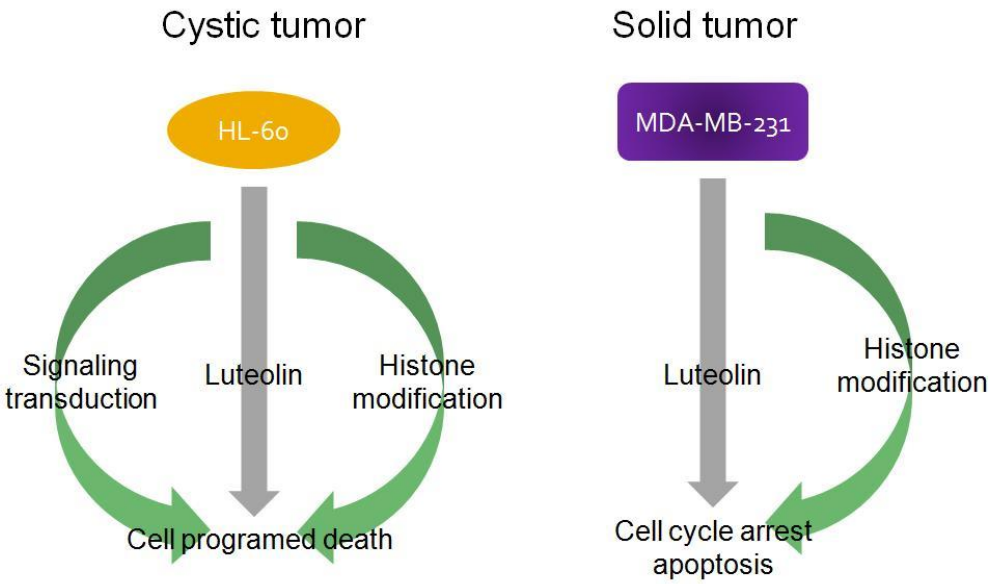
在 35μM 低濃度的情況下殺死半數的癌細胞，然而 luteolin 是否亦能有效作用在其他腫瘤細胞，因此我們進一步的比較木樨草素具有的抗癌活性使否特異性的針對血癌 (cystic tumor)，或是對於其他固態腫瘤 (solid tumor)，例如：MDA-MB-231 乳癌細胞也具有類似的抗癌活性。因此我們利用 MDA-MB-231 乳癌細胞作比較，同樣利用 MTT assay 比色定量分析 luteolin 是否能有效的抑制癌細胞生長，以 luteolin 0~100 μM 處理 12 小時，結果發現，隨著處理劑量的增加，會促使 MDA-MB-231 乳癌細胞死亡，並在濃度 75 μM 左右，造成半數癌細胞死亡 (Fig. 14)。

十一、Luteolin 調控 MDA-MB-231 乳癌細胞細胞週期蛋白表現

前面實驗發現，MDA-MB-231 乳癌細胞處理 35 μM luteolin 並不會誘導細胞凋亡，但是可以發現 G2/M 期的細胞周期停滯，而 CDKI (cyclin dependent kinase inhibitor) 會經由競爭抑制 cyclin-CDK 來調控細胞周期進行，進一步分析發現，luteolin 會增加細胞周期抑制蛋白 p21 的表現，而且透過的是 p53-independent 的途徑 (Fig. 15)，因此我們證明 luteolin 在抑制 MDA-MB-231 細胞生長是經由調控細胞週期蛋白 p21 而促使癌細胞停滯於 G2/M 期；並發現其表現受到 histone H3 acetylation 的調控(Fig.16-17)。

雖然在本研究中，我們發現 luteolin 會去影響組蛋白乙醯轉移酶與組蛋白去乙醯基酶而改變組蛋白乙醯化的現象，但是在測試 luteolin 對於上述兩種酵素的活性卻沒有影響，因而我們推論 luteolin 在影響組蛋白乙醯化的現象很可能主要是透過訊息傳遞路徑增加轉錄因子與染色體結合而間接影響到與轉錄因子結合的組蛋白乙醯轉移酶與組蛋白去乙醯基酶，進而增加凋亡基因的乙醯化，增加其轉錄活性。

結論



圖

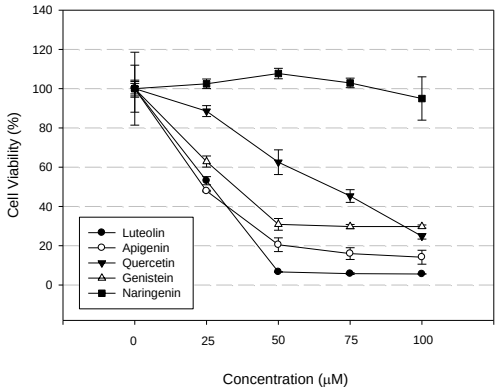


Figure 1. The effect of flavonoids on cell viability of HL-60 cells. HL-60 cells were treated with five different flavonoids at various concentration for 24 hrs then measure the absorbance after incubated with MTT for 4 hours. The experiments were performed in triplicate and per well were 5×10^4 cell numbers. Data presented as mean± S.D. of three independent experiments.

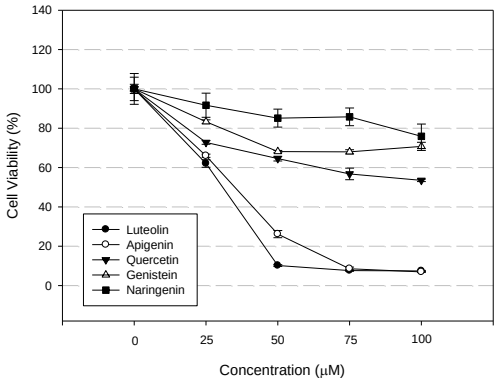


Figure 2. The effect of flavonoids on Cell Viability of U937 cells. U-937 cells were treated with five different flavonoids at various concentration for 24 hours then measure the absorbance after incubated with MTT for 4 hours. The experiments were performed in triplicate and per well were 5×10^4 cell numbers. Data presented as mean ±S.D. of three independent experiments.

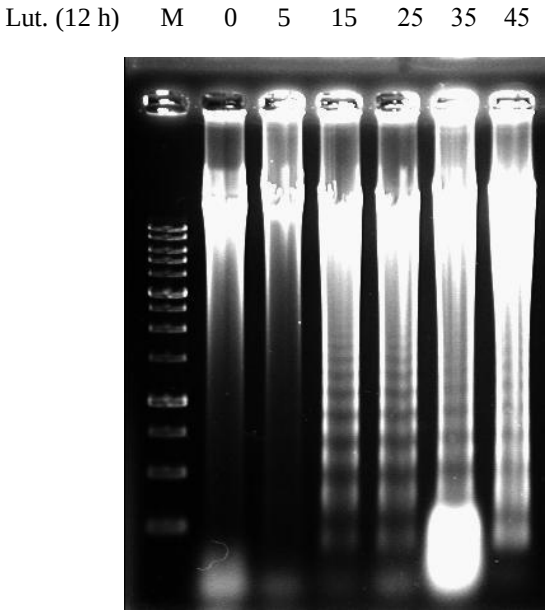


Figure 3. Induction of apoptosis by luteolin in HL-60 cells. HL-60 cells were treated with different concentration of luteolin for 12 hours. HL-60 cells were harvested for DNA fragmention assay by electrophoresis. The DNA were separated on a 1.5% agarose gel stained with ethidium bromide(EtBr), and photographed under ultraviolet light illumination.

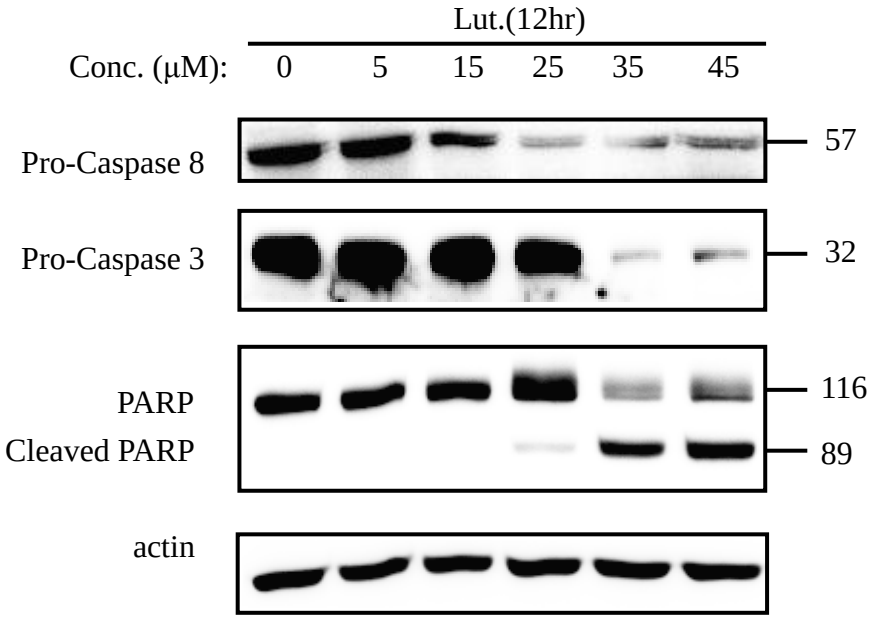


Figure 4. The effect of luteolin on protein expression of caspase 8, caspase 3 and PARP with various concentrations. HL-60 cells were treated with indicated concentration of luteolin for 12 hours. The total protein extracts were prepared for western blot analysis using indicated antibodies.

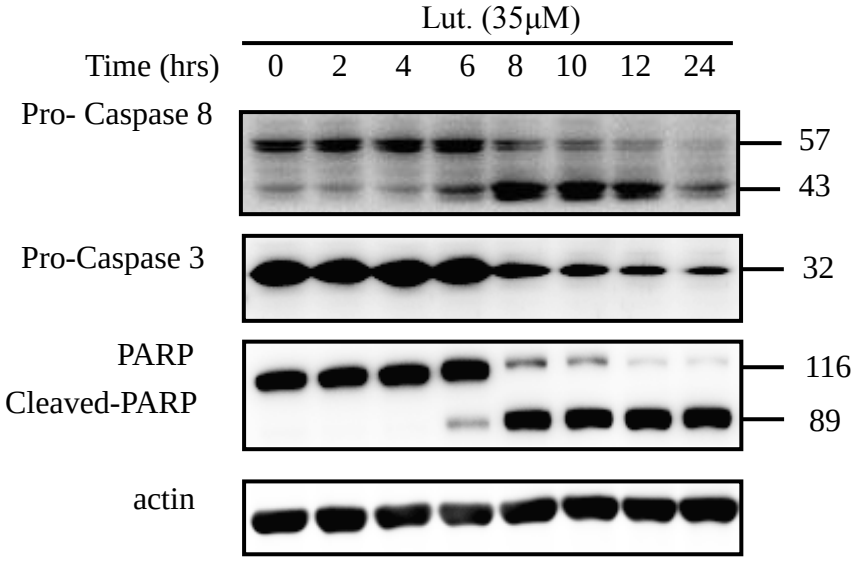


Figure 5. The effect of luteolin on protein expression of caspase 8, caspase 3 and PARP. HL-60 cells were treated with 35 μM luteolin for indicated times. The total protein extracts were prepared for Western blot analysis using indicated antibodies.

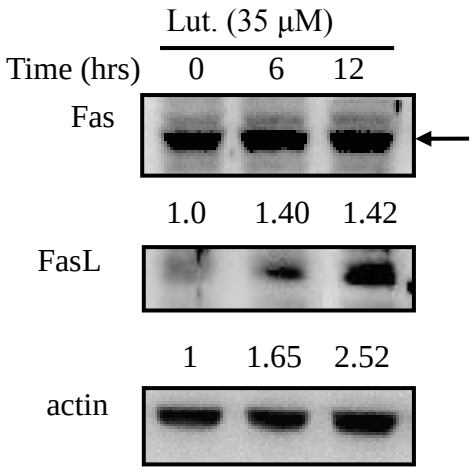


Figure 6. The effect of luteolin on protein expression of Fas and FasL. HL-60 cells were treated with 35 μM luteolin for 6 and 12 hours. The total protein extracts were prepared for western blot analysis using indicated antibodies.

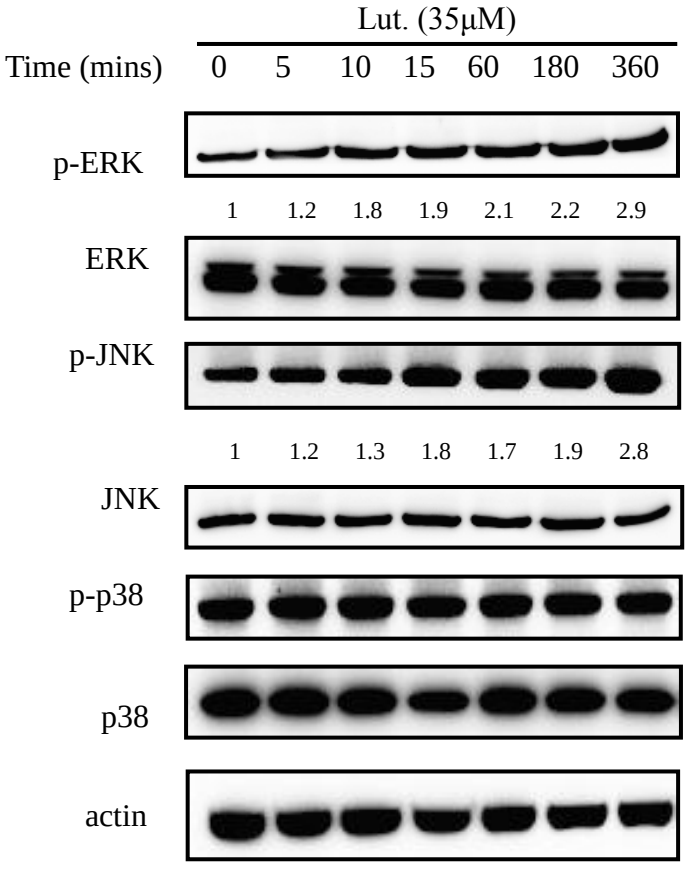


Figure 7. The effect of luteolin on MAPK pathway in lutolin stimulated HL-60 cells. The HL-60 cells were treated with 35μM luteolin for indicated time. The cell lysates were subjected to Western blot analysis using either phosphospecific antibodies against phospho-ERK, JNK/SAPK and p38, which react with active forms, and anti-ERK, JNK/SAPK and p38 antibodies, that recognized corresponding total non-phosphorylated enzymes.

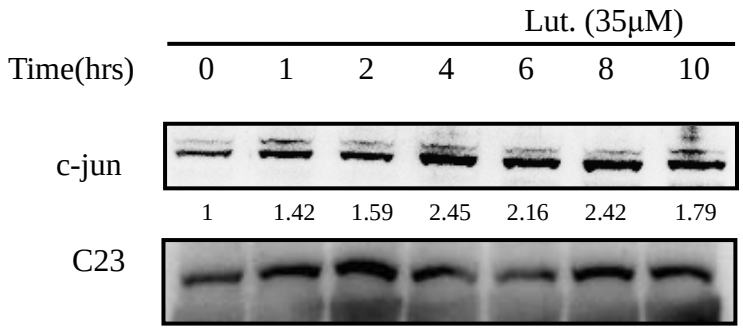


Figure 8. The effect of luteolin on c-Jun translocated from cytosol to nucleus in HL-60 cells. The HL-60 cells were treated with 35μM luteolin for indicated time. The nulear protein were extracted ans subjected to western blot analysis.

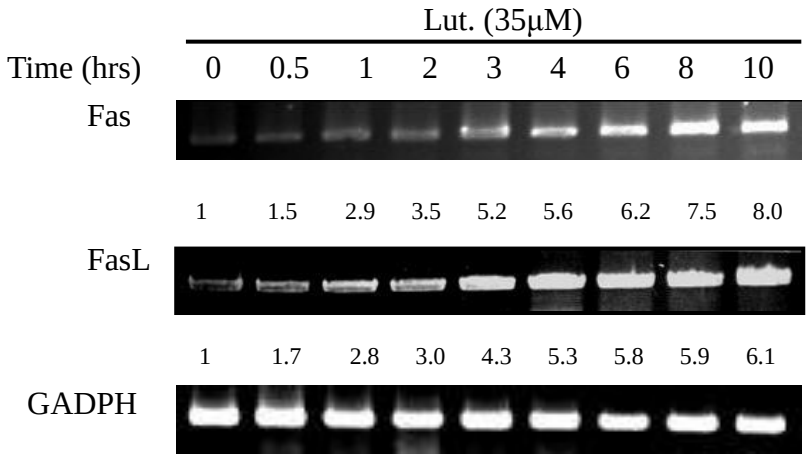
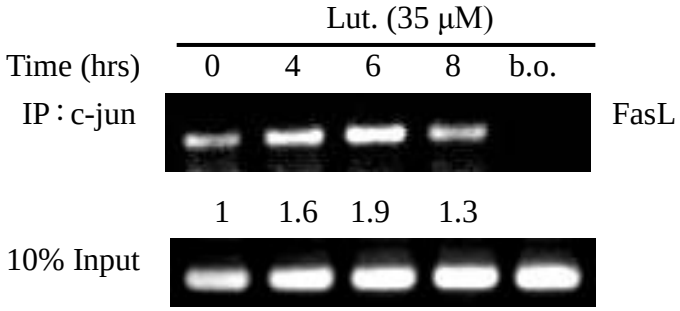


Figure 9. The effect of luteolin on mRNA level of Fas and FasL. HL-60 cells were treated with 35 μM luteolin with indicated time. Total RNA from HL-60 cells was analyzed by reverse-transcription (RT)–PCR using Fas, FasL and GADPH primers.

(A)



(B)

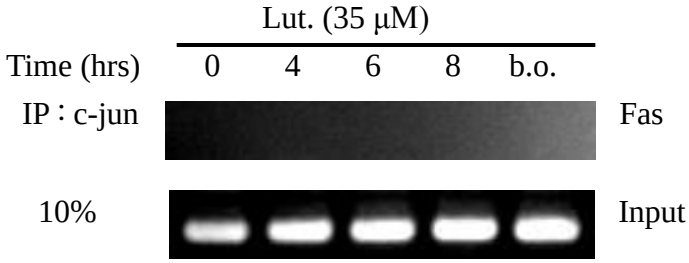


Figure 10. Luteolin induced p300 recruitment at FasL promoter regions but not Fas. Soluble precleared chromatin was obtained from HL-60 cells treated with or without 35 μM luteolin for 4, 6 and 8 hours immunoprecipitated (IP) with an anti-p300 and anti-c-jun antibodies. (A) The FasL promoter sequence was detected by PCR with specific primers. (B) The Fas promoter sequence was detected by PCR with specific primers. Input DNA was amplified from initial preparations of soluble chromatin (before immunoprecipitations). Beads only (b.o.) as negative control.

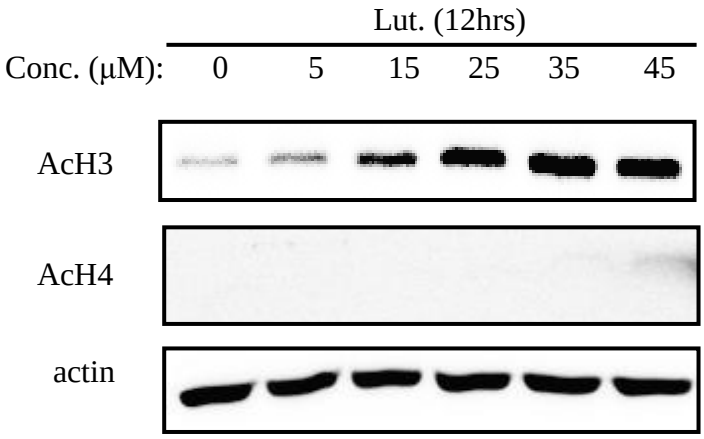


Figure 11. Luteolin induced acetylation of histone H3 in HL-60 cells. HL-60 cells were treated for 12 hours with indicated concentrations. Acetylation of histone H3(AcH3) and H4(AcH4) of the cell lysate were analyzed by immunoblotting assay.

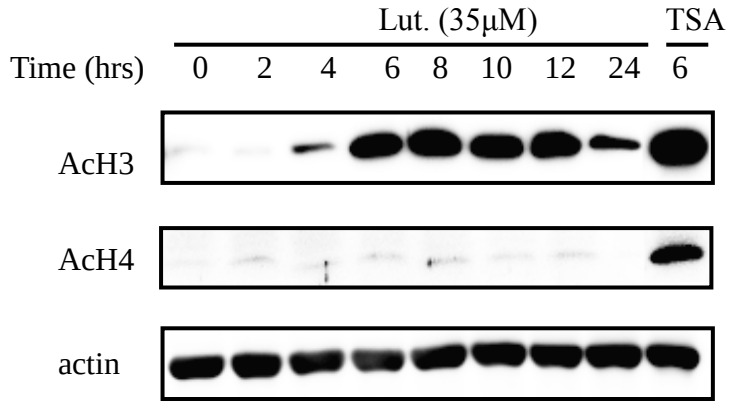


Figure 12. The time course effect of luteolin induced acetylation of histone H3 in HL-60 cells. HL-60 cells were treated with 35 μM luteolin and 0.3 μM TSA (trichostatin A) for indicated times. The total protein extracts were prepared for western blot analysis using indicated antibodies.

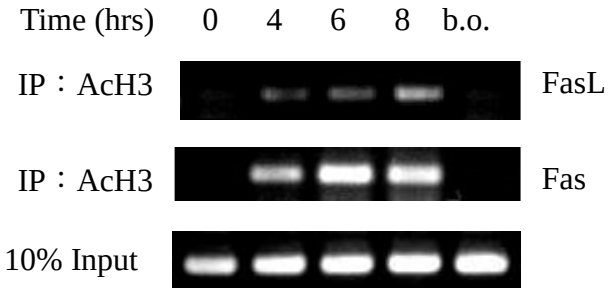


Figure 13. Luteolin induced acetylation of histone H3 at Fas and FasL promoter regions. Soluble precleared chromatin was obtained from HL-60 cells treated with or without 35 μM luteolin for 4, 6 and 8 hours immunoprecipitated (IP) with an anti-AcH3 antibody. The FasL and Fas promoter sequences were detected by PCR with specific primers. Input DNA was amplified from initial preparations of soluble chromatin (before immunoprecipitations). Beads only (b.o.) as negative control.

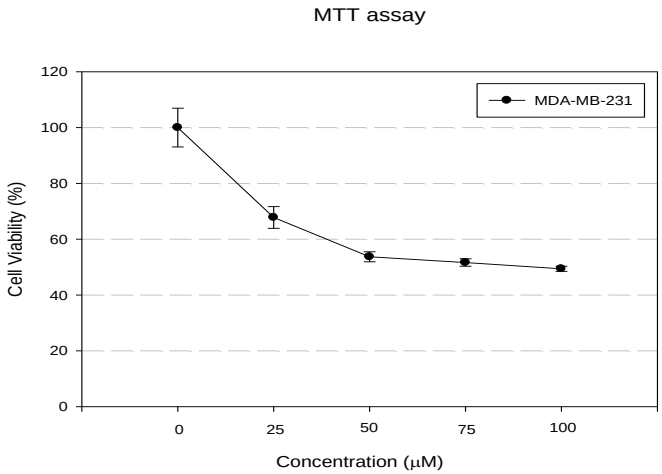


Figure 14. The effect of luteolin on cell viability of MDA-MB-231 cells. MDA-MB-231 cells were treated with luteolin at various concentration for 24 hours then measure the absorbance after incubated with MTT for 4 hours. The experiments were performed in triplicate and per well were 2×10^4 cell numbers. Data presented as mean± S.D. of three independent experiments.

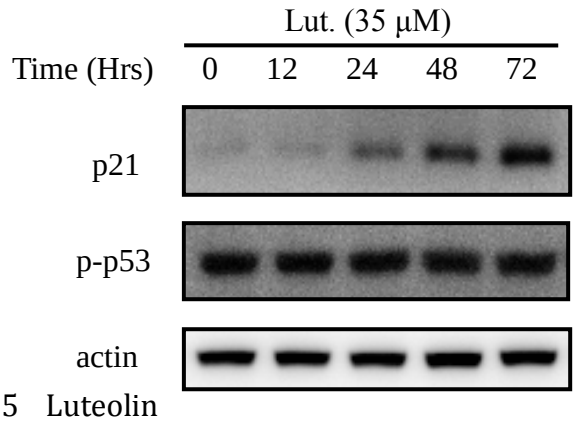


Fig. 15 Luteolin induced protein expression of p21 MDA-MB-231 cells. MDA-MB-231 cells were treated with 35 μM luteolin for 0, 12, 24, 48 and 72 hours.

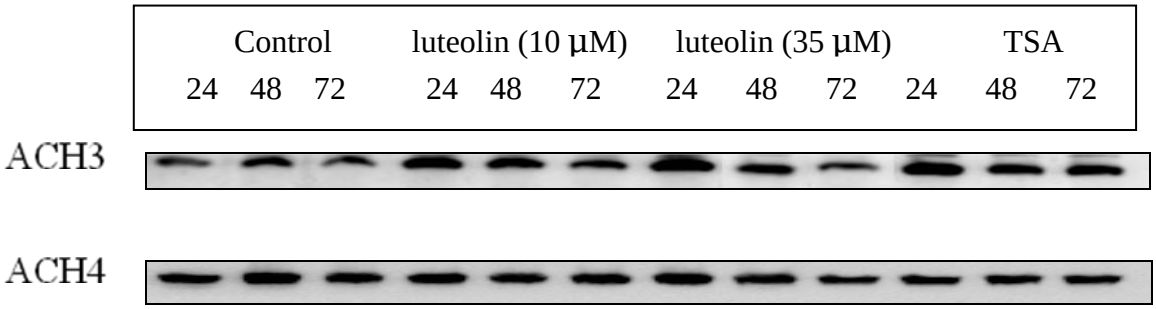


Figure 16. The effect of luteolin on AcH3 protein expression of MDA-MB-231

Fig.17 The association of luteolin (35 uM) induced AcH3 and p21 protein expression in MDA-MB-231.

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

v 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：

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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）

本研究由 *In vitro* and *in vivo* 研究發現 quercetin, apigenin, and luteolin 等 flavonoid 衍生物皆具有抗癌活性, 不管對血癌或 solid tumor 如 breast cancer 皆具有抑制增生之作用,其中 apigenin and luteolin, 於 C -ring 中無 hydroxyl group 作用反而比 quercetin 強,可能與某些訊息路徑調控之酵素 affinity 有關,另外發現三者皆可促進組蛋白乙醯化,且此乙醯化與促進 apoptosis 及 cell cycle arrest 有關, 本研究更清楚了解 flavonoid 衍生物其抗癌活性與 epigenetic modulation 之相關性,換言之透過 histone modification 不需改變 DNA sequence 即可改變 gene expression, specific epigenetic alteration 可能可提供高度有效的 chemoprevention strategies.

附　　錄

Quercetin induces FasL-related apoptosis, in part, through promotion of histone H3 acetylation in human leukemia HL-60 cells

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Keywords: Quercetin, Fas ligand, apoptosis, histone acetylation.

Running title: Quercetin induces leukemia apoptosis involving histone acetylation

Abstract. Quercetin, a naturally occurring flavonoid abundant in fruits and vegetables, has been demonstrated as a multipotent bioflavonoid with great potential for prevention and treatment of cancer. Apoptosis is thought to be an important response to most chemotherapeutic agents in leukemia cells. However, the underlying mechanism of induction of apoptosis by quercetin involving epigenetic regulation is poorly understood. In the present study, by evaluation of fragmentation of DNA, poly (ADP-ribose) polymerase (PARP) and procaspases, we found that quercetin was able to induce apoptosis of human leukemia HL-60 cells in a dose dependent manner. Quercetin triggered the extrinsic apoptosis pathway through activation of caspase 8 and induction of Bid cleavage, Bax conformation change, and cytochrome c release. Furthermore, quercetin induced Fas ligand (FasL) expression involving activation of extracellular signal-regulated kinase (ERK) and Jun N-terminus kinase (JNK) signaling pathway. In addition to activation of c-Jun, quercetin increased histone H3 acetylation which resulted in the promotion of the expression of FasL. Quercetin exhibited potential for the activation of histone acetyltransferase (HAT) and the inhibition of histone deacetyltransferase (HADC), both of which contributed to histone acetylation. However, only the activation effect on HAT was associated with the ERK and JNK pathway. These results demonstrated that quercetin induced FasL-related apoptosis by transactivation through activation of c-jun/AP-1 and promotion of histone H3 acetylation in HL-60 cells.

Introduction

Apoptosis plays a critical role in normal development, homeostasis and in the defense response against pathogens. Inappropriate suppression or activation of apoptosis can lead to a variety of diseases. This kind of cell death is thought to be an important response to most chemotherapeutic agents in leukemia cells.

Apoptosis is mediated through two major pathways, the extrinsic and intrinsic pathways, which both lead to the activation of caspases. The extrinsic pathway is triggered at the plasma membrane by the activation of the death receptor (Fas) and subsequently the activation of caspase 8. In some cells, caspase 8 directly activates downstream effector caspases such as caspase 3 while in other cell types caspase 8 mediates apoptosis *via* the proteolytic cleavage of the pro-apoptotic Bid protein. Following Bid cleavage and its translocation to mitochondria, truncated Bid (t-Bid) induces oligomerization and conformational changes in Bak and Bax, resulting in the release of cytochrome c and the procession of effector caspases (1, 2). The intrinsic pathway is triggered by various apoptotic stress signals and is characterized by mitochondrial dysfunction, which leads to the release of mitochondrial pro-apoptotic factors from their intermembrane space into the cytosol. The release of cytochrome c from mitochondria represents a critical event in initiating the activation of the caspase cascade. This occurs through its interaction with Apaf-1, and the subsequent processing and activation of the cell death protease, caspase 9. Activated caspase 9 triggers the catalytic maturation of effecter caspases such as caspase 3, which triggers oligonucleosomal DNA fragmentation (3, 4).

Cell responses to apoptotic-inducing agents have been associated with the inactivation of survival kinases and the activation of apoptotic kinases. One of the most relevant aspects in the regulation of apoptosis is the involvement of mitogen-activated protein kinases (MAPKs), a family of proline-directed serine/threonine protein kinases that mediate intracellular signal transduction in response to various stimuli. Activation of the pathways rapidly alters the pattern of gene expression. To date, three major MAPKs have been identified: the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinases (JNK) and p38 MAPK. All of these kinases have been reported to be associated with the activation of apoptosis (5). Increasing evidence indicates that in addition to directing the phosphorylation of upstream transcription

factors and co-activators which control immediate genes, MAPK cascades also act directly on chromatin proteins such as histone H3 to modify chromatin concomitant with gene induction (6, 7). However, the involvement of MAPKs- mediated histone H3 acetylation in apoptosis is unclear.

Epigenetic changes including histone acetylation, histone methylation, and DNA methylation are now thought to play important roles in the onset and progression of cancer in numerous tumor types (8). Dietary components selectively activate or inactivate gene expression by epigenetic regulation has been implicated as chemoprevention agent or developed for the treatment of cancer. HDAC inhibitors are being explored as cancer therapeutic compounds because of their ability to alter several cellular functions known to be deregulated in cancer cells (9). Recent investigators suggest that dietary components, including diallyl disulfide and sulforaphane possessing the ability to inhibit HDAC enzyme have been associated with cancer prevention (9). Quercetin (3, 3',4', 5, 7- petahydroxyflavone), which is found in fruits, vegetables, herbs and red wine, has been reported to exhibit antioxidative, anticarcinogenic, and anti-inflammatory effects (10-12). The molecular mechanisms behind the effects are largely unknown. It has been reported that quercetin strongly inhibits neoplastic cell transformation and inhibits the enzymes involved in cancer cell proliferation and cell signal transduction pathways including protein kinase C, tyrosine kinase, and DNA topoisomerase II. Quercetin mediates apoptosis by induction of stress proteins including heat shock proteins, disruption of microtubules and mitochondrial release of cytochrome c. Quercetin also augments TRAIL-induced apoptotic death involving the ERK signal transduction pathway (13). Although MAPK activation during quercetin treatment has been studied in a variety of cell types, the presence of epigenetic regulation is not clear. In this study, we observed that quercetin induced apoptosis in HL-60 cells by enhancing the expression of FasL, in part, through promotion of histone H3 acetylation.

Materials and methods

Cell culture. The HL-60 human promyelocytic leukemia cell was obtained from ATCC and maintained in a logarithmic growth phase in RPMI 1640 supplemented with sodium pyruvate, 10% fetal bovine serum (FBS; Invitrogen-Gibco), 1% penicillin/streptomycin and 1% non-essential amino acids. Cell lines were maintained at 37 ℃ in an incubator with 5 % CO_2 and 95% air.

Antibodies and reagents. Anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}), anti-phospho- JNK/SAPK (Thr^{183}/Tyr^{185}), and anti-phospho-p38 (Thr^{180}/Tyr^{182}) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-ERK1/2, JNK/SAPK, p38, caspase-3, -8, -9, PARP, Fas, FasL, Bid, t-Bid, cytochrome c, Tom20, c-fos, c-jun, C23, tubulin and β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-acetylated H3 and anti-acetylated H4 were obtained from Upstate Biotechnology (Lake Placid, NY). The MEK/ERK inhibitor (PD98059), and JNK inhibitor (SP600125), were obtained from Calbiochem (La Jolla, CA). Other chemicals such as quercetin were purchased from Sigma-Aldrich (St. Louis, MO).

DNA fragmentation assay. The cells were rinsed with ice-cold PBS and harvested by pipetting. The cell pellets were resuspended and incubated in 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 0.5% SDS, and 0.1 μg/ml proteinase K at 60 ℃ overnight. The digested cells were extracted for DNA with phenol/chloroform (1:1) and chloroform/isoamyl alcohol (1:24). The extracted DNA was precipitated and digested in 10 mM Tris-HCl (pH 5.0) containing 1 mM EDTA and 10 μg/RNase for 1 h at 37℃. Ten micrograms of DNA per sample was resolved by electrophoresis in a 1.8% agarose gel impregnated with ethidium bromide (0.5 μg/ml), and the DNA pattern was examined by ultraviolet transillumination.

Preparation of total cell extracts and immunoblot analysis. Cells were washed with PBS plus zinc ion (1 mM) and lysed in radio immunoprecipitation assay (RIPA) buffer (50 mM Tris-buffer, 5 mM EDTA, 150 mM NaCl, 1% NP 40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 81 μg/ml aprotinine, 170 μg/ml leupeptin, and 100 μg/ml PMSF; pH 7.5). After mixing for 30 min at 4 ℃, the mixtures were centrifuged (10000 × g) for 10 min, and the supernatants were collected as whole-cell extracts. The protein extracts were quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). The samples containing 50 to 100 μg of proteins were boiled in Laemmli sample buffer, separated on SDS polyacrylamide gel, electrophoretically transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL) and blotted with the indicated primary antibodies. Proteins were visualized with horseradish peroxidase–conjugated secondary antibodies (Zymed Laboratory, Inc., South San Francisco, CA) followed by chemiluminescence detection.

Preparation of mitochondria and cytosolic fractions. Mitochondrial fraction of the cells was isolated using a mitochondria isolation kit obtained from Pierce as per manufacturer's instruction. Cells (2×10^7) were pelleted by centrifugation at 850*g* for 2 min and were resuspended in 800 μl of reagent A in a microcentrifuge tube. Then, cells were incubated in ice for 2 min and subsequently homogenized in a precooled Dounce tissue grinder applying 40 to 50 strokes. Reagent C (800 μl) was added to the homogenized solution and thoroughly mixed by repeated inversion. The entire mixed solution was centrifuged at 700*g* for 10 min, and the pellet was discarded. The supernatant was further centrifuged at 12,000*g* for 15 min, and the pellet was considered as intact mitochondria. This fraction was further lysed in lysis buffer and subjected to Western blot analysis.

Immunofluorescence staining. After treatment of cells with quercetin, cells were harvested, washed with PBS and fixed with 3.7% paraformaldehyde for 10 min at room temperature. The fixed cells were

permeabilized with 0.2% Triton X-100 in PBS for 3 min and non-specific binding was blocked by incubation with 2% BSA in PBS for 30 min. Cells were then incubated for 30 min at room temperature with the primary antibody: mouse monoclonal anti-Bax (conformation specific clone 6A7, Sigma-Aldrich). Excess antibody was removed by washing the cover slips three times with PBS-2% BSA. Cells were then incubated with the mouse-conjugate FITC secondary antibody for 30 min at 37℃. After washing three times with PBS-2% BSA, cover slips were mounted, and then viewed under a fluorescence microscope.

DAPI staining. After treatment of cells with quercetin, cells were pipetted and collected and then washed once with ice-cold PBS. Cells were attached to the slide by cytospin (500 rpm, 5 min). They were then air-dried, fixed, and stained with the DAPI (10μg/ml). The stained cells were examined by fluorescence microscopy (Magnification × 400).

Reverse transcriptase-PCR. Total cellular RNA was extracted from cells using the TRI reagent method (Molecular Research Center). For the reverse-transcription reaction, 10 μL of reaction mixture (1 μg of total RNA, 1 μL of random decamers, 1 μL of 10x RT buffer, 2 μL of deoxynucleotide triphosphate mix, 0.5 μL of RNase inhibitor, 0.5 μL of reverse transcriptase, and 4 μL of nuclease-free water) was prepared for each sample. The mixtures were incubated at 44℃ for 1 h and then at 92℃ for 10 min to inactivate the reverse transcriptase. The first-strand cDNAs were synthesized with a RETROscript kit (Ambion) and served as templates for the PCR. A high-fidelity PCR master kit from Roche was used to perform PCR. The following primers were used: human Fas sense 5'-CACTCT- GCAACCTCTCTCCC-3' and antisence 5'-AGAGTGTGTGCACAAGGCTG-3'; human FasL sense 5'-TCAATGAAACTGGGCTGTACTTT-3' and antisence 5'-AGAGTT CCTCATGTAGACCTTGT-3'; human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense 5'-ACCACAGTCCATGCCATCAC-3' and antisense 5'-TCCACCACCCTGTTGCTGTA-3'.

PCR products were resolved by electrophoresis on a 1.8% agarose gel with 0.5 μg/mL ethidium bromide and photographed using an AlphaEase FC imaging system (Alpha Innotech).

Nuclear extract preparation. HL-60 cells were lysed by adding 25 μl NP-40 10% and gently passed through a 27-gauge needle. The nuclei were collected by centrifugation at 600 × *g* for 5 min and resuspended in 50 μl of 20 mM HEPES, pH 7.9, containing 400mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 0.7 μg/ml pepstatin, 1 μg/ml leupeptin and 10 μg/ml aprotinin. The tubes were placed at 4℃ on a rotator shaker for 30 min and centrifuged at 12000 × *g* for 5 min at 4℃. The supernatants were used as nuclear extracts and frozen at 70 ℃ until used.

Histone deacetylase and histone acetyltransferase assay. Assays were performed using the colorimetric HDAC and HAT activity assay from BioVision (BioVision Research Products, Mountain View, CA, USA) according to manufacturer instructions. Briefly, for HDAC activity, 50 μg of nuclear extracts were diluted in 85 μL of ddH_2O; then, 10 μL of 10× HDAC assay buffer was added followed by the addition of 5 μL of the colorimetric substrate; samples were incubated at 37° for 1 h. Subsequently, the reaction was stopped by adding 10 μL of lysine developer and left for an additional 30 min at 37℃. Samples were then read in an ELISA plate reader at 405 nm. For HAT activity, the adjusted weight of nucleic extracts (50 μg) was mixed with HAT substrate, followed by mixture with an enzyme mix. The samples were incubated at 37℃ for 1 hour. Samples were then read in an ELISA plate reader at 440 nm.

Chromatin Immunoprecipitation (CHIP) assays. CHIP assay was performed according to the manufacturer's instructions (Upstate). Briefly, HL-60 cells (1×10^6) with or without pretreatment with PD98059 or SP600125 were administrated with quercetin (100 μM). Then cells were fixed with 37% formaldehyde, sonicated and immunopreciptiated with anti-Ac-H3. DNA isolated from the

immunopreciptiated sample was amplified by PCR using FasL primers, 5’-CTGTAAATTATGGTGATCGG-3’ (forward), 5’-AACTCT- AACAAAATTGTTGTTCAG-3’ (reverse), flanking the AP-1 consensus sequence. The PCR was carried out as follows: an initial denaturation at 94℃ for 3 min, 36 cycles of 94℃ 30 sec, 55 ℃ 30 sec, and 72℃ for 10 min. The PCR product of FasL promoter was 206 bp.

Data analysis. Statistical significances were analyzed by one-way analysis of variance (ANOVA) with the post hoc Dunnett’s test. *P* values less than 0.05 were considered statistically significant (Sigma-Stat 2.0, Jandel Scientific, San Rafael, CA, USA).

Results

Apoptotic induction by quercetin in HL-60 cells.

In our preliminary study, quercetin induced cell death of HL-60 cells in a dose and time dependent manner (data not shown). We further clarified the type of cell death. The HL-60 cells were treated with 0-100 μM quercetin for 12 h. As shown in Fig. 1A, after treatment with quercetin, the DNA showed the typical fragmentation patterns formed by inter-nucleosomal hydrolysis of chromatin. This demonstrates that quercetin exhibited apoptosis-inducing effects in HL-60 cells. In order to ascertain the effects of quercetin-induced apoptosis on the key aspect of apoptotic initiation *via* activation of caspase cascade in HL-60 cells, we performed a western blot analysis to evaluate executioner caspases-3 and PARP, an intracellular biosubstrate of caspase-3. It was clearly shown that quercetin induced activation of caspase-3 and cleavage of PARP, both of which constitute hallmarks for apoptosis (Fig. 1B).

Activation of extrinsic apoptosis pathway by quercetin in HL-60 cells. The extrinsic apoptosis pathway involves binding of a ligand to one of the tumor necrosis factor families of death receptors, followed by activation of caspase 8 and caspase 3. Moreover, the intrinsic and extrinsic pathways are linked through the ability of caspase 8 to cleave Bid, which in turn leads to the conformation change of Bax in mitochondrial membrane and the release of cytochrome *c* from the mitochondria (3). We questioned whether the death receptor Fas/FasL system was implicated in the apoptosis of HL-60 cells by quercetin. As shown in Fig. 2A, treating cells with quercetin induced the increase of the expression of FasL protein after 3 h. By contrast, the expression of Fas protein was not modified by the treatment of quercetin. In addition, quercetin activated caspase-8 and cleaved Bid protein to its truncated form, t-Bid (Fig. 2B). Furthermore, we examined the effect of quercetin on conformation- changed Bax in mitochondrial membrane and cytochrome c release from mitochondria. By immunofluorescence staining with anti-Bax (clone 6A7), quercetin exhibited the induction of conformation changed- Bax (Fig. 2C). In addition, quercetin increased the release of cytochrome c from mitochondria (Fig. 2D). These results demonstrate that quercetin induced activation of extrinsic apoptosis cascade in HL-60 cells.

Involvement of ERK and JNK in quercetin-induced apoptosis in HL-60 cells. MAPKs family proteins have been implicated in the proliferation, differentiation and death of cells (7). They are, in general, subdivided into three different pathways, namely the ERK, p38 kinase, and JNK signaling pathways (14). In view of evidence that ERK, JNK/SAPK and p38 MAPK play a critical role in cell fate, the effects of quercetin on the activation of MAPKs were examined. We treated HL-60 cells with quercetin, then used western blot to examine the phosphorylation of ERK, JNK and p38 MAPK. Our results showed that exposure to quercetin increased both ERK and JNK phosphorylation (Fig. 3A and 3B). However, p38 MAPK was not activated by

treatment with quercetin in HL-60 cells. To further confirm these results and to examine the role of MAPKs in quercetin-induced apoptosis, we pretreated HL-60 cells with specific MAPK inhibitors before exposure to quercetin. Our results showed that pretreating PD98059 (MEK/ERK inhibitor, 50 μM) and SP600125 (JNK inhibitor, 20 μM) before exposure to quercetin significantly attenuated the quercetin-induced activation of the ERK and JNK. In addition, both the MEK/ERK inhibitor, PD98059, and JNK1/2 inhibitor, SP600125, abolished quercetin-induced cleavage of caspase 8, caspase 9, and caspase 3 (Fig. 3C). These results indicate that the ERK and JNK pathways were involved in quercetin-induced apoptosis.

Induction of FasL mRNA and c-Jun activation by quercetin involving ERK and JNK pathways. As shown in Fig. 2A, the Fas/FasL signaling pathway plays a crucial role in the quercetin-induced apoptosis in HL-60 cells. However, treatment with 100 μM of quercetin did not modify the expression of the Fas receptor while it increased that of FasL. Thus, this study attempted to determine whether quercetin-induced FasL protein expression results from up-regulation of FasL mRNA by transcriptional regulation. Incubation of HL-60 cells with 50 and 100 μM of quercetin for 3 and 6 h resulted in induction of FasL mRNA in a dose- and time-dependent manner. By contrast, expression of Fas mRNA was constitutive (Fig. 4A). These results show that quercetin induces FasL expression *via* transcriptional activation of the FasL gene in HL-60 cells.

AP-1 is a collection of dimmers composed of the Jun, Fos or ATF families of bZIP (basic region-leucine zipper) DNA binding proteins. During transcription activation, these dimmers bind to a common cis acting element known as the AP-1 site in nuclear. Previously, it has been shown that activation of AP-1 mediating FasL expression triggers cell apoptosis (15). We therefore determined the effect of quercetin on the nuclear accumulation of c-Jun and c-Fos proteins. Cells were cultured in the presence or absence of 50 and 100 μM of quercetin for 3- 9 h, and nuclear protein was extracted. Nuclear extracts were separated by SDS-PAGE, and a

western blot analysis was performed for either c-Jun or c-Fos. Quercetin did not increase the accumulation of c-Fos protein; however, c-Jun was significantly increased after 6 h of quercetin exposure (Fig. 4B). We next determined whether quercetin induced ERK- and JNK phosphorylation was involved in c-Jun translocation. HL-60 cells were pretreated with either PD98059 (MEK inhibitor) or SP600125 (JNK inhibitor) for 1 h, then cells were stimulated with 100 μM of quercetin for 6 h, nuclear protein was extracted, and a western blot analysis was performed for c-Jun. Indeed, quercetin increased the nuclear c-Jun, whereas, there were significant decreases in the nuclear c-Jun in cells pretreated with PD98059 and SP600125 (Fig. 4C). Taken together, these data confirm previous findings which indicate that both the ERK and JNK MAP kinases are linked to AP-1 activation (16-17).

Effects of ERK and JNK pathways on quercetin-induced histone H3 acetylation. In Fig. 4A, it is interesting to observe that treatment with 100 μM quercetin induced FasL mRNA at 3 h, however, induction of c-Jun translocation required approximately 6 h. The discrepancy between FasL mRNA expression and c-Jun translocation suggests that other gene regulation programs may be required to activate the FasL gene. Previously, it has been shown that the acetylation and deacetylation of histones alter chromatin structure, which is suggested to play an important role in transcriptional regulation (18). To evaluate whether quercetin altered the acetylation state of histone H3 and H4, HL-60 cells were administered with 25-100 μM of quercetin or 100 ng/ml of TSA, a histone deacetylase inhibitor, for the indicated time, and equal amounts of cell lysate protein were immunoblotted with specific antibodies for acetylated H3 and H4, or β-actin. The data showed that quercetin (75 μM and 100 μM) increased histone H3 acetylation apparently, but did not affect histone H4 acetylation, while TSA increased both proteins (Fig. 5A). We further detected the time course effect (0-12 h) of quercetin on histone H3 acetylation. The results show that quercetin induced histone H3

acetylation with treatment for 3 to 12 h (Fig. 5B).

The MAPK cascade has been shown to increase histone acetylation in other systems (19-20). The question arises as to whether MAPK is involved in the increase of histone acetylation by quercetin. To examine this possibility, we used selective inhibitors of MAPK. Blockade of the ERK pathway with a MEK/ERK inhibitor (PD98059; 50 μM) decreased H3 acetylation in the 100 μM quercetin-treated cells. Likewise, treatment with JNK inhibitor (SP600125; 20 μM) reduced H3 acetylation (Fig. 5C). Consistent with these results, cleavage of PARP induced by quercetin was also almost completely abrogated by PD98059 and SP600125. These data indicate that quercetin-induced H3 acetylation and apoptosis are dependent, at least in part, on ERK1/2 and JNK signaling.

Effects of quercetin on HDAC and HAT. Because histone acetylation is regulated by a balance of opposing histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities, we next determined whether quercetin affected the activity of HDAC and HAT and the role of ERK and JNK pathways in quercetin induced modulation of HDAC and HAT. Nuclear extracts were prepared from treatment with different concentrations of quercetin in the absence or presence of MEK/ERK or JNK inhibitors, and then the total nuclear HAT and HDAC activities were analyzed. The results indicated that quercetin (100 μM) increased HAT activity (Fig. 6A) and decreased HDAC activity (Fig. 6B). The presence of MEK/ERK and JNK inhibitors did not significantly affect the quercetin-decreased HDAC activity (Fig. 6B) but significantly blocked quercetin- increased HAT activity (Fig. 6A). Taken together, these experiments suggest that the quercetin induced histone H3 acetylation through modulation of both HDAC and HAT activity, however, ERK and JNK signaling cascades are only involved in quercetin-induced HAT activation.

Involvement of Ac-H3 in quercetin-induced FasL up-regulation. To evaluate the promotion of the

transactivation of FasL by histone H3 acetylation, CHIP assay was performed in HL-60 cells with or without treatment of quercetin. Using anti-Ac-H3 antibody followed by PCR with primers specific for the FasL promoter, the data showed that quercetin promoted histone H3 acetylation resulting in up-regulation of FasL, whereas with pretreatments of PD98059 and SP600125 decreased the expression of FasL (Fig.7). These results suggest that quercetin induced up-regulation of FasL was associated with ERK and JNK-mediated epigenetic regulation.

Discussion

The relationship between diet and cancer has been implicated in several epidemiological studies. The results indicate that dietary phytochemicals have antineoplastic potential. Previous studies have demonstrated that quercetin induces apoptosis in a wide range of human cancer cells (12-13). A better understanding of the mechanisms by which quercetin induces apoptosis is necessary for its further development as a promising chemoprevention agent. In the present study, quercetin induced apoptosis and activated ERK and JNK signaling pathways in HL-60 cells (Fig.1 and Fig. 3). Quercetin also increased expression of FasL in HL-60 cells, suggesting that the extrinsic apoptosis pathway is induced by quercetin (Fig.2).

The Fas/FasL pathway has been implicated as an important cellular pathway regulating the induction of apoptosis in diverse cell types and tissue. ERK and JNK have been identified to contribute to death receptor transcription-dependent apoptotic signaling via c-Jun/AP-1, leading to transcriptional activation of FasL (21). In addition to c-Jun, the ERK and JNK signaling pathways have been found to activate other transcription factors as well as to alter histone acetylation, which contribute to AP-1 activity (5, 6). Our data showed that

quercetin induced FasL mRNA expression occurred earlier than nuclear translocation of c-Jun (Fig.4). This suggests that other gene regulation programs may be activated. By CHIP assay, it showed that up-regulation of FasL by quercetin was associated with histone H3 acetylation (Fig.7). Quercetin may mediate recruitment of chromatin remodeling complexes, coactivators, and transcription factors to promote transcription of target genes.

Epigenetic modifications, such as histone acetylation and DNA methylation, are widely recognized as having a substantial role to play in both normal cellular physiology and disease processes, particularly in cancer where inappropriate gene-expression has long been known to play a fundamental role in the etiology of the disease (22). Increasing evidence suggests that induction of histone hyperacetylation is responsible for the antiproliferative activity and reversal of neoplastic characteristics through selective induction of genes (23). According to our results, quercetin exhibited the property of activating HAT which mediated by the ERK and JNK signaling pathways. In addition, quercetin exhibited a weak inhibitory effect on HDAC activity which was independent with ERK and JNK signaling pathways. In Fig. 5, TSA, a histone deacetylation inhibitor, induced histone H3 and H4 acetylation while quercetin only induced histone H3 acetylation. Previously, it has been reported that histone deacetylation inhibitors (HDI) induce apoptosis through accumulation of excessive DNA damage in leukemia cells (24). Whether quercetin causes DNA damage in leukemia cells will be determined in the future. From data of histone acetylaltion (Fig.5), it implicates there are different mediation in chromatin remodeling by quercetin and HDI. It is suggested that chemoprevention potential of dietary flavonoids may be mediated by epigenetic regulation. The association of flavonoid structure and potential of epigenetic regulation needs further investigation.

In conclusion, quercetin induced the expression of FasL through transactivation by the activation of

ERK and JNK signaling pathway and the promotion of histone H3 acetylation in HL-60 cells. In other words, quercetin affected gene expression mediated leukemia apoptosis via targeting signaling pathway and chromatin remodeling. These results provide an important link to relevance for quercetin as a chemopreventive agent.

Acknowledgement

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The authors have declared no conflict of interest.

References

1. Desagher S A, Nichols Osen-Sand A, Eskes R, Montessuit S, Lauper S, Maundrell, K., Antonsson B and Martinou JC: Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. J Cell Biol 144: 891-901, 1999.

2. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X: Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 94: 481-490, 1998.

3. Wang X: The expanding role of mitochondria in apoptosis. Genes Dev 15: 2922-2933, 2001.

4. Thornberry NA: Caspases: key mediators of apoptosis. Chem Biol 5: R97-103, 1998.

5. Platanias LC: MAP kinase signaling pathways and hematologic malignancies. Blood 101: 4667-4679, 2003.

6. Yang SH, Sharrocks AD, and Whitmarsh AJ: Transcriptional regulation by the MAP kinase signaling cascades. Gene 320: 3-21, 2003.

7. Clayton AL and Mahadevan LC: MAP kinase-mediated phosphoacetylation of histone H3 and inducibe gene regulation. FEBS Lett 546: 51-58, 2003.

8. Ellis L, Atadja PW, and Johnstone RW: Epigenetics in cancer: targeting chromatin modifications. Mol Cancer Ther 8: 1409-1420, 2009.

9. Ho E, Clarke JD, and Dashwood RH: Dietary sulforaphane, a histone deacetylase inhibitor for cancer prevention. J Nutr 139: 2393-2396, 2009.

10. Bischoff SC: Quercetin: potentials in the prevetnion and therapy of disease. Curr Opin Clin Nutr Metab Care 11: 733-740, 2008.

11. Wei YQ, Zhao X, Kariya Y, Fukata H, Teshigawara K and Uchida A: Induction of apoptosis by quercetin: involvement of heat shock protein. Cancer Res 54: 4952-4957, 1994.

12. Gupta K, Panda D: Pertubation of microtubule polymerization by quercetin throug tubulin binding: a novel mechanism of its antiproliferative activity. Biochemistry 41: 13029-13038, 2002.

13. Kim YH, Lee DH, Jeong JH, Guo ZS and Lee YJ: Quercetin augments TRAIL-induced apoptotic death: Involvement of the ERK signal transduction pathway. Biochem Pharmacol 75: 1946-1958, 2008.

14. Seger R and Krebs EG: The MAPK signaling cascade. FASEB J 9: 726-735, 1995.

15. Kasibhatla S, Brunner T, Genestier L, Echeverri F, Mahboubi A and Green DR: DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1. Mol Cell 1: 543-551, 1998.

16. DeSilva DR, Feeser WS, Tancula EJ and Scherle PA: Anergic T cells are defective in both jun NH2-terminal kinase and mitogen-activated protein kinase signaling pathways. J Exp Med 183: 2017-2023, 1996.

17. Frost JA, Geppert TD, Cobb MH and Feramisco JR: A requirement for extracellular signal-regulated kinase (ERK) function in the activation of AP-1 by Ha-Ras, phorbol 12-myristate 13-acetate, and serum. Natl Acad Sci USA 91: 3844-3848, 1994.

18. Johnstone RW: Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. Nat Rev 1: 287-299, 2002.

19. Ait-Si-Ali S, Carlisi D, Ramirez S, Upegui-Gonzalez LC, Duquet A, Robin P, Rudkin B, Harel-Bellan A, and Trouche D: Phosphorylation by p44 MAP Kinase/ERK1 stimulates CBP histone acetyl transferase activity in vitro. Biochem Biophy Res Commun 262: 157-162, 1999.

20. Kawasaki H, Schiltz L, Chiu R, Itakura K, Taira K, Nakatani Y and Yokoyama KK: ATF-2 has intrinsic histone acetyltransferase activity which is modulated by phosphorylation. Nature 405: 195-200, 2000.

21. Park GY, Joo M, Pedchenko T, Blackwell TS, and Christman JW: Regulation of macrophage cyclooxygenase-2 gene expression by modifications of histone H3. Am J Phys 286: L956-962, 2004.

22. Cosgrove MS and Wolberger C: How does the histone code work? Biochem Cell Biol 83: 468-476, 2005.

23. Rosato RR and Grant S: Histone deacetylase inhibitors in cancer therapy. Cancer Biol Ther 2: 30-37, 2003.

24. Gaymes TJ, Padua RA, Pla M, Orr S, Omidvar N, Chomienne C, Mufti GJ, and Rassool FV: Histone deacetylase inhibitors (HDI) cause DNA damage in leukemia cells: a mechanism for leukemia-specific HDI-dependent apoptosis? Mol Cancer Res 4: 563-573, 2006.

Legends for figure

Fig. 1. Quercetin-induced apoptosis in HL-60 cells. (A) Agarose gel electrophoresis of DNA from quercetin-treated HL-60 cells. HL-60 cells were treated with the indicated concentrations of quercetin for 12 h and genomic DNA was extracted, separated on an agarose gel and visualized under UV light by ethidium bromide staining. (B) Cells were incubated with 100 μM of quercetin for 12 h and cell lysates were assayed by Western blot for the cleavage of pro-caspase-3 and PARP in HL-60 cells. β-actin was used as a loading control. Data are a representative of the two independent experiments.

Fig. 2. Effect of quercetin on the protein of FasL, caspase 8, t-Bid, Bax and cytochrome c in HL-60. (A) HL-60 cells were treated with 100 μM of quercetin for indicated time, and then total cell extracts were harvested. Western blot analysis was carried out with anti-Fas and anti-FasL antibodies, and anti-β-actin was used as a loading control. (B) Western blot analysis was carried out with anti-caspase-8, anti-Bid, and anti-t-Bid antibodies. β-actin was used as a loading control. (C) HL-60 cells were treated with 100 μM of quercetin for 6 h and then immunestained with anti-Bax (6A7) to detect conformation-changed Bax. In addition, nuclear of HL-60 cells was stained with DAPI. Scale bar= 50 μm. (D) Cytosolic release of cytochrome c was measured by Western blot analysis in cytosolic and mitochondrial fraction. The tubulin and the Tom 20 were used as a cytoplasmic and a mitochondrial loading control, respectively.

Fig. 3. Effect of quercetin on phosphorylation of MAPKs and impact of MAPKs inhibitors on quercetin-induced apoptosis in HL-60 cells. (A) Representative western blots show the time-dependent phosphorylation of ERK1/2, JNK1/2 and p38 MAPK by quercetin. HL-60 cells were incubated with 100 μM for the indicated time points. Protein extracts were prepared and analysed on western blots

probed with specific antibodies to ascertain the phosphorylation of MAPKs. Membranes were stripped and reprobed with total ERK, JNK and p38 antibodies as loading controls. (B) Representative western blots show the dose-dependent phosphorylation of ERK, JNK and p38 MAPK by quercetin. HL-60 cells were incubated with the indicated concentrations of quercetin for 6 h. (C) HL-60 cells were pre-incubated with PD98059 and SP600125 for 1 h and then treated with quercetin (Que). Cells were also incubated with each inhibitor only. Protein extracts were prepared and analyzed on western blots probed with specific antibodies to ascertain the phosphorylation of MAPKs and cleavage of caspase-8, -9, and -3. Representative results are shown from two experiments yielded equivalent findings.

Fig. 4. Effect of quercetin on nuclear c-Jun. (A) HL-60 cells were treated with 50 and 100 μM of quercetin, and total cellular RNA was extracted at the indicated times. Expression of Fas, FasL, and GAPDH mRNAs was determined by quantitative RT-PCR using specific primers. (B) Time course and dose-dependent of quercetin effect on the translocation of c-Fos and c-Jun to nuclear. Cells were treated with 50 and 100 μM of quercetin for the indicated times and (C) cells were treated with 100 μM quercetin, 50 μM of PD98059 and 20 μM of SP600125 alone or in combination for 6 h. Then, cells were harvested by centrifugation at 600 x *g* for 10 min at 4℃, and the nuclear fractions were prepared as described in the section of Materials and Methods. To determine c-Fos and c-Jun translocation, the resulting nuclear fractions were analyzed by immunoblotting with antibodies specific for c-Fos, c-Jun, and C23 as nuclear protein control. Representative results are shown from three experiments yielded equivalent findings.

Fig. 5. Quercetin -induced apoptosis involving increase acetylation of histone H3 through ERK and JNK pathways in HL-60 cells. (A) Cells were treated with indicated concentrations of quercetin and 100

ng/mL of TSA for 6 and 12 h and the expression of Ac-H3 and the acetylation of H4 (Ac-H4) were assessed. (B) By immunoblotting analysis, time course effect of quercetin (100 μM) on the acetylation of histone H3 (Ac-H3) was assessed. (C) Cells were treated with 100 μM of quercetin (Que), 50 μM of PD98059 and 20 μM of SP600125 alone or in combination for 6 h. The expression Ac-H3 and cleaved PARP were analyzed by the immunoblotting analysis.

Fig. 6. Association of ERK- and JNK- pathways with HDAC activity and HAT activity by treatment of quercetin. Cells were treated with 100 μM of quercetin, 50 μM PD98059 and 20 μM SP600125 alone or in combination for 6 h. (A) HAT and (B) HDAC activity were measured as described in Materials and Methods.

Fig. 7. Involvement of hyperacetylation of histone H3 in quercetin-induced FasL expression in HL-60 cells. Soluble precleared chromatin was obtained from HL-60 cells pretreated with or without 50 μM of PD98059 (MEK inhibitor) and 20 μM of SP600125 (JNK inhibitor) for 2 h. Then, cells were treated with or without 100 μM of quercetin for 6 h. ChIP analysis of histone H3 acetylation (Ac-H3) at the loci was performed by immunoprecipitation (IP) with an antibody against Ac-H3. The FasL promoter sequences were detected by PCR with specific primers. To control input DNA, FasL promoter was amplified from initial preparations of soluble chromatin (before immunoprecipitation). PCR products obtained at 36 cycles are shown. A sample without the addition of DNA was used as negative control (NC).

無研發成果推廣資料

96 年度專題研究計畫研究成果彙整表

計畫主持人：曾翠華	計畫編號：96-2320-B-040-011-MY3
計畫名稱：槲黃素及其相關衍生物之抗癌活性及對癌細胞組蛋白乙醯化與其所調控基因表現之影響研究	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	2	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	0	2	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%		
國外	論文著作	期刊論文	0	2	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%	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	4	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)

本研究由 In vitro and in vivo 研究發現 quercetin, apigenin, and luteolin 等 flavonoid 衍生物皆具有抗癌活性, 不管對血癌或 solid tumor 如 breast cancer 皆具有抑制增生之作用,其中 apigenin and luteolin, 於 C -ring 中無 hydroxyl group 作用反而比 quercetin 強,可能與某些訊息路徑調控之酵素 affinity 有關,另外發現三者皆可促進組蛋白乙醯化,且此乙醯化與促進 apoptosis 及 cell cycle arrest 有關, 本研究更清楚了解 flavonoid 衍生物其抗癌活性與 epigenetic modulation 之相關性,換言之透過 histone modification 不需改變 DNA sequence 即可改變 gene expression, specific epigenetic alteration 可能可提供高度有效的 chemoprevention strategies.