2014 年臺灣國際科學展覽會
優勝作品專輯

作品編號　090002

參展科別　醫學與健康科學

作品名稱　以中藥活血藥方透過 GDNF 媒介路徑作為抑制乳癌轉移之新穎策略

得獎獎項　大會獎：三等獎

候補作品：1

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關鍵字　乳癌、轉移、中草藥

作者簡介

我叫卜聿珊，目前就讀於復興實中雙語班。從小就喜歡做實驗的我在高一時非常幸運的考上了中研院的「高中生命科學研究人才培育計畫」。這對我來說非常的重要，這個計畫讓我原本乏味的高中生活變得有趣，我認識了許多志同道合的朋友，並進入實驗室做專題研究。

實驗室不只是做實驗的地方，實驗室對我來說是另一個家，學長姐就像哥哥、姊姊一樣的照顧我，指導教授也對我非常的好。這次能有幸參加國際科展複審也多虧了學長耐心的教導與陪伴，當然還有教授的指教。希望未來還能繼續在研究的這條路發展。

摘要

乳癌為最常見的婦女癌症，轉移後之乳癌具有極差的預後與較低存活率，直至目前尚未具有效抑制乳癌轉移的藥物，因此研發一個有效抗轉移的藥物是極為迫切的。許多研究指出促發炎因子如 GDNF、STAT3、JAK 和腫瘤的轉移有密切關聯，因此本研究使用一個含有多味具抗發炎效果的中藥複方 CR-1，探討其是否能應用於抗乳癌轉移。在動物實驗結果我們看到 CR-1 能夠有效的抑制腫瘤的生長，為了進一步研究將此複方之成分，依循中藥水煎與酒煎的概念處理分為:水層 (CR-1-WF) 與酒精層 (CR-1-EF)。經由 Cytokine array 指出 CR-1-WF 處理過後的乳癌細胞，GDNF、CCL5、IL8 等與轉移高度相關的促發炎因子表現量有明顯下降。在癒傷實驗和 transwell migration assay 我們發現 CR-1-WF 能夠顯著抑制乳癌細胞的移動。這些實驗結果皆顯示未來 CR-1 有可能成為一個抗乳癌轉移的藥物。

Abstract

Breast cancer is the most common cancer among women; breast cancer metastasis is associated with poor prognosis and high death rate, however, an efficient anti-metastasis therapy is still rare, urging the need of finding an efficient anti-metastasis drug. Many studies indicated that proinflammatory cytokines/chemokines such as GDNF, STAT3, and JAK have high connection with tumor metastasis. Therefore, in this study we used a Traditional Chinese Medicine formula CR-1 which contains many anti-inflammatory Chinese herbs as an anti-metastasis strategy for breast cancer. In animal model we observed that the group treated with CR-1 has a significant tumor growth delay. We firstly separated CR-1 into CR-1-WF (water fraction) and CR-1-EF (ethanol fraction) by traditional method. To further investigate the underlying mechanism behind the effects of CR-1, we examine whether CR-1 affect the expression of cytokines/chemokines. Interestingly, cytokine array showed that CR-1-WF suppressed the production of GDNF, CCL5, IL8, which are claimed to be highly associated with tumor progression and metastasis. Furthermore, in the wound-healing assay and transwell migration assay, we saw that CR-1-WF significantly inhibit migration of MDA-MB-231 breast cancer cell. Consistently, result of western blot showed that the downstream signals of GDNF like STAT3 and AKT are also inhibited by CR-1-WF. With these results we suggest that CR-1-WF inhibited migration of breast cancer cell via GDNF mediated pathway. The promising results warrant future study of CR-1 and its potential to become a novel strategy for anti-metastasis of breast cancer.

壹、 前言

乳癌為最常見的婦女癌症，也是癌症發生率最高的癌症，佔世界癌症死亡比例的 14 %。乳癌可分為 luminal A/B (ER $^{+}$) 型、HER2-enriched (HER2^{+}) 型、basal-like (三陰性) 型。大約有 20 % ～ 30 % 的乳癌病患死於轉移性復發。根據統計 luminal A 型的乳癌相較於其他種類的乳癌有較低的 15 年內轉移性復發率，大約為 27.8 %，其他種類的乳癌為 40 % ～ 50 %。

關於癌症轉移的機制至今始終不是很明確，轉移後並產生抗藥性的乳癌患者病情難以預測，因此一個能夠有效抑制乳癌轉移的方法是極為迫切的。儘管目前轉移性乳癌的主要成因還不是很明確，但有越來越多的研究指出腫瘤轉移的進展是由許多因素所影響的，腫瘤的級數 (stage)、腫瘤的增生、血管增生以及其目標組織周圍的微環境。

近期的研究也將發炎環境與腫瘤移動性、侵略性連繫在一起，一些與其相呼應的研究指出細胞激素和趨化素在乳癌轉移的過程中可能扮演著一個重要的角色。目前已有透過阻擋血管新生路徑來達到抗轉移的治療方式，但一個能夠有效針對轉移發炎路徑的治療方式還不多。

天然藥物之所以能夠被廣泛接受是因為他們是從天然物萃取出來的。一些天然藥物例如中草藥已被大量的使用在傳統的治療方式，中草藥不僅被廣泛的使用在全球各地更已在亞洲地區使用長達千年之久。傳統中藥使用方式的原理主要是專注在全面性以及穩定調理的治療方式，因此傳統中藥通常是由許多味草藥所組成的複方，以達到最好的治療效果。然而，在現今西方醫學以對抗療法為主的概念下，許多中草藥的複方無法達到藥物發展的標準。雖然在傳統及現代醫學的統合上缺乏系統性的研究，但近期的一些研究已經發現了一些中草藥的機制，並為中藥複方成為新型治療方式揭開一道曙光。

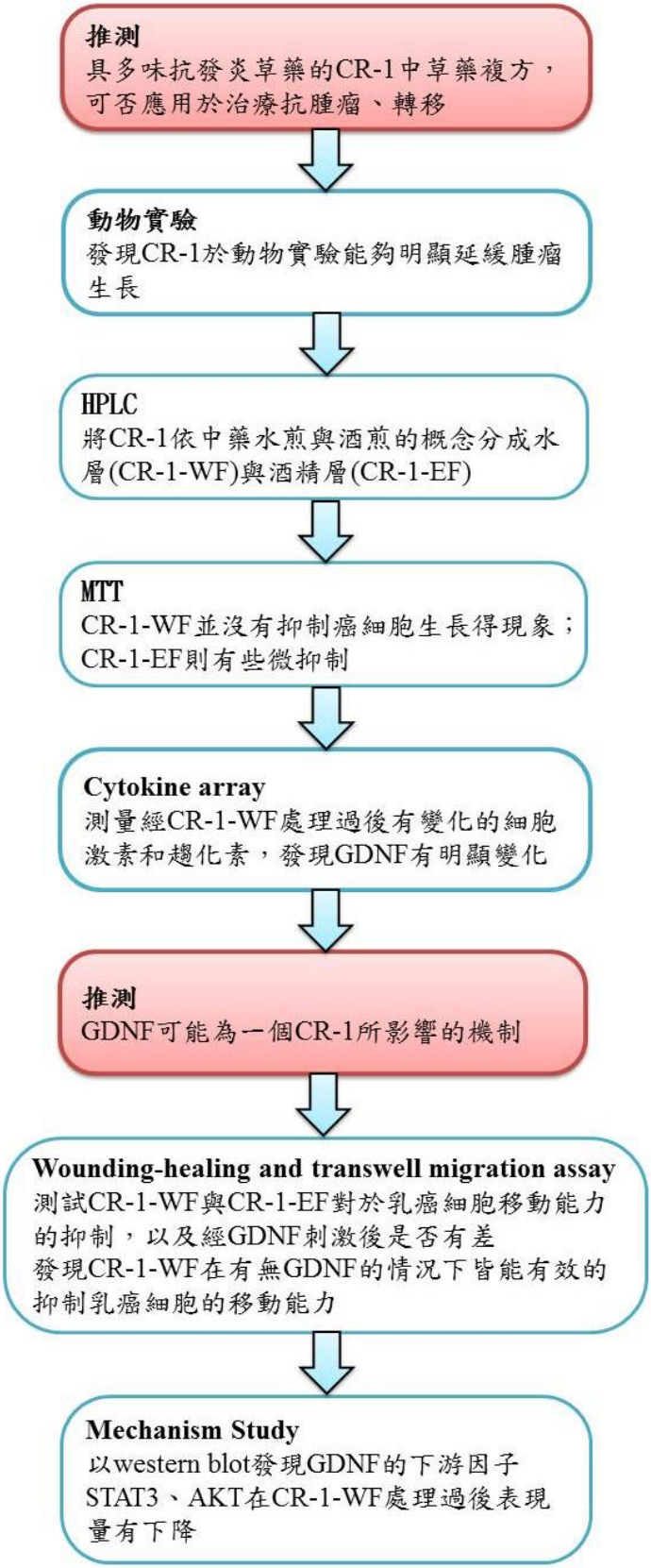
例如一個以黃耆為主的中草藥配方 (PG-2) 近期已由台灣食品和藥物管理局 (TFDA) 批准為新型藥物，用於和以鉑為基礎的化療方式 (Platinum-Based Chemotherapy) 結合，以減輕其毒性以及提高治療效果。

由於促發炎反應的細胞激素和趨化素在腫瘤生長、轉移的訊號傳遞中扮演了一個重要的角色，因此將具有抗發炎效果的草藥作為一個抗腫瘤轉移的新型治療是一個值得研究的方向。

本研究是要尋找參與腫瘤轉移路徑的促發炎因子並探討被歸類為有助活血的傳統中藥複方是否為一個有效的抗發炎藥物且可以有效的抑制腫瘤轉移。實驗中我們使用一個以紅花為主的複方 (CR-1)，一個傳統的外敷傷藥，作為一個新的抗轉移藥物。該藥方乃由紅花、大黃、黃芩、黃連、黃柏、當歸、川芎、生地、乳香、末藥、黃花蜜菜、紅花蜜菜、冰片、白介子及半夏 15 味藥所組成的複方。

我們發現 CR-1 於細胞實驗只顯示輕微的抗腫瘤效果。然而 CR-1 的水層卻有效的抑制 GDNF 的產生 (一個與癌症轉移、抗藥性有關的趨化素)，因而有效的抑制癌轉移以及抑制 AKT 和 STAT3 的活化。藉由研究傳統中藥的抗發炎效果，我們闡明了此一發現可應用於新的抗乳癌轉移臨床實驗。

貳、研究流程


推測
具多味抗發炎草藥的CR-1中草藥複方，可否應用於治療抗腫瘤、轉移
動物實驗
發現CR-1於動物實驗能夠明顯延緩腫瘤生長
HPLC
將CR-1依中藥水煎與酒煎的概念分成水層(CR-1-WF)與酒精層(CR-1-EF)
MTT
CR-1-WF並沒有抑制癌細胞生長得現象；CR-1-EF則有些微抑制
Cytokine array
測量經CR-1-WF處理過後有變化的細胞激素和趨化素，發現GDNF有明顯變化
推測
GDNF可能為一個CR-1所影響的機制
Wounding-healing and transwell migration assay
測試CR-1-WF與CR-1-EF對於乳癌細胞移動能力的抑制，以及經GDNF刺激後是否有差
發現CR-1-WF在有無GDNF的情況下皆能有效的抑制乳癌細胞的移動能力
Mechanism Study
以western blot發現GDNF的下游因子STAT3、AKT在CR-1-WF處理過後表現量有下降

參、研究材料與方法

一、藥材

本研究所使用的以紅花為主的複方 (CR-1) 是由15味藥所組成的,紅花、大黃、黃芩、黃蓮、黃柏、乳香、末藥、當歸、川芎、生地、半夏、黃花蜜菜、紅花蜜菜、冰片及白介子。並將此複方再依中藥水煎與酒煎的概念處理之後分為，水層 (CR-1-WF) 與酒精層 (CR-1-EF) 。

二、高壓液相層析 (HPLC)

分析的樣品為 CR-1-WF、CR-1-EF，使用分析管柱 TSKgel ODS-100S (5 m, 250mm x 4.6mm)，水：乙腈 = 6：4，流速為 1mL/min，檢視的波長為 254nm。

三、細胞株 (Cell Line)

本研究所採用的 4T1 小鼠乳腺癌細胞是由 ATCC 購得。MDA-MB-231 是美國安德森癌症中心所贈與的。4T1 培養於 RPMI-1640 + 10 % FBS (Fetal Bovine Serum) (Invitrogen)，37 ℃、5 %二氧化碳培養箱中。MDA-MB-231 則是培養於 DMEM/F12 (Sigma-Aldrich) + 10 % FBS (Fetal Bovine Serum) (Invitrogen)，37 ℃、5 %二氧化碳培養箱中。4T1 細胞內表現冷光酵素 (Luciferase) 以配合活體即時影像檢測系統觀察癌細胞在小鼠體內之發展。

四、實驗動物

由國家實驗研究實驗動物中心 NLAC (National Laboratory Animal Center) 購得 20 隻 4 週齡的 BALB/c 母鼠。飼養於無特定病源動物房，老鼠每五隻一籠，定期更換墊料並給予飼料和蒸餾水採自由覓食，光照為 12 小時亮 12 小

時暗，溫度保持在 22 ± 2 ℃。所有動物實驗將遵守實驗動物護理和使用委員會 IACUC (Institutional Animal Care and Use Committee) 的規範。

五、細胞存活 (Cell Viability)

將細胞種入 96 孔盤 (1×10^4)，放入培養箱內過夜，使細胞能伏貼孔盤底部。以 CR-1 水層／酒精層 (200，100，50，25，12.5，6.25 g/mL) 分別對細胞進行藥物處理 12，24，48，72 小時。

以 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay 測量細胞存活狀況。

六、癒傷實驗 (Wound-healing)

將 culture-insert (Ibidi) 至於 24-well 盤中使其黏於每個 well 中心位子，以此代替傳統用 tip 在細胞中間畫線的方式。將 MDA-MB-231 (2×10^4) 懸浮於沒有 FBS 的培養基中種入 24-well 盤中，放入培養箱內過夜，使細胞能伏貼孔盤底部。將 culture-insert 以鑷子拔起，移除培養基並以 PSB 沖洗一遍，加入新的培養基，並以不同濃度的 CR-1-WF、CR-1-EF 對細胞進行藥物處理。於藥物加入後的第 0，4，8，12 小時以顯微鏡觀察細胞移動及覆蓋傷口的情形並拍照紀錄。

七、細胞移動性 (Transwell Migration)

利用 8 m pore polycarbonate membrane transwell chambers (Corning)，來觀察細胞的移動能力。於 transwell 上層種入懸浮於 phenol-free DMEM/F12 + 0.5% charcoal-stripped FBS 裡的 MDA-MB-231(1×10^4)。並對細胞以不同濃度的 CR-1-WF、CR-1-EF 進行藥物處理。Transwell 下層則是加入正常的培養基 + 10 % FBS 或是正常的培養基 + 10 % FBS + GDNF。24 小時候以棉花棒將 transwell membrane 上層的細胞移除乾淨，並將 transwell membrane 下層的細

胞以 4 % PFA 固定，0.1 % 的結晶紫染色。最後以顯微鏡拍照，並計算 3 個不同視野內的細胞數平均後製成圖表。

八、細胞激素矩陣 (Cytokine Array)

以 RayBio Human Cytokine Antibody Array (AAH-CYT-5, RayBiotech Inc, GA, USA) 分析細胞所釋放出的細胞激素和趨化素。所有實驗步驟皆參照廠商所給的說明書。

九、西方墨點法 (Western Blot)

將 MDA-MB-231 (2 x 10^5) 種於 6-well 盤中，並培養於 phenol-free DMEM/F12 + 0.5 % charcoal-stripped FBS 裡，進行 24 小時的 starvation 處理。以 RIPA lysing buffer 萃取細胞的蛋白質後利用 SDS-PAGE 將蛋白質依大小分開，並將其轉置到 PVDF membrane 上。

將 PVDF membrane 浸泡於 5 % 的脫脂牛奶裡 1 小時，再將其浸入含有一抗 (Actin (NOVUS)、p-AKT (Cell signaling biotech)、p-ERK (Cell signaling biotech)、ERK (Cell signaling biotech)、p-STAT3 (Cell signaling biotech)) 的 5 % 的脫脂牛奶裡，至於 4 ^{o}C 冷房放至隔夜。於室溫浸入含有二抗的 5 % 的脫脂牛奶裡 1 小時。

十、動物實驗 (Animal Model)

4 週齡的 BALB/c 母鼠飼養於動物房內，使其適應環境 1 ~ 2 週開始實驗。本實驗為原位移植 (orthotopic)，將 4T1 乳腺癌細胞 (1 × 10^6 cells/100 L/mouse) 稀釋在 PBS 裡，注射入 BALB/c 右邊第四對乳房的乳腺 (mammary fat pad)。注射一週，待腫瘤體積長大至 50 mm^3，將老鼠隨機分成兩組：控制組 (塗抹凡士林)；CR-1 組。藥塗抹在腫瘤上再以透氣膠帶貼起來，每天換藥，持續三週後停藥一週，於第五週將老鼠犧牲。實驗期間每週以非侵入性活體

影像系統 (IVIS System) 觀察癌細胞在小鼠體內的發展,並測量腫瘤的體積。犧牲之後將原位瘤、肺、和脾取出，將其浸泡在福馬林放至隔夜，替換 PBS 浸泡一天，再將其脫水、包埋、切片、染色做進一步分析。

肆、研究結果

CR-1 對於小鼠原位癌移植的腫瘤生長抑制

由於中草藥以人為治療依歸講求全面性之治療，我們猜測 CR-1 除其本身所具之抗腫瘤毒性外，亦可能透過調節身體等系統性作用影響腫瘤的正常生長。為了瞭解 CR-1 是否可以延緩乳癌於動物體內生長我們使用 BALB/c 母鼠進行 4T1 小鼠乳腺癌細胞的原位癌移植，並觀察 CR-1 的抗腫瘤效果。CR-1 或者凡士林（控制組）是以塗抹的方式給藥，藥塗抹於腫瘤上。每天都會幫小鼠換藥，並每週觀察腫瘤的生長狀況。

由圖、4 可見 CR-1 在實驗中止點之前對於腫瘤生長都有明顯的延緩效果（實驗中止點為控制組的腫瘤大小皆達到 500 mm^3 時）。於實驗結束時，相較於控制組，我們可在 CR-1 組看到顯著的腫瘤生長抑制 ($p< 0.001$) (圖、4)。此一結果顯示 CR-1 於小鼠乳腺癌原位移植的實驗中有良好的效果。

為了進一步評估腫瘤的進展狀況，我們將組織進行 Ki-67 免疫染色。Ki-67 是一個廣泛被用來鑑定腫瘤細胞增生程度的指標。Ki-67 免疫染色陽性反應範圍為 19-89.5% 、 CR-1 明顯的降低了 Ki-67 陽性反應比例(圖、4B)。此外 CR-1 藥物處理的小鼠並沒有觀察到肺轉移(圖、4C)。

CR-1 水層與酒精層的分離以及其質譜圖譜

為了進一步了解 CR-1 的機制與效果，我們將其分成水層與酒精層。並以高壓液相層析 (HPLC) 分析其質譜圖。由圖、3 可見，水層與酒精層的質譜圖有明顯的不同，因此我們認為水層與酒精層對於乳癌細胞會有不同的影響。接著我們以水層 (CR-1-WF)、酒精層 (CR-1-EF) 分別對 MDA-MB-231 進行藥物處理以測試效果。如圖、5 所示，CR-1-EF 對於癌細胞有些微的生

長抑制，而 CR-1-WF 則沒有。這些結果顯示，CR-1 的機制可能是影響細胞的訊息傳遞，而非直接抑制癌細胞增生或對細胞進行毒殺。

CR-1 對於 MDA-MB-231 所釋放出的細胞激素和趨化素的影響

由於促發炎反應因子在乳癌的進程上扮演著一個關鍵的角色以及其具有促進乳癌轉移的特性，讓我們推測發炎訊號及其調控的下游因子可成為一個新的治療標的。有鑑於細胞激素和趨化素在腫瘤進程上扮演著關鍵的角色，因此我們猜測 CR-1 是透過影響發炎反應間接影響腫瘤的生長。為了證明 CR-1 是否為藉由抗發炎反應而達到抗腫瘤的效果，我們以細胞激素矩陣來檢測 MDA-MB-231 在無 CR-1 時及有 CR-1 時所產生的細胞激素和趨化素的差異。由圖、6 我們可以看到有加 CR-1 時 GDNF、MIF、IL-8、和 CCL5/RANTES 的表現量有明顯的下降，而從近期研究中我們知道這些都是和腫瘤侵略性、轉移高度相關的因子。分析過後發現 GDNF 的下降最為明顯，因此我們將進一步研究 GDNF 在 CR-1 的調控裡扮演著什麼樣的角色。

CR-1-WF 對於 GDNF 表現量及癌細胞移動能力的抑制

由於乳癌患者的病情通常在乳癌轉移後就變得非常難以預測，並且在治療過後沒有良好的治療效果，因此我們想要驗證 CR-1-WF 是否能抑制轉移。本實驗選用 MDA-MB-231 人類乳癌細胞做為 transwell migration 的實驗細胞株是因為他具有極高的轉移特性。

分別以濃度為 25 g/ml、50 g/ml、100 g/ml 的 CR-1-WF 或 CR-1-EF 對細胞進行藥物處理 0、4、8、12 小時。在癒傷實驗 (Wound-healing)中，控制組在培養 12 小時後有明顯的移動。而我們發現給予 CR-1-WF 50 g/ml、100 g/ml 藥物處理的細胞結果顯示為有抑制細胞移動的能力 (圖、7 A.)，然而此一現象在 CR-1-EF 處理的實驗組中並沒被觀察到 (圖、8 A.)。

已有文獻指出 GDNF 與促進乳癌細胞的轉移能力有關，因此我們進一步的以實驗驗證 CR-1-WF 是不是因為抑制 GDNF 表現量進而抑制轉移。MDA-MB-231 在加入 GDNF (10 ng/mL) 後移動能力有顯著的增加，然而最重要的是，經過 CR-1-WF 藥物處理可以看到其對 GDNF 刺激有顯著的抑制(圖、7 B.)。

我們也有使用了 transwell assay 來測試 CR-1-WF、CR-1-EF 對於 MDA-MB-231 移動能力的影響。實驗結果與癒傷實驗 (Wound-healing) 的結果雷同，經藥物 CR-1-WF 處理過後可以明顯的將 GDNF 刺激逆轉。因此我們總結，CR-1-WF 的抗轉移機制可能是透過影響 GDNF 或其下游訊號傳遞的路徑。

CR-1-WF 抑制了 GDNF 的訊號傳遞路徑

先前有文獻指出，GDNF 結合 RET 之活化路徑導致乳癌細胞可透過傳遞 RET 之下游訊息傳遞路徑如 MAPK extracellilar-regulated kinase 1/2 (ERK 1/2)、phosphatidylinositide 3-kinases/protein kinase B (AKT/PKB)，(JAK/STAT)進而增加乳癌的轉移與抗藥性的能力。我們因此推論 CR-1-WF 作為一種抗發炎藥物可能具有抑制 GDNF 媒介之訊息路徑，並產生抑制 GDNF 表現與抗轉移之效果。

我們進一步研究 CR-1-WF 是否有能力如 GDNF 抑制劑般抑制 GDNF 下游訊號如 AKT、 ERK1/2、STAT 的活化。如圖、10 A. 所示，磷酸化的 AKT 訊號經 GDNF 刺激 (5 ～ 10 分鐘) 後有增加，不過很意外的在 GDNF 刺激過後並沒有看到 STAT3 的活化。由圖、10 B.可以看見即使在 GDNF 刺激過後 CR-1-WF 仍然可以非常有效的抑制 AKT、STAT3 的磷酸化。不過由圖、10 B.所示，ERK 1/2 的活化並沒有被 CR-1-WF 影響。這些結果顯示，CR-1-WF

可以抑制 GDNF 的下游訊號，因此推論 CR-1-WF 再添加 GDNF 時依然可以抑制轉移是因為 CR-1-WF 破壞了 GDNF 所調控的 AKT、STAT3 活化。

伍、研究討論

一般大眾對免疫的認知為，免疫是身體對抗病原菌的機制，然而慢性的促發炎與一般的發炎是不一樣的。這類慢性發炎反應因子的產生是藉由免疫細胞、腫瘤細胞之間的訊息傳遞，並與腫瘤轉移有密切關聯。長期的促發炎反應被認為是一個使腫瘤轉移、對治療產生抗藥性的原因。在本實驗中我們以一個跌打損傷藥的中草藥複方 CR-1 作為一個新的抗乳癌轉移治療方式。

我們於實驗結果發現，經 CR-1 處理過後的癌細胞並沒有明顯的生長抑制，但於動物實驗時 CR-1 卻能有效的減緩腫瘤的生長速度。雖然目前對於 CR-1 調控的抗腫瘤機制還不是很明瞭，但值得注意是 CR-1 處理過後，癌細胞的移動能力被減弱了。由 Cytokine array 我們看到 CR-1 抑制了許多與腫瘤惡化、轉移高度相關的細胞激素和趨化素的表現量如 GDNF、CCL5、IL-8，而這些發現與中醫的全面性治療觀念相合。然而這些推測都需要進一步的驗證，發炎因子為一個促使腫瘤發展的原因，但不排除當 CR-1 達到夠高濃度時還會影響其他能夠控制細胞移動性的機制。在動物實驗中所觀察到的抗腫瘤現象也有可能是透過其他機制所產生的。

反觀現今乳癌的治療方式，目前針對各種乳癌皆已有有效藥物如 Lumial A/B 型有雌激素受體結抗劑、HER2 型有 anti-HER2 抗體、三陰性乳癌則以化學治療為主，但轉移性的乳癌始終無法有效治癒並具極差的預後。因此低毒性並具抗發炎活性之中草藥，或可為一個乳癌轉移治療的選擇。

如前面所說，雖然 CR-1-WF 能夠有效的抑制癌細胞的移動，不過這個現象也許不能只單由抑制 GDNF 的表現量來解釋。雖然 CR-1-WF 可以有效抑制 GDNF 的下游訊息傳遞因子如 AKT 和 STAT3，且當我們將 GDNF 外加回去時 CR-1-WF 的抑制作用並不會被抵銷，但是 CR-1-WF 並無法完全的抑制乳癌細胞的移動。因此我們認為 CR-1 所調控的抗腫瘤效果，可能是因為許多細胞激素和趨化素被影響後的反應。

陸、結論與應用

由本實驗結果顯示 CR-1 確實能夠有效的延緩腫瘤於小鼠體內的生長，並且在 wound-healing 與 transwell migration assay 中看到 CR-1-WF 對乳癌細胞移動能力的抑制。CR-1-WF 也抑制促發炎因子 GDNF、IL8、CCL5 的表現量。

雖然已有初步結果顯示 CR-1-WF 應該是透過 GDNF 所調控的路徑來抑制乳癌的轉移，但確切參與之機制還需要更深入探討，因 CR-1 於小鼠實驗的抗腫瘤效果也有可能是因為其他機制所造成的。而我們也會進一步對其他被 CR-1-WF 所抑制的細胞激素和趨化素如 IL8、MIF、CCL5 作深入研究，CR-1-WF 抑制腫瘤的轉移可能是因為多個細胞激素和趨化素被影響的總結果。總結來說，我們的研究結果讓中草藥複方在未來可能可以發展成，抗癌症轉移的藥物。

Introduction

Breast cancer is the most prevalent diagnosed women cancer disease and is the first leading cause of cancer death accounting for 14 % all cancer death worldwide (Jones et al.). Breast cancer is a heterogeneous disease including multiple subtypes such as luminal A/B (ER +), HER2-enriched (HER2+), and basal-like (triple-negative) (Perou et al., 2000). Approximately 20 % to 30 % of patients with breast cancer relapse from early breast cancer and that of patients are commonly die from distant recurrence (2005). Additionally, only luminal A type of breast cancer show the less relapse rate (27.8 %) compared with other subtypes have 40 % to 50 % relapse rates according to 15-year distant relapse rate estimates (Kennecke et al., 2010). The mechanism by which cancer disseminate distant organs are not clearly known, but the metastatic breast tumor that are resistant to standard therapy show a poor prognosis, indicating a need for an anti-metastasis strategy for metastatic breast tumors is quite urgent. Although a comprehensive understanding of major cause of metastatic breast cancer is not known, accumulating evidence pointed that metastatic tumor progress is multifactorial depended on tumor grade, proliferation, angiogenesis, and microenvironment surrounding targeted tissues (Qian and Pollard, 2010). Further, recent studies have linked inflammatory environment to tumor migration and invasion (Qian and Pollard, 2010). Consistent with these findings, some studies have suggested an important role for cytokines/chemokines in promoting breast cancer metastasis (Qian and Pollard, 2010). Although current anti-metastasis therapy that block angiogenesis pathway have been described, an effective therapy for targeting metastatic inflammatory pathway is still rare.

Natural products are popular worldwide because they are derived from natural

sources. Indeed, some of natural products such as Chinese Herbal Medicine (CHM) have been widely used in ethnopharmacological applications. CHM has been widely used in worldwide and also has been used in clinic up to thousand years in Asia. The principle of concept of Traditional Chinese Medicine (TCM) is focused on the “holistic” function and “homeostatic” regulation. Therefore, TCM generally comprises many herbs as “formula” or “combo” in the purpose of improvement of therapeutic outcome. As a result, under the concept of allopathy (so-called “anti-”), many TCM formulas cannot meet the drug development criteria. Although there are few systemic studies on the integration of traditional and current medicine, recent review studies have revealed insight into the underlying mechanism in CHM and have shed light on the opportunity for developing CHM formula as novel therapeutics. For example, *Astragalus*-based herbal formula (PG-2) is recently approved by TFDA (Taiwan Food and Drug Administration) as a new drug, applied for reducing toxicity and increasing efficacy in combination with platinum-based chemotherapy (McCulloch et al., 2006).

Considering the role of proinflammatory cytokine and chemokine in transformation, tumorigenesis, and metastasis signaling, it is of great interest to identify natural herbs that exert anti-inflammatory activity as a new anti-metastatic therapeutic formula. The goal of this study was to identify the proinflammatory targets participated in tumor metastatic pathways and to investigate whether TCM formula classified as the blood-activating medicine is as a potent inhibiting agent for anti-inflammation and is effective to inhibiting metastasis. Here, we describe the *Carthamus*-based formula (CR-1), a folk medicine used in Taiwan for bone bruises external formula, as a new strategy for anti-metastasis. CR-1 is a mixture herbal formula consisting of 15 Chinese herbs that are *Carthamus tinctorius*, *Rheum officinale*, *Scutellaria baicalnsis*, *Coptis chinensis*, *Angelica sinensis*, *Ligusticum chuanxiong*, *Rehmannia glutinosa*, *Boswellia*

thurifera, *Cortex Phellodendri*, *Commiphora molmol*, *Wedelia chinensis*, *Alternanthera sessilis*, *Dryobalanops aromatic*, *Sinapis alba*, and *Pinellia ternate*. We found that CR-1 only show moderate anti-tumor activity *in vitro* and *in vivo*. Additionally, the water partition of CR-1 suppressed the production of glial derived neurotropic factor (GDNF), a chemokine associated with migration and resistance, causing the inhibition of cancer cell metastasis and significantly suppressing AKT and STAT3 activation. By studying the anti-inflammatory effects of TCM, we highlight the new clinical application of our finding.

Materials and Methods

Materials

All commercial chemical reagents were purchased from sigma-aldrich. The recombinant protein GDNF was purchase from ProSpec. The 96-well plates and cultural dishes were purchased from Cloning. The CHM materials were purchase from commercial company.

Cell Lines

4T1 cell lines were obtained from the American Type Culture Collection. MDA-MB-231 cell line was a gift provided from MD Anderson Cancer Center (USA). 4T1 cells were cultured in RPMI-1640 supplemented with 10 % FBS (Invitrogen) at 37 ℃ with 5 % CO_2. MDA-MB-231 cells were cultured in DMEM/F12 (Sigma-Aldrich) supplemented with 10 % FBS (Invitrogen) at 37 ℃ with 5 % CO_2.

High-performance liquid chromatography (HPLC) analyses

Sample analysis was carried out on an LC-20A VP HPLC system (Shimadzu Inc.) consisting of a quaternary pump (LC-20AT), an on-line degasser (DGU-14A), an autosampler (SIL-20AD), a photodiode-array detector (SPD-M20A) and a Class VP for data collection. Liquid chromatography was performed using an Agilent Poroshell 120 SB-C18 column (150 mm × 4.6 mm, i.d., 2.7 m, Agilent Technologies, Palo Alto, CA, USA). The sample injection volume was 10 l. The mobile phase was prepared by mixing acetonitrile (solvent A) with 4 mM ammonium acetate (solvent B, pH value being adjusted to 3.5 using acetic acid). Flow rate of column was 0.6 ml/min, column temperature was ambient temperature, and the detection wavelength was 254 nm. Gradients of elution sequentially were 22 % A to 24 % A for 0 to 3 minutes, 24 % A to

25 % A for 3 to 9 minutes, 25 % A to 28 % A for 9 to 10 minutes, 28 % A to 49 % A for 10 to 14 minutes and held for 7 minutes, then 49 % A to 52.5 % A for 21 to 25 minutes and 52.5 % A to 60% A for 25 to 30 minutes. Over the next 20 minutes, the percentage of mobile phase A increased linearly to 100 %. The mobile phase was filtered through a 0.22 m Millipore filter and degassed before use. From the dry extract, 1 mg was dissolved in 1 ml of methanol and filtered through a 0.45 m membrane filter before loading on the HPLC column.

Cell viability

Cells (1×10^4) were loaded in 96-well culture plates and incubated in medium for stabilization. After overnight incubation, cells were treated with the water partition or ethanol partition of CR-1 at 200, 100, 50, 25, 12.5, 6.25 g/ml for 12 h, 24 h, 24 h, 48 h, or 72 h. The cell viability was assessed by 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) assay.

Wound-healing assay

In the scratch wound healing experiment, we utilized the culture-inserts purchased from Ibidi to form the scratch. MDA-MB-231 cells (2×10^4) were seeded into 24-well culture plate and allowed cells to adhere overnight in the starved condition medium (0.5% charcoal-stripped FBS in phenol-free DMEM/F12 medium). After overnight incubation, culture-inserts were removed, cells washed with PBS and replaced with normal culture medium containing indicated concentrations of CR-1-WF and CR-1-EF. Cells migrate and cover the scratch was observed at 0 h, 4 h, 8 h, 12 h time points under the microscopic fields. The microscopic observations were imaged with NIKON ECLIPSE TS100F, and pictures were captured by NIKON DIGITAL SIGHT DS-L1.

Transwell migration assay

The migration and metastatic activity was assessed by using transwell migration assay. In the upper chamber, the cells (1×10^4) were loaded into the 8 m pore polycarbonate membrane transwell chambers (Corning) in the starved cultural medium (0.5 % charcoal-stripped FBS in phenol-free DMEM/F12 medium). And the cells were treated with CR-1-WF or CR-1-EF at indicated concentrations. Otherwise, lower chambers were filled with normal condition medium containing 10 % FBS in the presence or absence of GDNF (Prospec). After incubation for 24 h, cells remaining on the upper membrane were removed with cotton swab. The membranes were treated with 4 % PFA for fixing, and the cells were stained in 0.1 % crystal violet. Migrated cells were counted from randomly three different microscopic fields.

Western blot analysis

MDA-MB-231 cells (2×10^5) were seeded in 6-well plates and starved with starved cultural medium (0.5 % charcoal-stripped FBS in phenol-free DMEM/F12 medium) for 24 hours. Total cell lysates was harvest and suspended in RIPA lysing buffer. The total lysta proteins were separated by SDS-PAGE, and transferred it into PVDF membranes. The membrane were blocked with 5 % skim milk, and hybridized with primary antibodies including Actin (NOVUS), p-AKT (Cell signaling biotech), p-ERK (Cell signaling biotech), ERK (Cell signaling biotech), p-STAT3 (Cell signaling biotech) for overnight at 4 ^{o}C, and incubate with secondary antibodies for 1 hour at room temperature. The bands were visualized by ECL (Milipore).

Human cytokine antibody array

We conducted RayBio Human Cytokine Antibody Array (AAH-CYT-5, RayBiotech Inc, GA, USA) to analyze cytokines and chemokine production. This array contains human cytokines, chemokines and inflammatory factors. All the procedures

were performed following the manufacturer instructions. The levels of cytokine expression were analyzed by using ImageQuant densitometry software and normalized by the density of positive controls.

***In vivo* experiment**

20 female BALB/c mice (4-week-old) obtained from National Laboratory Animals Center, Taipei, Taiwan were given standard feed and drinking water ad libitum and kept on a 12 hour light/dark cycle at 22 ± 2 ℃ under specific pathogen-free conditions. All animal work will be done in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC). Academia Sinica, Taiwan. The in vivo experimental model was performed as described previously (Yoneda et al., 2000). 4-week-old female BALB/c mice were allowed to adapt for 1 ~ 2 weeks in the animal room before experiment. 4T1 cells (1×10^6 cells) were suspended in 100 l PBS and injected into the right forth mammary fat pad of each mouse. A week after injection, the mice carried tumor at size over 50 mm^3, were than randomly divided into experimental and control groups. Control group treated with Vaseline and CR-1 group. Treatment was applied on to the tumor and covered with tape, treatment were applied every day. Treatment was applied for 3 weeks, stop for 1 week, and sacrificed on the 5th week. During experiment tumor growth is tracked with IVIS system every week, and tumor volume measured.

Statistical analysis

Data were generally presented as the mean ± SEM of at least three determinations. Statistical analysis for comparing two independent groups were used Student's t test. The significant difference is presented as asterisk symbol (*) depending on the p values< 0.05.

Results

Carthamus-based formula inhibit growth of allograft breast tumors in BALB/c mice through non-invasive delivery approach

Considering the holistic function of CHM, we therefore hypothesized that, in addition to its intrinsic anti-tumor activity, systemic regulation of tumor growth would also be affected. To determine whether CR-1 can delay breast tumor growth *in vivo*, we evaluate the antitumor activity using breast tumor grown from 4T1 orthotropic allograft breast cancer cells in BALB/c mice. CR-1 or Vaseline (control group) was delivered from the patch formulation. Mice were treated with patch continuously and monitored time to tumor growth weekly. As shown in Figure 4A, treatment with CR-1 significantly delayed tumor growth time to end point, depending on the control tumors reach a volume of 500 mm^3 (Teicher, 2006). At this end time statistically significant tumor growth inhibition was observed at the CR-1-treated group, compared with Vaseline-treated group ($p < 0.001$) (Figure 4A). This observation indicates that CR-1 show a significant benefit in a xenograft model of breast cancer. To assess the state of tumor progression, we next evaluate the tumor proliferation with Ki-67 IHC score. Because Ki-67 is widely used in the measurement of tumor cell proliferation and that is highly correlated with patient's prognosis (Jones et al.), we used Ki-67 as the biomarker for evaluating the effect of CR-1 treatment. As shown in Figure 4B, IHC Ki-67 score range from 19 - 89.5 %. The IHC Ki-67 of tumor showed that treatment with CR-1 reduced the Ki-67 positive cells. Ki-67 mean of control is 68 %, but mean of treatment with CR-1 group is 41 %. In addition, compared with the control group, treatment with CR-1 did not observe 4T1 cancer cells metastasis to the lung organ (Figure 4C).

The fractionation of *Carthamus*-based formula with water and ethanol and the separation of HPLC chemical profile

To better understand the effects and mechanisms of CR-1, we fractionate the CR-1 with water or ethanol. High-performance liquid chromatography (HPLC) analyses were applied to analyze the chemical profile of water and ethanol fractionation of CR-1. In our experience, the separation of CR-1 fractionation was used reverse phase HPLC, and acetonitrile and water (the ratio=4:6) were chosen as the eluting solvent. The flow rate was set at 1 ml/min to separate the HPLC profile. As shown in Figure 3. the HPLC traces at 254 nm revealed the profile of water and ethanol fractionation of CR-1 and the separation of CR-1 water and ethanol fraction exhibited the different chemical profile. These data imply that the response of water and ethanol fraction may exert different functions in breast cancer cells. The effects of water fraction (CR-1-WF) and ethanol faction (CR-1-EF) were then assessed in breast cancer MDA-MB-231 cells. As shown in Figure 5, the slight growth inhibitory activity were observed upon CR-1-EF treatment for 24, 48, 72 hr, whereas these effects did not show in the treatment with CR-1-WF. These findings suggest that rather than function directly on killing of breast cancer or inhibiting of cell proliferation, the underlying mechanism behind CR-1 may act on the complementary signaling independently of proliferation.

The *Carthamus*-based formula alter the cytokine and chemokine expression profiles in MDA-MB-231 cells

The pivotal role of proinflammation in the progression of breast cancer and its ability to trigger breast cancer metastasis suggested that inflammation and their downstream signaling act as a potential therapeutic target. Because cytokines and chemokines are the critical factors in tumor progression, we therefore hypothesize

whether CR-1-WF act indirectly on tumor growth through inflammatory signaling. For this, we sought to determine whether CR-1-WF exert antitumor activity via anti-inflammation mechanisms, by testing the panel of cytokine production in the presence or absence of CR-1-WF. Using an antibody cytokine array, we analyzed the effects of CR-1-WF on the levels of cytokine expression in MDA-MB-231 cells. As shown in Figure 6, we detected an decrease of cytokines and chemokines including GDNF, MIF, IL-8, and CCL5/RANTES that are highly associated with tumor invasion and metastasis, as evidenced by recent studies (Gil et al., 2010; Kim et al., 2012; Velasco-Velazquez et al., 2012). As analyzed by the densitometry of array blots, we noticed a significant decrease of GDNF compared to untreated group and gained a great interest of the role of GDNF in CR-1-WF-mediated effects (Figure 7 B.).

The CR-1-WF suppresses the level of GDNF expression and contributes to its ability of migration

As poor prognosis and worse therapeutic outcome in patients suffering breast cancer disease commonly occur in metastatic breast tumors, we sought to determine whether CR-1 can affect the ability of cell migration. In our experiment, we selected MDA-MB-231 cells as the model cell line for migration assay owing to its metastatic activity. The cells were treated with CR-1-WF and CR-1-EF at 25 g/ml, 50 g/ml, or 100 g/ml doses for 0 h, 4 h, 8 h, and 12 h. In a scratch wound-healing assay, MDA-MB-231 cells showed an obvious migration after 12 h exposure of culture medium containing 5 % FBS. We found that treatment with CR-1-WF at the concentration of 50 g/ml and 100 g/ml show a moderate suppression of migration (Figure 7 A.). However, in contrast, treatment with CR-1-EF had no significant function on the suppression of MDA-MB-231 cell migration (Figure 8 A.). Since GDNF has been linked to the stimulation of metastatic ability of breast cancer (Morandi et al.,

2011), we examined whether the decrease in GDNF expression by treating with CR-1-WF resulted in the suppression of migration. Addition of GDNF (10 ng/ml) increased the migration of MDA-MB-231 but, most importantly, CR-1-WF strongly inhibited the migration induced by GDNF stimulation (Figure 7 B.). The effects of CR-1-WF and CR-1-EF on the ability of migration were also assessed in MDA-MB-231 cells using chamber transwell assay. Similarly, transwell assay showed that the effects of addition of GDNF on induction of migration in MDA-MB-231 cells were significantly reversed by treating with CR-1-WF. We conclude that the anti-metastatic effects of CR-1-WF, at least in part, may act on the GDNF or downstream of GDNF signaling pathway.

The GDNF mediated signaling was inhibited by CR-1-WF.

Previous studies have shown that GDNF-RET activation leads to increased metastasis and resistance of breast cancer cells through transactivation of downstream signaling of RET, a known receptor of GDNF, such as MAPK extracellilar-regulated kinase 1/2 (ERK 1/2), phosphatidylinositide 3-kinases/protein kinase B (AKT/PKB) and Janus-activated kinase/signal transducer and activator of transcription pathways (Morandi et al., 2011). We hypothesized therefore that CR-1-WF, as an anti-inflammation agent, might markedly suppress GDNF-mediated signaling via single or multiple mechanisms, contributing to the functions of anti-metastasis and suppression of GDNF in breast cancer cells. We then examined whether CR-1-WF, like GDNF inhibitor, affects downstream of GDNF signaling such as AKT, ERK1/2, and STAT activation. As shown in Figure 10 A., the phosphorylation of AKT signaling was escalated from 5 min to 30 min by treating with GDNF. However, unexpectedly, the data did not observe the activation of STAT3 after GDNF stimulation. Treatment with CR-1-WF showed the significant inhibition of AKT and STAT3 phosphorylation even in

the presence of GDNF stimulation (Figure 10 B.). But, these inhibitory effects of CR-1-WF did not influence ERK1/2 activation (Figure 10 B.).These results demonstrated the inhibitory effects of CR-1-WF on downstream signaling of GDNF, suggesting that the inhibition of migration when addition of GDNF might be due to the possibility that CR-1-WF dampen the GDNF-mediated AKT and STAT3 activation.

Discussion

In general, inflammation is the phenomenon that is thought to an intrinsic defense mechanism against pathogen. However, the chronic pro-inflammation differs from general immune response in that the reciprocal production of inflammatory cytokines is initiated via crosstalking by immune cells or tumor cells and therefore tightly linked to metastasis (Quail and Joyce, 2013). The long-term proinflammation is thought to allow tumor cells metastasis and endow tumor cells with an increased capacity against current anti-cancer therapy (Qian and Pollard, 2010; Quail and Joyce, 2013). Consistently, anti-inflammation drug such as NSAIDs have been demonstrated their ability to prevent colon cancer, providing the significant benefit in clinic (Ruegg et al., 2003). We show here that the folk medicine CR-1 using Chinese herbal medicine for bone bruises formula may serve as a novel therapy for metastatic breast cancer.

Interestingly, treatment with CR-1 significantly retarded the tumor growth *in vivo*, but CR-1-WF showed non-toxic effects *in vitro*. The exact mechanisms underlying the CR-1-mediated anti-tumor function is current unknown. Notably, a decreased of cancer cell viability was observed in the treatment with more than 100 g/mL of CR-1-WF, arguing that CR-1 may not act by directly killing cancer cells. Rather, we suggest that CR-1-WF suppresses myriad of cytokines and chemokines highly associated with tumor malignant and metastasis; possible candidates are GDNF, CCL5, and IL-8. These findings might agree the fundamental theory of Chinese Medicine that performs “holistic” effect toward human disease. This suggestion remains to be carefully proven, since the inflammatory cytokines are one of reasons to drive tumor progress and other unknown mechanisms may also controls cancer cell viability mediated by CR-1 at high enough concentrations *in vitro* system. It remains possible that the anti-tumor activity

observed *in vivo* occurs via other mechanisms.

In view of current breast cancer therapy, there is existence of effective drugs for each subgroup such as estrogen receptor antagonist for Luminal A/B group, anti-HER2 antibody for HER2-enriched group, and chemotherapy for TNBC group (Perou et al., 2000). However, metastatic breast cancer still presents as incurable disease with poor prognosis. Therefore, CHM with relative less toxicity and anti-inflammation activity may serves as an option to treat metastasis of breast cancer.

As described in our finding, migration assay showed that the inhibition of migration by CR-1WF, which cannot be only explained by suppressing the levels of GDNF. Although the downstream signaling of GDNF pathway such as AKT and STAT3 were inhibited by CR-1-WF treatment, and addition of GDNF did not alter the effects of CR-1-WF, still CR-1-WF treatment cannot achieve to the complete inhibition of breast cancer cell migration. We propose, therefore, that the anti-metastasis effects mediated by CR-1 reflect the convergent conclusion affected by other chemokines/cytokines. Collectively, our findings may offer a new option to conduct traditional Chinese medicine as a strategy for anti-metastasis.

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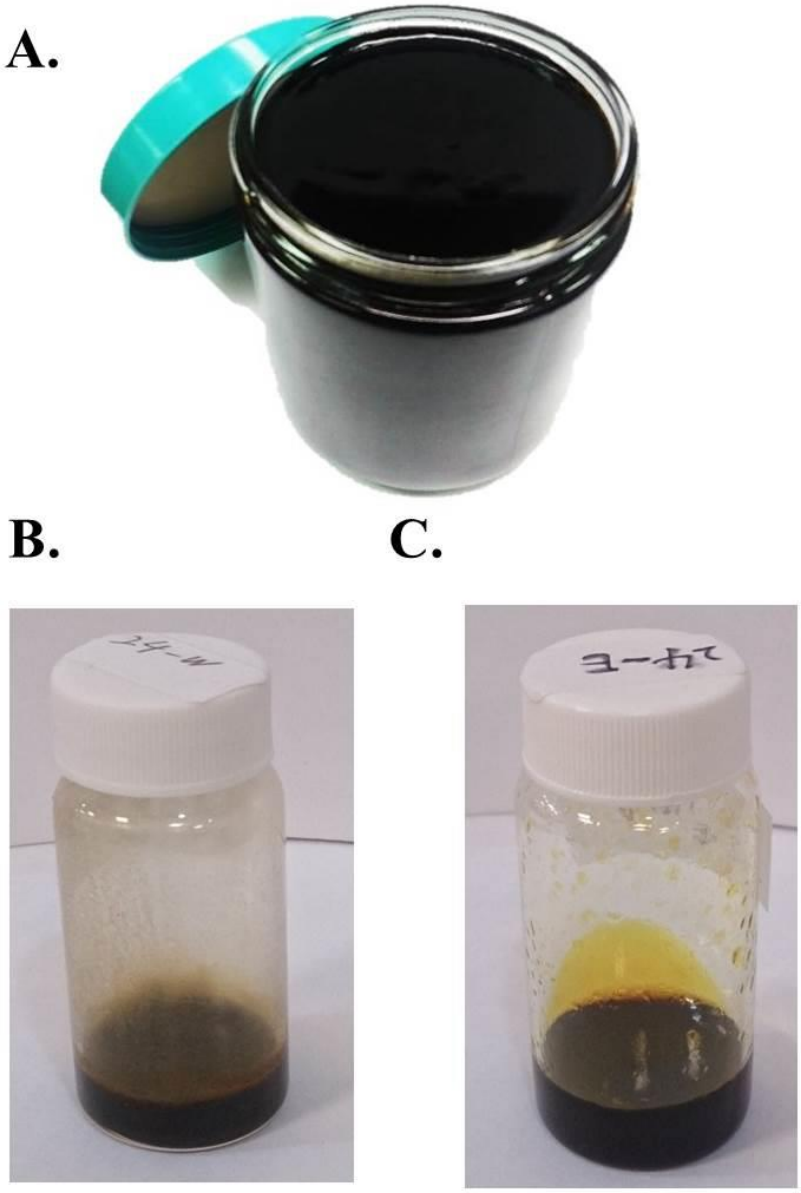
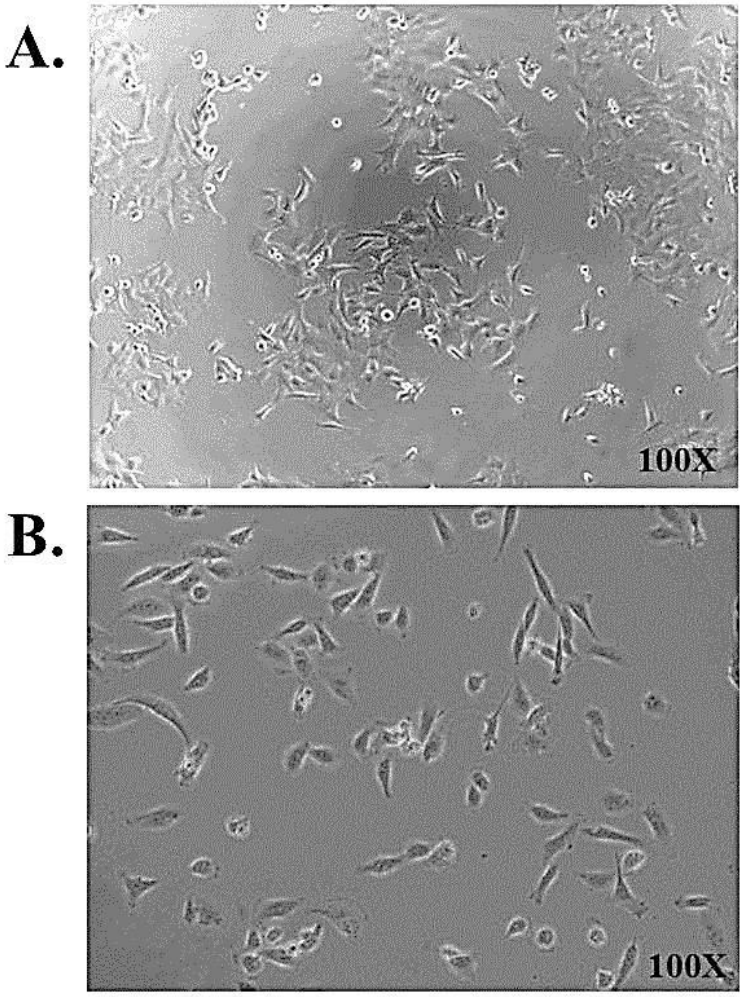
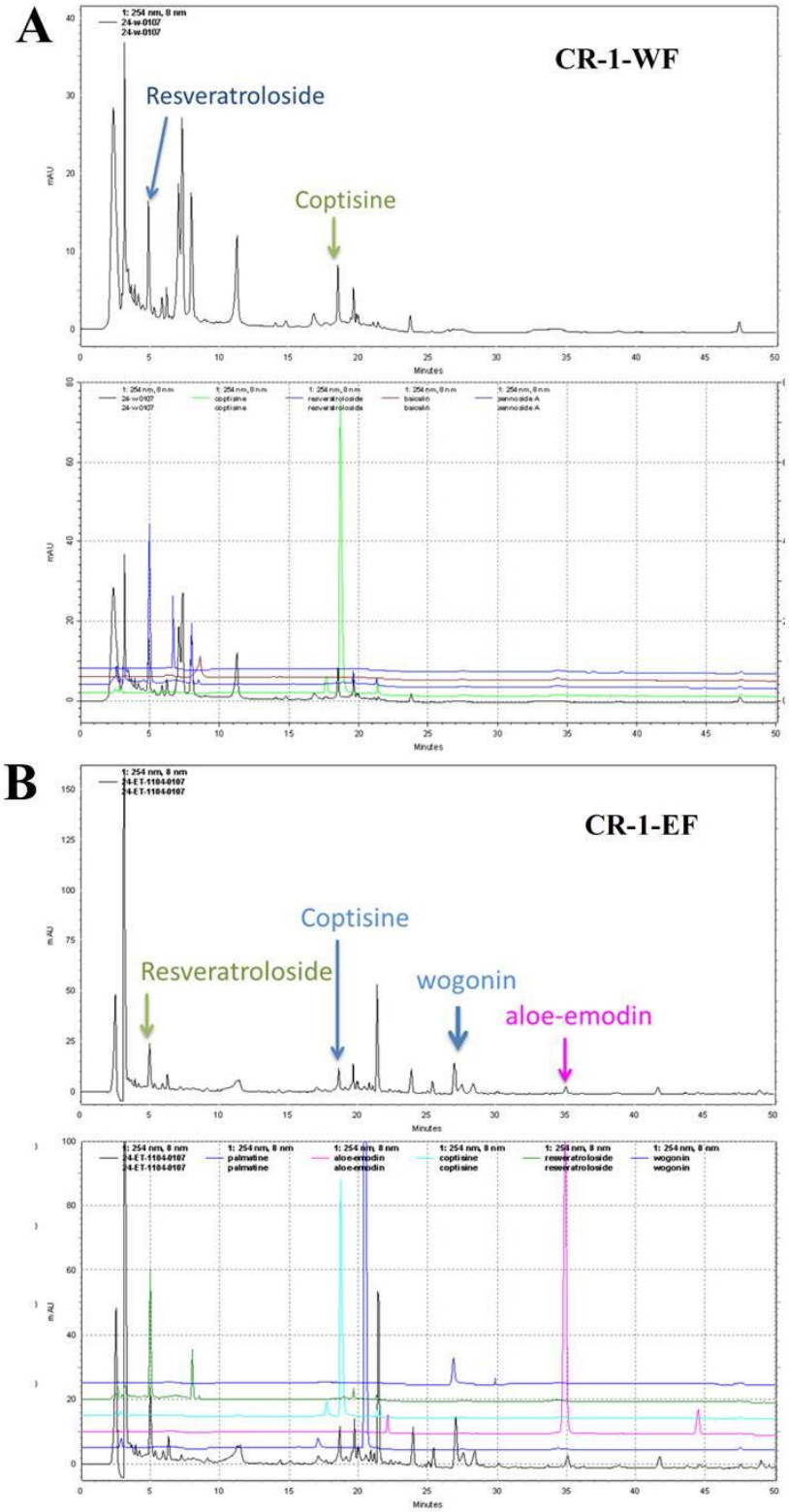
柒、附圖與附表



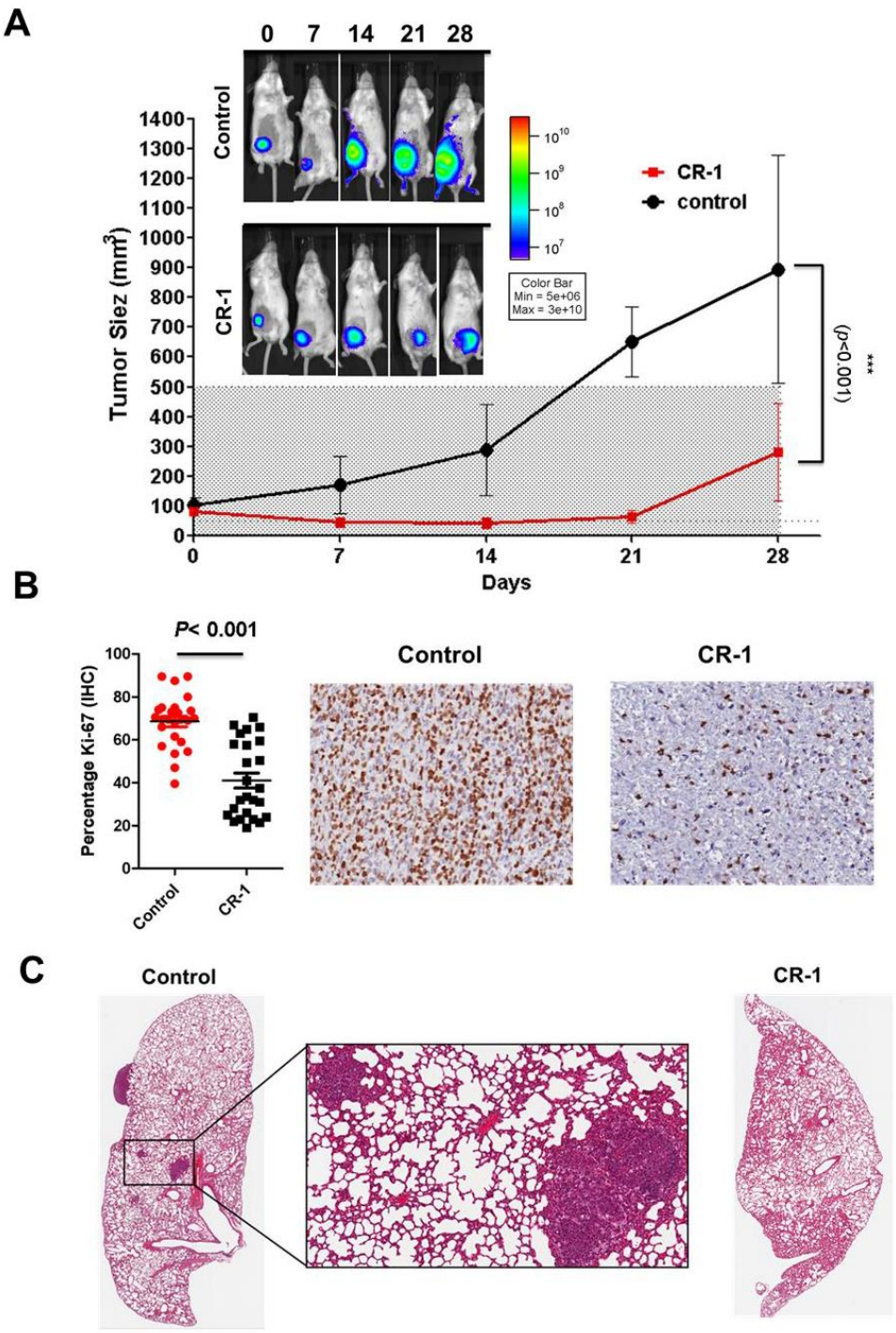
圖、1 (Figure 1) (A.) 由紅花、大黃、黃芩、黃連、當歸、黃柏、川芎、生地、乳香、末藥、黃花蜜菜、紅花蜜菜、冰片、白介子及半夏所組成的 CR-1 複方 (B.) CR-1 經過再一次處理的水層成分 (C.) CR-1 經過再一次處理的酒精層成分



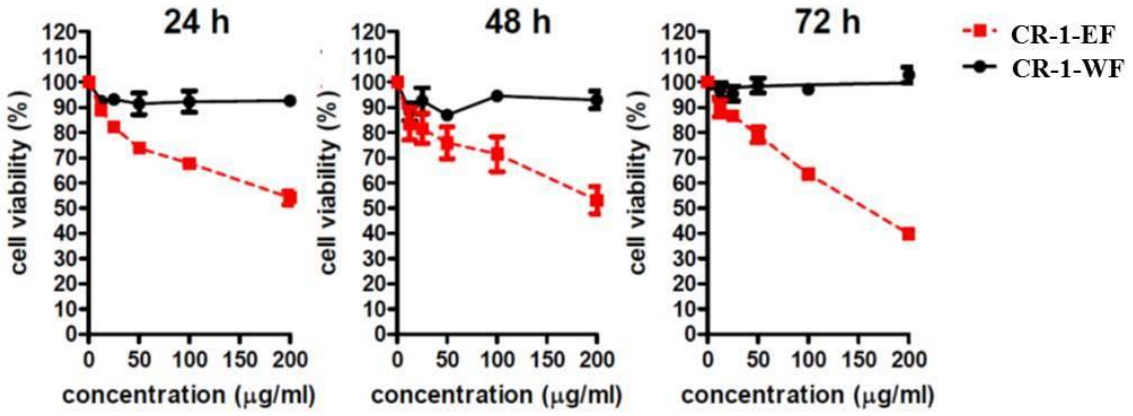
圖、2 (Figure 2) (A.) 4T1 小鼠乳腺癌細胞 (B.) MDA-MB-231 人類乳癌細胞



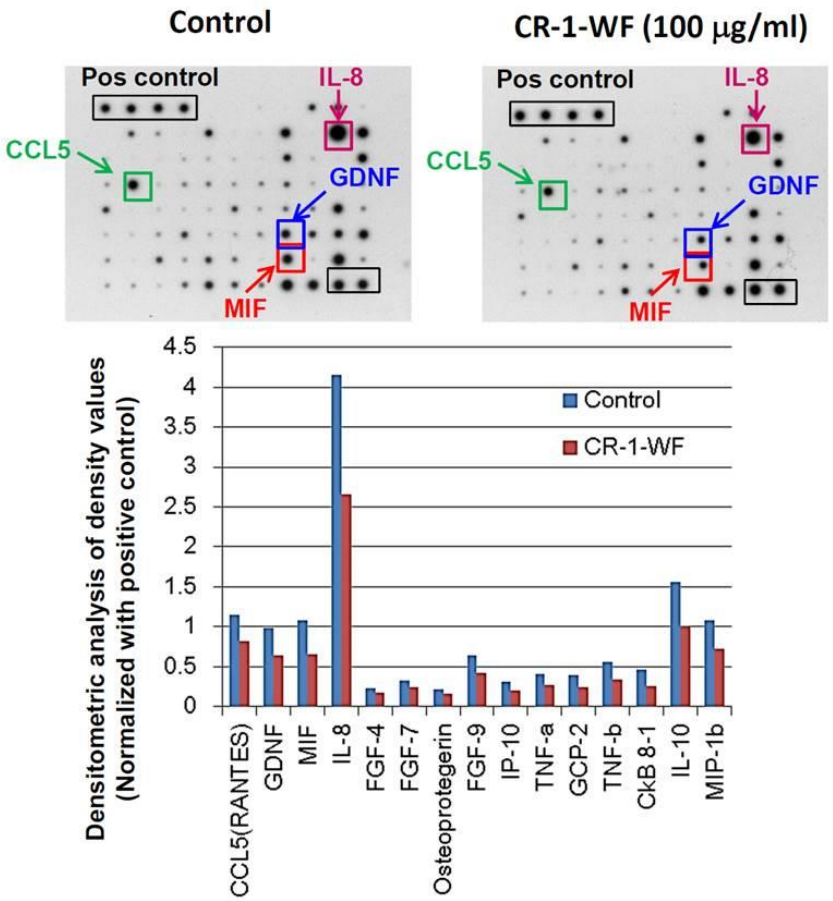
圖、3 (Figure 3) (A.) 水層的質譜圖，(B.) 酒精層的質譜圖



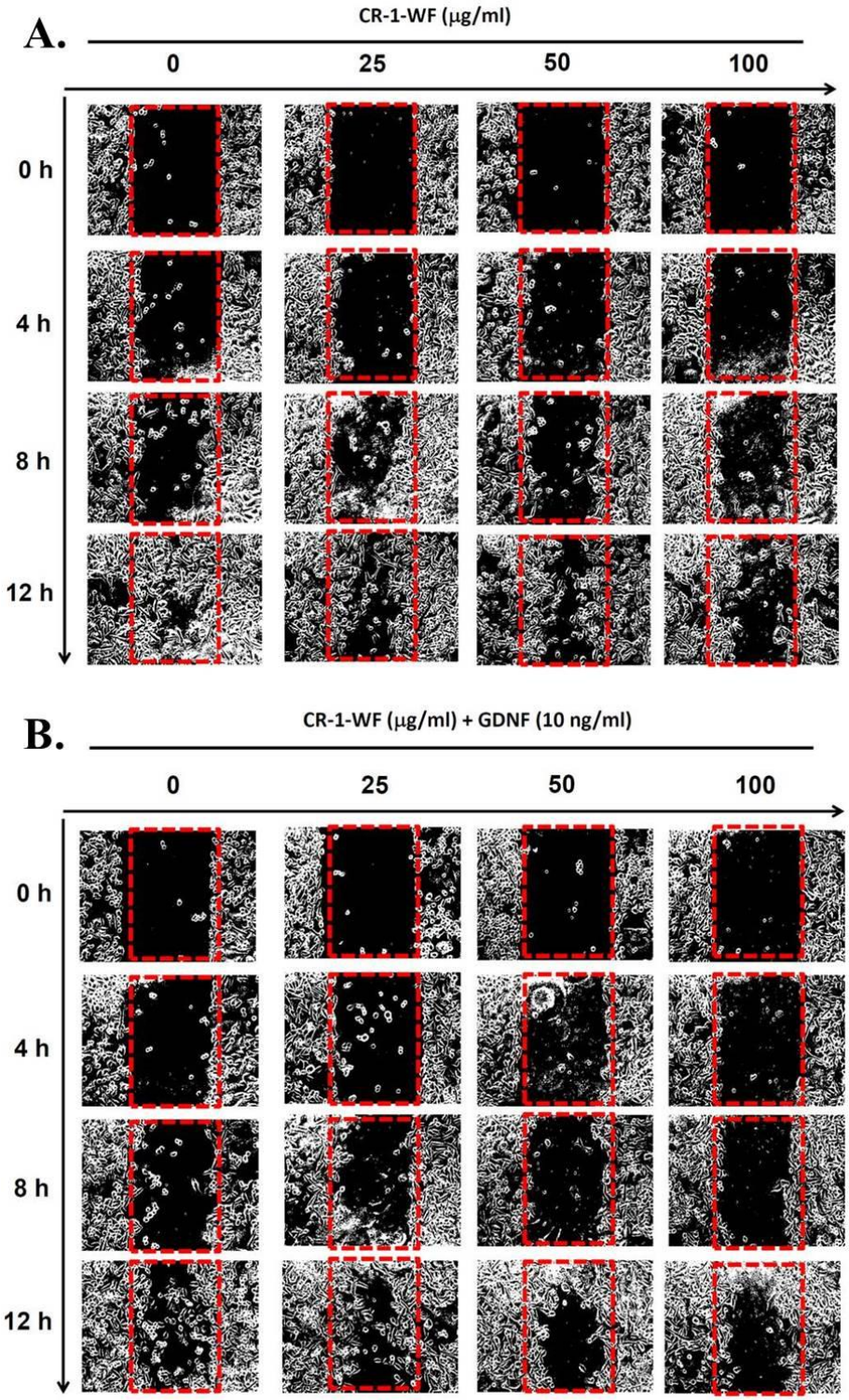
圖、4 (Figure 4) 將 4T1 小乳腺癌細胞以原位移植的方式注射到 BALB/c 的右邊第四對乳腺以 IVIS 系統觀察腫瘤於小鼠體內的生長情形。(A) 由圖可見 CR-1 藥物處理的小鼠腫瘤生長有明顯延緩。(B) 控制組的 Ki-67 陽性反應細胞為 68%，而 CR-1 藥物組則是 41%。(C) 在控制組的肺觀察到轉移，而 CR-1 藥物組的並沒有。



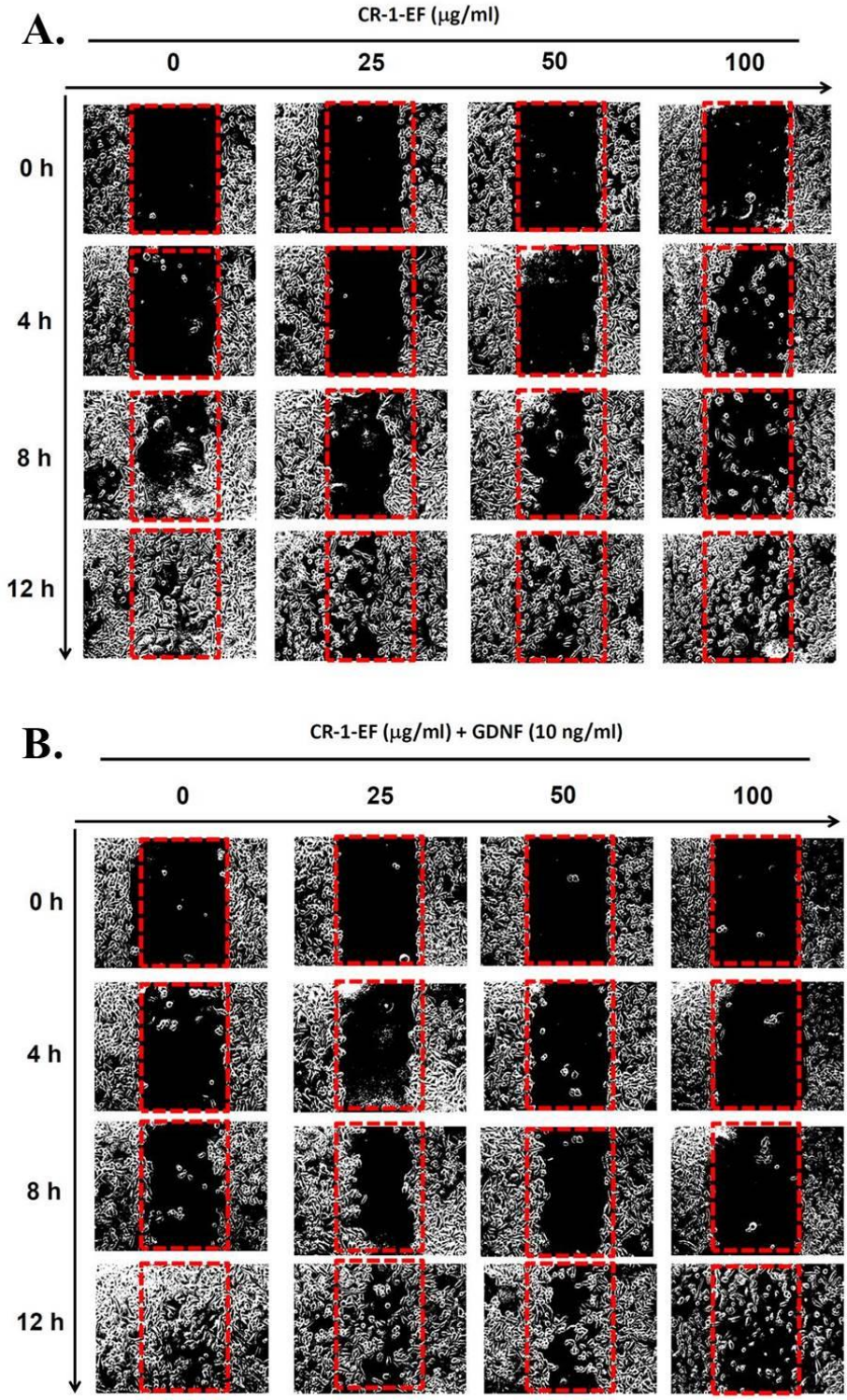
圖、5 (Figure 5) 以 MTT assay 分析 MDA-MB-231 經 CR-1-WF、CR-1-EF 處理過後的生長狀況，可以看到 CR-1-WF 對癌細胞生長並沒有抑制，而 CR-1-EF 則有些微抑制效果。



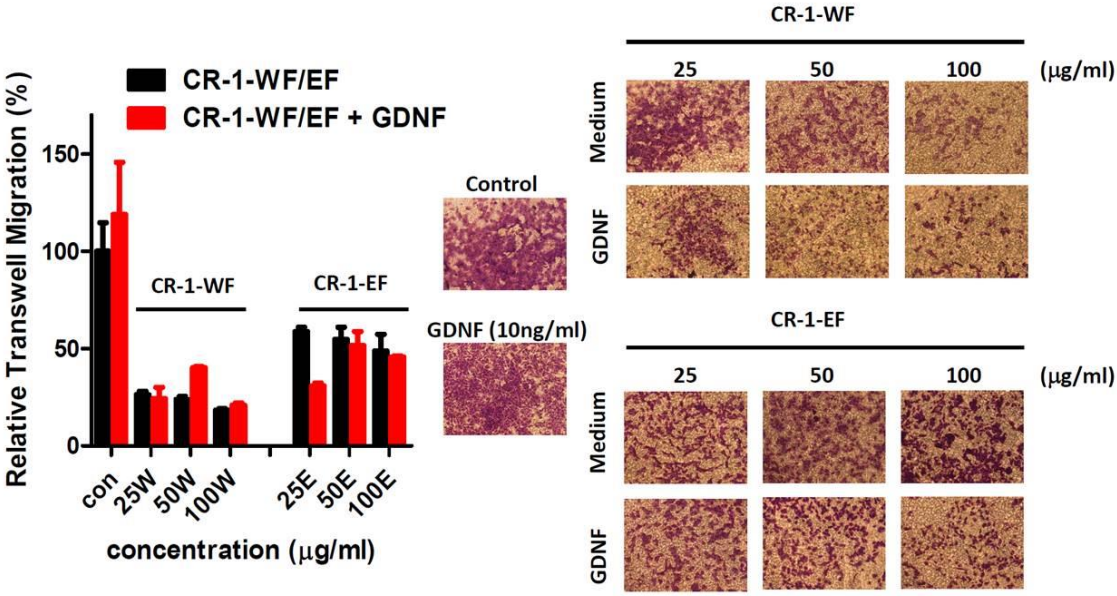
圖、6 (Figure 6) 以 CR-1-WF 處理過後的 MDA-MB-231 乳癌細胞所分泌的細胞激素和趨化素與控制組有明顯差異的有 GDNF、IL-8、MIF、CCL 等與腫瘤發展、轉移高度相關的細胞激素和趨化素。



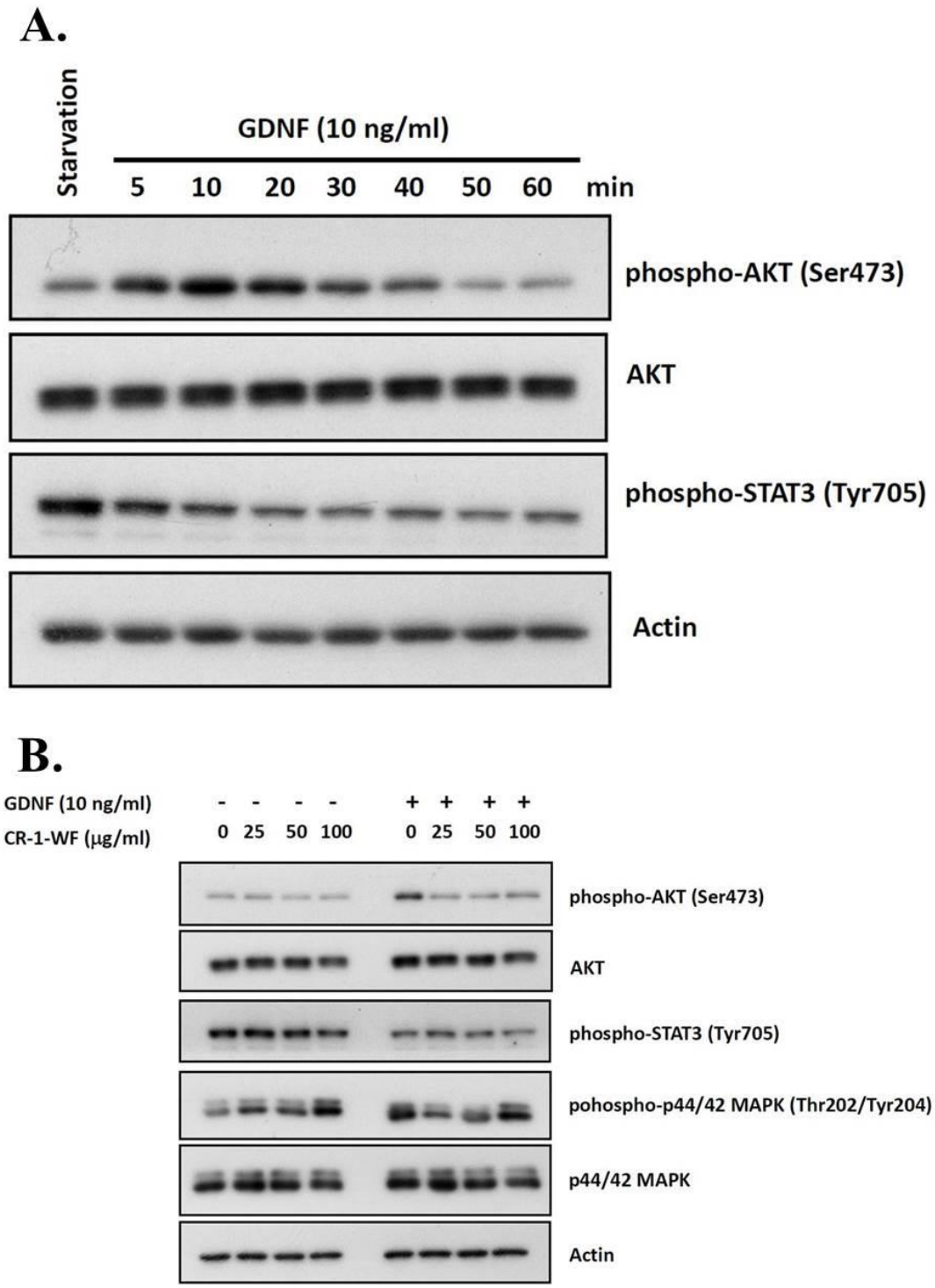
圖、7 (Figure 7) 於加藥後每 4 小時以顯微鏡拍照觀察的結果顯示，加入 CR-1-WF 的 cell migration 有顯著的抑制，即使在額外加入 GDNF 後 CR-1-WF 還是能有效的抑制轉移。



圖、8 (Figure 8) 於加藥後每 4 小時以顯微鏡拍照觀察的結果顯示，加入 CR-1-EF 後對 cell migration 並沒有明顯的抑制效果，在額外加入 GDNF 後效果也是差不多。



圖、9 (Figure 9) 在 transwell migration assay 中 CR-1-WF 在有或沒有 GDNF 的情況下，皆能顯著的抑制乳癌細胞移動。而 CR-1-EF 則沒有顯著差異。



圖、10 (Figure 10) (A.) MDA-MB-231 人類乳癌細胞以 GDNF 刺激 5 ～ 60 分鐘後對於 p-AKT、AKT、p-STAT3 的影響，actin 為 control。(B.) MDA-MB-231 人類乳癌細胞以 CR-1-WF 處理過後以及再以 GDNF 刺激 30 分鐘後對於 AKT 和 STAT3 皆有抑制表現量的效果。

評語

探討中藥複方 CR-1 是否能應用於抗乳癌轉移：

1. 對於 WF 及 EF 其中之成分應分析；

2. 除 GDMF pathway 外其他路徑是否有影響。