# 科技部補助專題研究計畫成果報告
# 期末報告

## 白藜蘆醇（resveratrol）與其甲氧基化結構衍生物抑制癌細胞上皮細胞-間質細胞轉換與侵襲性之分子機制研究(第 3 年)

計 畫 類 別 ：個別型計畫
計 畫 編 號 ：NSC 100-2320-B-040-009-MY3
執 行 期 間 ：102 年 08 月 01 日至 103 年 07 月 31 日
執 行 單 位 ：中山醫學大學生物醫學科學學系（所）

計 畫 主 持 人：陳威仁
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中 華 民 國 103 年 10 月 31 日

中 文 摘 要 ： Resveratrol（白藜蘆醇）為天然的抗氧化劑及自由基的清除者，是漿果類植物如藍莓、葡萄等中主要的芪類植物抗毒素(stilbene phytoalexin)，在早期的小鼠表皮腫瘤發生模式實驗中即被證實具有預防癌症發生的能力。如今，多種resveratrol 的甲氧基取代結構衍生物被開發，理由是因為甲氧基取代會增加 resveratrol 的 hydrophobicity，增強resveratrol 的甲氧基取代結構衍生物被細胞吸收的能力，並加強其抗癌藥效。而癌細胞的轉移是造成癌症病人死亡的最主要原因，近期的研究發現癌細胞侵襲力的獲得可能與癌細胞型態上的上皮細胞-間質細胞轉換有極為密切的相關。此外，Wnt-β-catenin 訊號路徑的失調被認為是癌細胞上皮細胞-間質細胞轉換的主要機制之一。因此，抑制 Wnt-β-catenin 訊號路徑活化所造成的癌細胞上皮細胞-間質細胞轉換或許能夠降低癌症的轉移與致死率。

我們研究成果中發現，resveratrol 的甲氧基取代結構衍生物之一的 3,5,4‘-trimethoxystilbene (MR-3)，較resveratrol 更能在低濃度的情況下增加 MCF-7 乳癌細胞株上皮細胞型態標記 E-cadherin 的表現，並同時抑制間質細胞型態標記 Snail 與 uPA 之表現，顯示 MR-3 可能會抑制 MCF-7乳癌細胞的上皮細胞-間質細胞轉換。另外，MR-3 也降低了β-catenin 在細胞及細胞核內的表現，而 β-catenin 所調控的 c-myc、cyclin D1 基因表現量也同步下降，暗示 MR-3可能是透過抑制 Wnt/β-catenin 訊號路徑的活化來減少MCF-7 乳癌細胞株之上皮細胞-間質細胞轉換並降低其侵襲性表徵。進一步探索其分子作用機轉，發現 MR-3 會抑制 PI3K-Akt 訊號路徑的活化，降低 glycogen synthase kinase(GSK)-3β 被磷酸化而失活的機會，由此推測 MR-3 可能是藉由保留 GSK-3β 的活性，來抑制 Wnt/β-catenin 訊號路徑的活化並抑制 MCF-7 乳癌細胞株之上皮細胞-間質細胞轉換。故透過本實驗來瞭解 resveratrol 與其甲氧基取代結構衍生物對抗癌症轉移的機制，並配合其低毒性和非致癌性之特性，將有助於設計出抗癌效果比 resveratrol 更強的癌症化學治療劑或預防劑，並期望為保健食品開發成新一代的癌症化學預防藥劑的分子基礎做出一定的貢獻。

中文關鍵詞： 白藜蘆醇； 3,5,4‘-trimethoxystilbene (MR-3)； 上皮細胞-間質細胞轉換； Wnt/β-catenin； 肝糖合成激酶(GSK)-3β

英 文 摘 要 ： The molecular basis of epithelial-mesenchymal transition (EMT) functions as a potential therapeutic

target for breast cancer because EMT may endow breast tumor-initiating cells with stem-like characteristics and enable the dissemination of breast cancer cells. We have recently verified the antitumor activity of 3,5,4’-trimethoxystilbene (MR-3), a naturally methoxylated derivative of resveratrol, in colorectal cancer xenografts via an induction of apoptosis. The effect of MR-3 on EMT and the invasiveness of human MCF-7 breast adenocarcinoma cell line were also explored. We found that MR-3 significantly increased epithelial marker E-cadherin expression and triggered a cobblestone-like morphology of MCF-7 cells, while reciprocally decreasing the expression of mesenchymal markers, such as snail, slug, and vimentin. In parallel with EMT reversal, MR-3 downregulated the invasion and migration of MCF-7 cells. Exploring the action mechanism of MR-3 on the suppression of EMT and invasion indicates that MR-3 markedly reduced the expression and nuclear translocation of β-catenin, accompanied with the downregulation of β-catenin target genes and the increment of membrane-bound β-catenin. These results suggest the involvement of Wnt/β-catenin signaling in the MR-3-induced EMT reversion of MCF-7 cells. Notably, MR-3 restored glycogen synthase kinase-3β activity by inhibiting the phosphorylation of Akt, the event required for β-catenin destruction via a proteasome-mediated system. Overall, these findings indicate that the anti-invasive activity of MR-3 on MCF-7 cells may result from the suppression of EMT via down-regulating phosphatidylinositol 3-kinase (PI3K)/AKT signaling, and consequently, β-catenin nuclear translocation. These occurrences ultimately lead the blockage of EMT and the invasion of breast cancer cells.

英文關鍵詞： resveratrol； 3,5,4‘-trimethoxystilbene (MR-3)； epithelial-mesenchymal transition； Wnt/β-catenin； glycogen synthase kinase (GSK)-3β

附件一

# 科技部補助專題研究計畫成果報告

（□期中進度報告/☑期末報告）

白藜蘆醇 (resveratrol) 與其甲氧基化結構衍生物抑制癌細胞上皮細胞-間質細胞轉換與侵襲性之分子機制研究

Study on the mechanisms by which resveratrol and its methoxylated derivatives inhibitepithelial-mesenchymal transition and cancer cell invasion

計畫類別：☑個別型計畫　□整合型計畫

計畫編號：NSC 100-2320-B-040 -009 -MY3

執行期間： 100 年 8 月 1 日至 103 年 7 月 31 日

執行機構及系所：中山醫學大學生物醫學科學學系

計畫主持人：陳威仁

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碩士班研究生-兼任助理人員：蕭佩怡

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中　華　民　國 103 年 10 月 31 日

附件二

# 科技部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（簡要敘述成果是否有嚴重損及公共利益之發現）或其他有關價值等，作一綜合評估。

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☑ 達成目標

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☐ 因故實驗中斷

☐ 其他原因

說明：

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乳癌細胞的轉移是造成女性乳癌最主要的死亡原因，而近期的研究發現乳癌細胞侵襲力的獲得可能與乳癌細胞型態上的上皮細胞-間質細胞轉換 (epithelial-mesenchymal transition，EMT) 有極為密切的關聯，因為 EMT 可以賦予乳癌細胞呈現腫瘤起始細胞與癌症幹細胞的特性，進而促進乳癌細胞的擴散與轉移。因此，若能夠找出適當的癌症化學預防或治療試劑來預防乳癌細胞的轉移，或許是預防或治療女性乳癌的方法之一。本三年期計畫即以此為目標，研究白藜蘆醇 (resveratrol) 的甲氧基 (methoxy group) 取代結構類似物 3,5,4'-trimethoxystilbene (MR-3) 對抗乳癌細胞 EMT、轉移與侵襲性的能力。因為在我們近期的研究已指出 MR-3 能夠藉由誘導細胞凋亡，抑制大腸結腸癌細胞株 COLO 205 在小鼠中所形成異體移殖瘤；但 MR-3 是否能夠藉由抑制乳癌細胞的上皮細胞-間質細胞轉換，進而降低乳癌細胞的擴散與轉移則仍不得而知。因此我們以 MR-3 處理 MCF-7 人類乳癌細胞株，發現 MR-3 確實能夠增加 MCF-7 細胞中 epithelial-like marker E-cadherin 的表現，回復 cobblestone-like 細胞形態，並同時抑制 mesenchymal-like markers 如 snail，slug 與 vimentin 等之表現。此外，MR-3 逆轉 EMT 的同時，亦抑制了 MCF-7 細胞在 transwell assay 下之侵襲與轉移的能力。進一步探討 MR-3 逆轉 MCF-7 細胞 EMT 之作用機轉，發現 MR-3 會減少 β-catenin 在細胞核的堆積，降低 β-catenin 下游基因的表現，並同時增加 membrane-bound β-catenin。另一方面，MR-3 亦透過抑制 PI3K/Akt 的活化，來增加 GSK-3β 穩定性與活性，而 GSK-3β 已知會促成 β-catenin 經由 ubiquitin proteasome pathway 被分解。因此，我們推論 MR-3 可能是先藉由調節 PI3K/Akt/GSK-3β 訊號路徑的活性，進而抑制 β-catenin 進入細胞核與減少其下游基因的表現，來促使 MCF-7 細胞 EMT 的逆轉與降低其侵襲力。由此論文的研究成果，除瞭解 MR-3 對抗乳癌轉移與 EMT 之機制外，配合其低毒性和非致癌性等特性，將有助於評價 MR-3 作為乳癌治療劑與預防劑的可能性，甚至可以作為將來開發抗乳癌新藥的基礎。

# 目錄

**3,5,4'-Trimethoxystilbene, a natural methoxylated analogue of resveratrol, inhibits breast cancer cell invasiveness by downregulation of PI3K/Akt and Wnt/β-catenin signaling cascades and reversal of epithelial-mesenchymal transition**

# 科技部補助專題研究計畫成果報告

## 白藜蘆醇 (resveratrol) 與其甲氧基化結構衍生物抑制癌細胞上皮細胞-間質細胞轉換與侵襲性之分子機制研究

計畫編號：NSC 100-2320-B-040 -009 -MY3
執行期限：100 年 8 月 1 日至 103 年 7 月 31 日
主持人：陳威仁 中山醫學大學 生物醫學科學系
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**3,5,4'-Trimethoxystilbene, a natural methoxylated analogue of resveratrol, inhibits breast cancer cell invasiveness by downregulation of PI3K/Akt and Wnt/β-catenin signaling cascades and reversal of epithelial-mesenchymal transition**

### 1. 中文摘要

Resveratrol (白藜蘆醇) 為天然的抗氧化劑及自由基的清除者，是漿果類植物如藍莓、葡萄等中主要的芪類植物抗毒素 (stilbene phytoalexin)，在早期的小鼠表皮腫瘤發生模式實驗中即被證實具有預防癌症發生的能力。如今，多種 resveratrol 的甲氧基取代結構衍生物被開發，理由是因為甲氧基取代會增加 resveratrol 的 hydrophobicity，增強 resveratrol 的甲氧基取代結構衍生物被細胞吸收的能力，並加強其抗癌藥效。而癌細胞的轉移是造成癌症病人死亡的最主要原因，近期的研究發現癌細胞侵襲力的獲得可能與癌細胞型態上的上皮細胞-間質細胞轉換有極為密切的相關。此外，Wnt-β-catenin 訊號路徑的失調被認為是癌細胞上皮細胞-間質細胞轉換的主要機制之一。因此，抑制 Wnt-β-catenin 訊號路徑活化所造成的癌細胞上皮細胞-間質細胞轉換或許能夠降低癌症的轉移與致死率。

我們研究成果中發現，resveratrol 的甲氧基取代結構衍生物之一的 3,5,4'-trimethoxystilbene (MR-3)，較 resveratrol 更能在低濃度的情況下增加 MCF-7 乳癌細胞株上皮細胞型態標記 E-cadherin 的表現，並同時抑制間質細胞型態標記 Snail 與 uPA 之表現，顯示 MR-3 可能會抑制 MCF-7 乳癌細胞的上皮細胞-間質細胞轉換。另外，MR-3 也降低了 β-catenin 在細胞及細胞核內的表現，而 β-catenin 所調控的 *c-myc*、*cyclin D1* 基因表現量也同步下降，暗示 MR-3 可能是透過抑制 Wnt/β-catenin 訊號路徑的活化來減少 MCF-7 乳癌細胞株之上皮細胞-間質細胞轉換並降低其侵襲性表徵。進一步探索其分子作用機轉，發現 MR-3 會抑制 PI3K-Akt 訊號路徑的活化，降低 glycogen synthase kinase (GSK)-3β 被磷酸化而失活的機會，由此推測 MR-3 可能是藉由保留 GSK-3β 的活性，來抑制 Wnt/β-catenin 訊號路徑的活化並抑制 MCF-7 乳癌細胞株之上皮細胞-間質細胞轉換。故透過本實驗來瞭解 resveratrol 與其甲氧基取代結構衍生物對抗癌症轉移的機制，並配合其低毒性和非致癌性之特性，將有助於設計出抗癌效果比 resveratrol 更強的癌症化學治療劑或預防劑，並期望為保健食品開發成新一代的癌症化學預防藥劑的分子基礎做出一定的貢獻。

關鍵字: 白藜蘆醇; 3,5,4'-trimethoxystilbene (MR-3); 上皮細胞-間質細胞轉換; Wnt/β-catenin; 肝糖合成激酶 (GSK)-3β

## 2. 英文摘要

The molecular basis of epithelial-mesenchymal transition (EMT) functions as a potential therapeutic target for breast cancer because EMT may endow breast tumor-initiating cells with stem-like characteristics and enable the dissemination of breast cancer cells. We have recently verified the antitumor activity of 3,5,4’-trimethoxystilbene (MR-3), a naturally methoxylated derivative of resveratrol, in colorectal cancer xenografts via an induction of apoptosis. The effect of MR-3 on EMT and the invasiveness of human MCF-7 breast adenocarcinoma cell line were also explored. We found that MR-3 significantly increased epithelial marker E-cadherin expression and triggered a cobblestone-like morphology of MCF-7 cells, while reciprocally decreasing the expression of mesenchymal markers, such as snail, slug, and vimentin. In parallel with EMT reversal, MR-3 downregulated the invasion and migration of MCF-7 cells. Exploring the action mechanism of MR-3 on the suppression of EMT and invasion indicates that MR-3 markedly reduced the expression and nuclear translocation of β-catenin, accompanied with the downregulation of β-catenin target genes and the increment of membrane-bound β-catenin. These results suggest the involvement of Wnt/β-catenin signaling in the MR-3-induced EMT reversion of MCF-7 cells. Notably, MR-3 restored glycogen synthase kinase-3β activity by inhibiting the phosphorylation of Akt, the event required for β-catenin destruction via a proteasome-mediated system. Overall, these findings indicate that the anti-invasive activity of MR-3 on MCF-7 cells may result from the suppression of EMT via down-regulating phosphatidylinositol 3-kinase (PI3K)/AKT signaling, and consequently, β-catenin nuclear translocation. These occurrences ultimately lead the blockage of EMT and the invasion of breast cancer cells.

**KEYWORDS:** resveratrol; 3,5,4'-trimethoxystilbene (MR-3); epithelial-mesenchymal transition; Wnt/β-catenin; glycogen synthase kinase (GSK)-3β

## 3. 研究目的與背景介紹

Metastatic dissemination is conceived to be the late onset of breast cancer progression. However, an epidemiological study based on an analysis of more than12,000 breast cancer cases indicates that the initiation of metastasis may occur 5.8 years before the diagnosis of the primary tumor [1]. This study suggests that some types of precancerous cells may have the ability to invade and spread to distal tissues prior to the formation of large tumors. The candidates of such cells that have early metastatic potential may include circulating tumor cells (CTCs) found in the peripheral bloodstream or disseminated tumor cells (DTCs) located in the bone marrow. DTCs can be detected in about 31% of stage I to III breast cancer patients with micrometastasis and poor prognosis [2]. Recently validated by animal experiments, lung and bone *micrometastases* without the formation of primary breast cancer were monitored and accompanied by DTCs in wild-type mice transplanted with premalignant HER-2 transgenic glands [3, 4]. Thus, the emergence of CTCs and/or DTCs has been considered a diagnostic or prognostic index for evaluating malignant dissemination in patients with breast cancer.

Recent clinical evidence reveals that CTCs acquired from a subset of primary breast cancer patients exhibit mesenchymal and cancer stem cell-like characteristics [5, 6], suggesting that epithelial-mesenchymal transition (EMT) is likely to initiate cancer invasion and the metastatic spread of

primary breast tumor. EMT is a morphogenetic program involved in embryonic development, wound healing, and organ fibrosis. Executed by various types of epithelial carcinomas, EMT has been proposed to endow cancer cells with increased motility and invasiveness during tumor dissemination [7, 8] and acquisition of cancer stem cell-like characteristics [9]. A hallmark of epithelial tumor cells undergoing EMT is the decrease in the expression of E-cadherin, a cell adhesion receptor found in the basolateral membrane of polarized epithelia. E-cadherin connects the identical cell adhesion receptor to an adjacent cell, known as the adherens junction, in a calcium-dependent manner; it also plays a critical role in maintaining the integrity of the basal membrane and differentiated epithelial phenotype [10, 11]. The loss of E-cadherin expression and function has frequently been observed in breast cancer [12, 13], and its expression is often repressed by several EMT-related transcription factors, such as snail, slug, ZEB1, and ZEB2 [14-16]. In Asian females, aberrant E-cadherin expression is epigenetically silenced by promoter hypermethylation in invasive ductal breast cancer correlated with increased metastatic potential and poor prognosis [17]. E-cadherin gene mutation is also tightly associated with loss of heterozygosity at the long arm of chromosome16, which contains the E-cadherin locus in human lobular breast cancer [18, 19]; its dysfunction is customarily associated with the loss of differentiated function and an increase in invasive and mesenchymal phenotypes [20, 21]. These findings suggest that E-cadherin acts as an invasive suppressor with prognostic significance in breast cancer; the suppression of an EMT program may be an accessible tactic for the prevention of breast cancer cell invasion.

Resveratrol (3,5,4'-trihydroxystilbene; Fig. 1), a natural stilbene-based antioxidant present in various plants including grapes, peanuts, and berries [22], has been identified as a potential cancer chemopreventive agent [23]. The inhibitory role of resveratrol in EMT has recently been elucidated. Resveratrol suppresses epidermal growth factor (EGF)-induced EMT by inhibiting extracellular signal-regulated kinase (ERK)1/2 activation in human breast cancer cells [24]. Resveratrol also inhibits lipopolysaccharide (LPS)-triggered EMT in mouse melanoma, which is critical for inflammation-initiated metastasis [25]. Finally, the oral administration of resveratrol exerts repressive effects on xenografted tumor size and the EMT markers of head and neck cancer [26].

Although numerous studies have described the mechanisms by which resveratrol prevents cancer and have validated its significant anti-tumor effects [27], the systemic bioavailability of resveratrol remains very low (less than 1%) [28, 29]. Thus, to reduce the rapid biotransformation of resveratrol and improve its pharmacokinetics, researchers have developed natural and synthetic resveratrol analogues, in which free phenolic groups are protected by methoxylation. Given their structural similarity, the methoxylated derivatives of resveratrol possess anti-tumor effects that are as effective as those of resveratrol. For example, pterostilbene (trans-3,5-dimethoxy-4'-hydroxystilbene; Fig. 1), a natural 3,5-dimethoxyanalogue of resveratrol, exhibits a more efficient apoptotic ability than does resveratrol against leukemia cells [30]. Our previous study demonstrates that the natural resveratrol analogue, 3,5,4’-trimethoxystilbene (MR-3; Fig. 1), inhibits the growth of several human cancer cells and the xenograft tumor growth of colorectal cancer in SCID mice [31]. We have also recently reported that 3,5,3’,4’,5’-pentamethoxystilbene (MR-5; Fig. 1), a synthetically methoxylated analog of resveratrol, demonstrates a stronger growth-inhibitory effect than does resveratrol

against human breast carcinoma cell line MCF-7 through the induction of G1 cell cycle arrest [32].

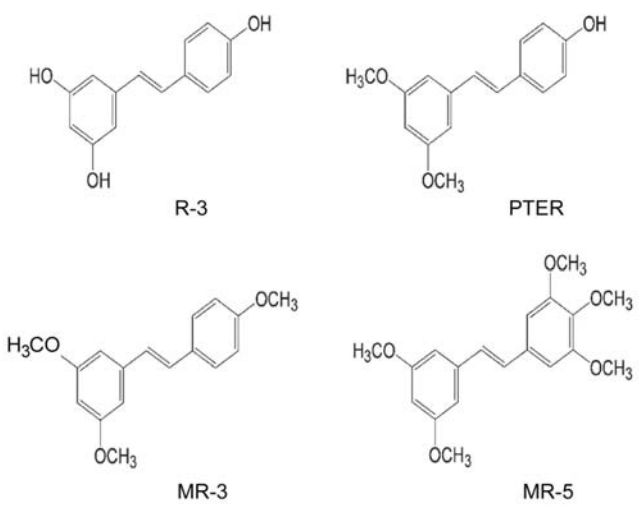


**Fig. 1.** Chemical structures of resveratrol and its methoxylated analogues.

Despite the progress made, however, the effects of these tested compounds on the EMT-resultant invasion of breast cancer carcinomas remain undefined. To address this issue, we initially assessed the expression profiles of EMT-related markers in MCF-7 cells after treatment with resveratrol or its methoxylated derivatives, pterostilbene, MR-3, and MR-5. We aim to evaluate which among the tested methoxylated analogues is more effective than resveratrol in inducing cadherin switching and EMT reversal; such inducement consequently blocks aggressive invasive phenotypes of breast carcinoma. To understand the mechanism of action of resveratrol, the upstream signaling pathway that executes the EMT program and subsequent invasion was explored in breast cancer cells exposed to the EMT-blocking methoxylated derivatives of resveratrol. Our results provide support for the potential use of methoxylated resveratrol analogue in preventing the EMT-induced malignant transformation of breast carcinoma.

## 4. 研究方法與材料

*Chemicals*

Resveratrol was synthesized using 4-methoxybenzyl alcohol and 3,5-dimethoxybenzaldehyde as precursors as described before [33]. A similar approach was used to synthesize its methoxylated derivatives including pterostilbene, MR-3 and MR-5. The purity of these compounds is > 97-99% by HPLC or GC. PI3K inhibitor LY294002 and GSK-3β inhibitor SB216763 and were obtained from Sigma (St. Louis, MO). β-Catenin siRNA were obtained from Cell Signaling Technology (New England Biolabs, Ipswich, MA). DAPI (4',6-diamidino-2-phenylindole) was obtained from Southern Biotech (Birmingham, AL).

*Cell culture and morphological observation*

Human MCF-7 breast carcinoma cells obtained from American Type Culture Collection were grown in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum (FCS) (Gibco BRL, Grand Island, NY), 100 units/mL of penicillin and 100 μg/mL of streptomycin, and kept at 37°C in a humidified atmosphere of 5% $CO_2$ in air according to ATCC recommendations. For all experiments, the cells were subjected to no more than 20 passages. Morphological changes of MCF-7 cells with MR-3 treatment were observed using Zeiss inverted phase contrast microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

*Transient transfection and dual-luciferase reporter assay*

The regulatory region of the E-cadherin gene was cloned by PCR amplification of genomic DNA and inserted into the HindIII/BglII sites of the pXP2 vector to generate the pXP2-E-cadherin (wt) construct. For promoter reporter assay, MCF-7 cells ($1 \times 10^6$) were plated onto 6-well plates and grown to 90% confluent and then co-transfected with pRL-CMV (containing the Renilla luciferase

reporter gene) and pXP2-E-cadherin using Lipofectamine TM2000 system (Invitrogen, Carlsbad, CA) for 6 h of incubation, and then the medium was replaced with 10% FCS-containing medium. After transfection, the MCF-7 cells were treated with resveratrol and its methoxylated analogues at the concentration of 20 μM. For luciferase activity, cells were lysed in lysis buffer (Promega, Madison, WI), and the firefly luciferase and Renilla luciferase activities were detected by dual luciferase assay kits (Promega, Madison, WI) .

*Western blotting*

After treatment with the tested agents as indicated in Figure legends, cells were lysed in a protein extraction buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 0.5 mM phenylmethanesulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min at 4°C. Total cellular proteins (50 μg) of each sample were resolved by SDS-polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore, Billerica, MA), and then incubated with primary antibody followed by secondary antibody conjugated with horseradish peroxidase. The immunocomplexes were visualized with enhanced chemiluminescence kits (Amersham, U.K.) and the density of the bands was quantified using the Fujifilm LAS-4000 luminescent image analyzer (Fujifilm UK Ltd, Bedford, UK). β-actin was used as an internal control for equivalent protein loading. The antibodies against Akt, phospho-Akt (Ser473), β-catenin, cyclin D1, phospho-β-catenin (Ser33/Ser37/Thr41), GSK-3β, phospho-GSK-3β (Ser9), c-Myc and snail were obtained from Cell Signaling Technology (New England Biolabs, Ipswich, MA). The antibodies against E-cadherin and vimentin were purchased from Millipore (Billerica, MA). The β-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Histone H1 antibody was from BioChain (BioChain Institute, Inc., Hayward, CA). The densities of the bands on the blots were quantitated with a computerized densitometer (ImageQuant LAS4000 Digital System) and analyzed by ImageQuant TL software.

*Wound healing assay*

A cell-based wound healing assay was performed following well-established methods [34]. Briefly, serum-starved MCF-7 cells were grown to 90% confluence and a linear wound was created in the confluent monolayer using a 200 μL micropipette tip. The cells were then washed with PBS to eliminate detached cells and diluted in serum-free DMEM. Then, various concentrations of MR-3 were added for 24 h and 48 h of incubation, and the wound edge movement was monitored with a microscope.

*In vitro migration and invasion assays*

Serum-starved MCF-7 cells ($2.5 \times 10^4$) treated with a variety of concentrations of MR-3 were seeded in the upper well of a transwell chamber (corning cat.no 3422) with a polycarbonate membrane containing 8-μm pores, and NIH-3T3 conditioned media were placed in the lower chamber as a source of chemoattractants. The migration was allowed to proceed for 48 h, followed by fixation with 4% paraformaldehyde for 30 min and staining with 1% crystal violet. The number of cells migrating the lower side of the filter was counted under microscopy in 10 random selective fields. The invasion assay was performed in similar experiments but with a coated invasion chamber that was coated with 100 μL of diluted Matrigel matrix (0.5 mg/mL) (BD Biosciences, Bedford, MA).

*Cell adhesion assay*

Cell adhesion assay was carried out by a colorimetric method based on staining cells with the dye crystal violet [35, 36]. Briefly, a 24-well culture

plate was coated with 10 μg/mL of type I collagen for 24 hours at 4°C, washed twice with PBS, and then plate were blocked with 1% BSA in DMEM for 1 hour. MCF-7 cells treated with various concentrations of MR-3 in DMEM containing 10% FCS were plated at a density of $1 \times 10^5$ cells per coated well, followed by 1 h incubation at 37°C. After washing unattached cells with PBS, adherent cells were fixed in ethanol, stained with 0.1% crystal violet, and then photographed under microscopy at 200×. The number of adherent cells was counted after being lysed in 0.2% Triton X-100, and the absorbance was measured at 550 nm.

*Cell viability assay*

MCF-7 cells were seeded at a density of $5 \times 10^3$ cells/mL into 96-well plates and grown overnight. Then the cells were treated with various concentrations of MR-3 for the indicated times. Control cells were treated with dimethyl sulfoxide (DMSO) to yield a final concentration of 0.05% (v/v). After incubation, the proliferating cell numbers were determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as follows: 20 μL of MTT solution (5 mg/mL, Sigma, St. Louis, MO) was added to each well and incubated for 24 h at 37 °C. Then the supernatant was aspirated, and the MTT-formazan crystals formed by metabolically viable cells were dissolved in 200 μL of DMSO. Finally, the absorbance was monitored by a microplate reader at a wavelength of 570 nm.

*Subcellular fractionation*

After a 24 h treatment with MR-3, MCF-7 cells were washed twice with cold phosphate buffered saline and resuspended in a hypotonic buffer (10 mM HEPES pH 7.6, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM PMSF, 1.5 mM $MgCl_2$, 1 mM PMSF, 1 μg/mL aprotinin and 1 μg/mL leupeptin) to prepare cytosolic and nuclear fraction. The resuspended cells were homogenized by sonication twice for 15 s each time. Cell lysates were subjected to ultracentrifugation at 3,000 × g for 5 min at 4°C and the supernatants containing cytosolic proteins were collected and stored at -20°C. The resultant pellet was then resuspended and lysed with hypertonic buffer containing 30 mM HEPES, 1.5 mM $MgCl_2$, 450 mM KCl, 0.3 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM PMSF, 1 μg/mL aprotinin and 1 μg/mL leupeptin. The supernatants containing nuclear proteins were obtained by centrifugation at 12,000 × g for 20 min at 4°C. Western blotting was used to detect the protein expression of β-catenin in equal volume of cytosolic or nuclear sample.

*Immunofluorescent staining*

MCF cells were plated on coverslips to 60% confluency and then exposed to the indicated concentrations of MR-3. After 24 h incubation, the cells were washed with cold PBS, fixed with 4% paraformaldehyde for 30 min at room temperature. Then the cells were immunolabeled with mouse anti-E-cadherin or anti-β-catenin antibody for 24 h, washed with cold PBS and incubated for 30 min with rabbit anti-mouse secondary antibody conjugated with fluorescein isothiocyanate and rhodamine (Sigma, St. Louis, MO) at room temperature. The cells were again washed with cold PBS and mounted on glass slides with Dapi-Fluoromount-G (Southern Biotech). The images were visualized with a fluorescence microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

*Reverse transcription-polymerase chain reaction (RT-PCR)*

Total RNA was isolated from MCF-7 cells using the TRIzol reagent (Life Technologies, Invitrogen Corporation, CA) according to the manufacturer's directions. Total RNA (5 μg) was

reverse-transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase and oligo (dT) 18 primer by incubating the reaction mixture (25 μL) at 40 °C for 90 min, and then the RT product was subjected to PCR analysis. PCR were performed in a final volume of 50 μL containing 2 μL of RT product, dNTPs (each at 200 μM), 1× reaction buffer, 1 μM concentration of each primer (c-Myc, forward 5'-CGGGTTCCCAAAGCAGAGGG-3', reverse 5'-GAAGCCCTGCCCTTCTCGAG-3'; cyclin D1, forward 5'-GCTGTCGGCGCAGTAGCAGC-3', reverse 5'-GCGCGGCGGATGGTTTCCAC-3'; GAPDH, forward 5'-GAAGGTGAAGGTCGGAGTC-3', reverse 5'-GAAGATGGTGATGGGATTTC-3'), and 50 units/mL Pro *Taq* DNA polymerase under the following conditions: 95°C for 5 min, followed by 30 cycles of amplification (95 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min) and 72 °C for 10 min. A 5 μL sample of each PCR product was electrophoresed into 2 % agarose gel and visualized by ethidium bromide staining. The mRNA expression levels of target gene of β-catenin were normalized to those of GAPDH. Values presented are the means ± S.E. of triplicate measurements.

*siRNA transfection*

MCF-7 cells were seeded in a 60 mm dish. When the cells reached confluence, the medium was replaced with serum-free Opti-MEM (Gibco BRL,Grand Island, NY). The cells were then transiently transfected with control siRNA and β-catenin siRNA (Cell Signaling Technology, Beverly, MA) for a final concentration of 100 nM per culture using Lipofectamine 2000 reagent in accordance with the instructions of the manufacturer's protocol (Life Technologies, Invitrogen Corporation, CA). The protein levels of β-catenin, E-cadherin and Snail were assessed 48 h post transfection using Western blotting.

*Immunoprecipitation*

Five-hundred microgram total cellular proteins were first precleared by being incubated with protein A/G-agarose beads (10 μL, 50% slurry, Santa Cruz Biotechnology, Santa Cruz, CA) in immunoprecipitation buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, , 1% Triton X-100, 0.5 mM phenylmethanesulfonyl fluoride, and 1 μg/mL leupeptin) for 4 h. The clarified supernatants were collected by microfugation, and then incubated with β-catenin for 4 h on a rotating platform at 4°C. The immunocomplexes were further precipitated with 20 μL of protein A/G-agarose beads on a rotating platform at 4°C. After incubation, the beads were washed twice with immunoprecipitation buffer and subjected to 10% SDS-PAGE. The immunoprecipitated proteins were visualized by Western blotting.

*Statistical analysis*

Quantitative data represent mean values with the respective standard error of the mean (SE) corresponding to 3 or more replicates. The significance between multiple groups and single control group was analyzed by one-way analysis of variance (ANOVA) using post-hoc test. Data were considered significantly different when $p < 0.05$.

## 5. 結果與討論

*MR-3 exerts reversion effect on EMT markers in MCF-7 human breast cancer cells*

To elucidate the role of resveratrol and its methoxylated analogues in the EMT program of breast carcinoma cells, we first examined the effect of the tested compounds (i.e., resveratrol, pterostilbene, MR-3, and MR-5) on the expression of EMT-representative markers in MCF-7 breast cancer cells by Western blot analysis. Fig. 2A shows that at a concentration of 20 μM, all the tested compounds resulted in an elevated expression of

epithelial marker E-cadherin, consequently repressing the expression of mesenchymal-related marker snail. However, only MR-3 had a markedly suppressive effect on the expression level of mesenchymal-related marker vimentin. Snail functions as a transcription repressor for E-cadherin expression by binding the E-box element to the *E-cadherin* gene promoter. To explore whether the downregulation of E-cadherin protein level by the tested compounds is associated with snail-mediated E-cadherin repression, dual-luciferase promoter assay was performed. Fig. 2B shows that unlike the three other compounds, MR-3 adequately restores E-cadherin promoter activity. The result reveals that MR-3, but not resveratrol, pterostilbene, and MR-5, exerted the opposite effect on the EMT profile in breast cancer cells. Significantly, MR-3 more effectively altered the EMT-related phenotypes than did resveratrol and other methoxylated compounds in MCF-7 cells. We therefore selected MR-3 as the lead compound for further study.

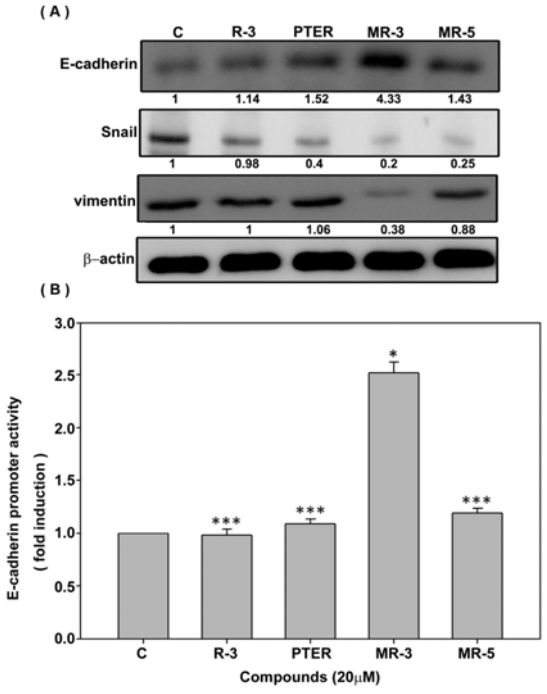


**Fig. 2.** Effects of resveratrol and its methoxylated derivatives on EMT profiles in human MCF-7 breast adenocarcinoma cells. (A) Western blot analysis of EMT-related markers, E-cadherin, snail, and vimentin, with β-actin functioning as an internal control in MCF-7 cells after 24 h of treatment with resveratrol and its methoxylated derivatives at a concentration of 20 μM. The relative protein levels below the blots were determined by densitometry analysis of the specific protein bands normalized to β-actin levels. All Western blot results are representative of at least three independent experiments. (B) After co-transfection with pRL-CMV and pXP2-E-cadherin in MCF-7 cells, the cells were treated with resveratrol and its methoxylated analogues at a concentration of 20 μM for 24 h. The promoter activity of E-cadherin was analyzed by luciferase reporter assays. Data were plotted by mean ± SE (n =3). The value of the control was set to 1. Asterisks represent statistically significant differences from the control group (*, $p < 0.05$; ***, $p < 0.001$).

*MR-3 causes an epithelial cobblestone morphological change from a fibroblast-like appearance in MCF-7 cells*

To eliminate the possibility that the suppression of the expression levels of mesenchymal-related markers snail and vimentin by MR-3 is due to cytotoxicity, cell viability was determined by MTT assay. Fig. 3A shows that a 24-h treatment of MCF-7 cells with MR-3 at low concentrations (0 μM to 20 μM) has no significant effect on MCF-7 cell survival despite the fact that a dose-response inhibition of cell growth was observed at high concentrations (40 μM to 100 μM) of MR-3 treatment. This result indicates that MR-3 decreases the snail and vimentin levels of MCF-7 cells via the induction of EMT conversion, rather than via growth inhibition.

To ascertain whether the reverse effect of MR-3 on EMT-related proteins is associated with epithelial-like reversal in MCF-7 cell morphology, we observed the morphological differences between untreated (control) and MR-3-treated cells. The

MCF-7 cells treated with MR-3 displayed a generally rounded shape and adhered to neighboring cells, whereas the control maintained a more fibroblast-like morphology with a loss of cell to cell contact (Fig. 3B, upper panel). Immunofluorescence staining shows that the expression of vimentin decreased in a dose-dependent manner in response to MR-3 (Fig. 3B, lower panel). Similar to the acquisition of epithelial morphological change and the reduction in the cellular distribution of vimentin, the exposure of MCF-7 cells to various concentrations (0 μM to 20 μM) of MR-3 resulted in a dose-dependent increase in E-cadherin expression, along with a gradual decrease in snail or slug expression (Fig. 3C). These findings provide strong evidence that MR-3 reverses EMT in MCF-7 cells.

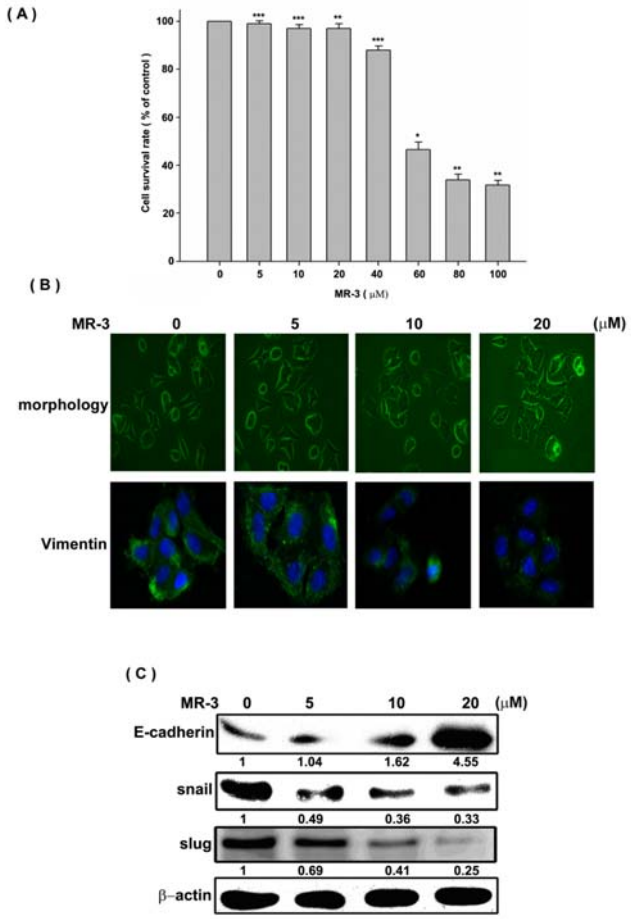


**Fig. 3.** Cell viability and reversal effects of MR-3 on EMT in cultured MCF-7 cells. (A) MCF-7 cells were treated with MR-3 at the indicated concentrations for 24 h, and then subjected to an MTT assay for cell viability. Each bar represents mean ± S.E (n =3). The asterisks represent statistically significant differences from the control group (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (B) The cells were treated with the indicated concentrations of MR-3 for 24 h. Morphological changes in response to MR-3 are displayed under an inverted phase contrast microscope. The immunofluorescence of indicated cells was labeled with Alexa Fluor 488 phalloidin (green) for vimentin filaments. Nuclei were stained with DAPI (blue). (C) Expression changes in EMT markers, including E-cadherin, snail, and slug were determined by Western blot analysis, with β-actin acting as internal control. The relative levels of protein expression normalized to β-actin are shown below the blots.

*MR-3 suppresses the aggressive invasion phenotype of MCF-7 cells*

Cancer cells undergoing EMT has been considered as an early event that leads to local invasiveness and distant metastasis. Thus, to validate whether EMT conversion by MR-3 is closely linked to the downregulation of MCF-7 cell invasion, we initially performed a scratch wound healing assay. This procedure enables the assessment of whether MR-3 and resveratrol affect cell motility. Fig. 4A illustrates that the stimulation of MCF-7 cells with 1% FCS produced a marked wound closure at the leading edge of the wound area, beginning at 24 h and lasting until 48 h. By contrast, the wounds treated with MR-3 exhibited significantly dose-related delays in healing under the same conditions. However, 20-μM resveratrol exerted a weak effect on wound closure. We then immediately determined the effects of MR-3 and resveratrol on the cell migratory and invasive capacities of MCF-7 cells by Transwell migration assay and Matrigel invasion assay. MR-3 caused a dose-dependent decrease in the percentage of Transwell migrated

cells, from ~78% to ~35% (Fig. 4B). For the assessment of invasion, the observed cells invading the site opposite the Matrigel-coated filter gradually decreased to a significant low percentage (~82% to ~40%) in response to MR-3-treatment under various concentrations. By contrast, 92% of the migrated cells and 87% of the invaded cells were observed in the 20-μM resveratrol-treated group. On the basis of these results, we suggest that MR-3 has a stronger anti-invasive activity than does resveratrol because of the ability of the former to reverse the EMT program.

Integrin-mediated adhesion to extracellular proteins, such as fibronectin and collagen, is a key mechanism by which mesenchymal cells acquire increased motility [37]. We propose that MR-3-induced EMT inhibition may affect cell adhesion to the extracellular matrix (ECM). Thus, cell-matrix adhesion assay was performed. The data show that MCF-7 cells adhering to the matrix decreased in a dose-dependent manner in response to MR-3 treatment (Fig. 4C). Collectively, the findings confirm that EMT-reversal mediated by MR-3 is closely associated with the downregulation of the aggressive invasion phenotype of MCF-7 cells.

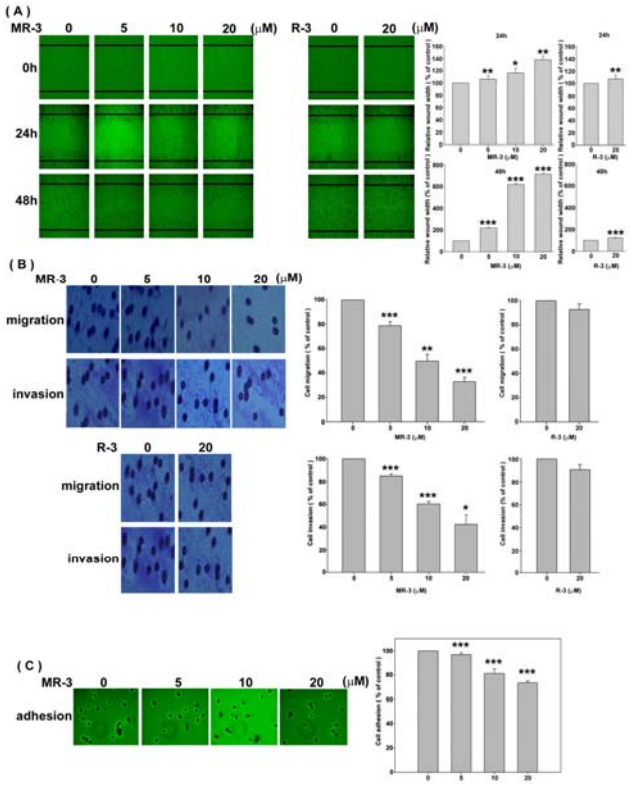


**Fig. 4.** Downregulation of the aggressive invasion phenotypes of MCF-7 cells by MR-3. (A) For the wound healing assay, 90% confluent cultures were scraped to form a wound, and then exposed to MR-3 and resveratrol at the indicated concentrations for 24 and 48 h. Photos of the wound were captured by microscopy. Relative distance of the wound width was measured and divided by the initial half-width of the wound. Results are expressed as the mean ± S.E. of three independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. the 0 h control (Fig. 4A, right panel). (B) For the migration and invasion assays, MCF-7 cells were seeded in a Transwell upper chamber and either coated with a diluted Matrigel (invasion assay) or not (migration assay). After attachment for 24 h, the cells were treated with various concentrations of MR-3 and 20 μM of resveratrol to allow migration and invasion for 48 h. Migrating and invading cells on the lower surface of the filter were stained and quantified under a microscope. (C) For the adhesion assay, MCF-7 cells pretreated with MR-3 (0, 5, 10, or 20 μM) for 24 h were plated on 24-well dishes coated with type I collagen (10 μg/mL), and cultured for 1 h. Adherent cells were stained and observed by microscopy. For quantification, the number of migrating, invasive, and adherent cells were counted in 10 randomly selected fields per well; each bar represents mean ± S.E (n =3). Asterisks represent statistically significant differences from the control group (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

*MR-3 restores membrane-bound β-catenin and suppresses the nuclear localization and function of β-catenin in MCF-7 cells*

Membrane-bound β-catenin linked to E-cadherin plays a crucial role in the maintenance of adherens junctions and functional epithelia. By contrast, free β-catenin functions as a transcriptional coactivator of the canonical Wnt signaling pathway, which contributes to EMT during tumorigenesis [38]. The dissociation of β-catenin from the cytoplasmic tail of E-cadherin may increase the nonjunctional pool of β-catenin in cytosol, thereby leading to epithelial cell migration [39]. Therefore, to elucidate whether the increase in E-cadherin in response to MR-3 in MCF-7 cells is accompanied by the reconstitution of the E-cadherin-β-catenin complex and the suppression of Wnt/β-catenin signaling, the expression, cellular distribution, and function of β-catenin were studied. An inhibition of β-catenin protein levels was observed in the MR-3-treated MCF-7 cells in a dose-dependent manner (Fig. 5A). Consistently, MR-3 reduced the nuclear translocation of β-catenin (Fig. 5B), parallel to the downregulation of mRNA and protein expression of β-catenin target genes, such as c-myc and cyclin D1 (Fig. 5C). In particular, the knockdown of β-catenin by specific siRNA converted the expression status of the EMT markers, including E-cadherin and snail, in MCF-7 cells compared with that of the scramble siRNA control (Fig. 5D). Subsequently, immunofluorescence staining was performed to determine the cell distribution of β-catenin and E-cadherin in MR-3-treated cells. The data show that MR-3 treatment enhanced the presence of membrane-bound β-catenin, as well as the upregulation of E-cadherin (Fig. 5E). Overall, these results indicate that MR-3 increases the β-catenin level in the plasma membrane and downregulates the free pool of nuclear β-catenin, suggesting that the modulation of Wnt/β-catenin signaling may be a possible course of action through which MR-3 may elicit the EMT reversal of MCF-7 cells.

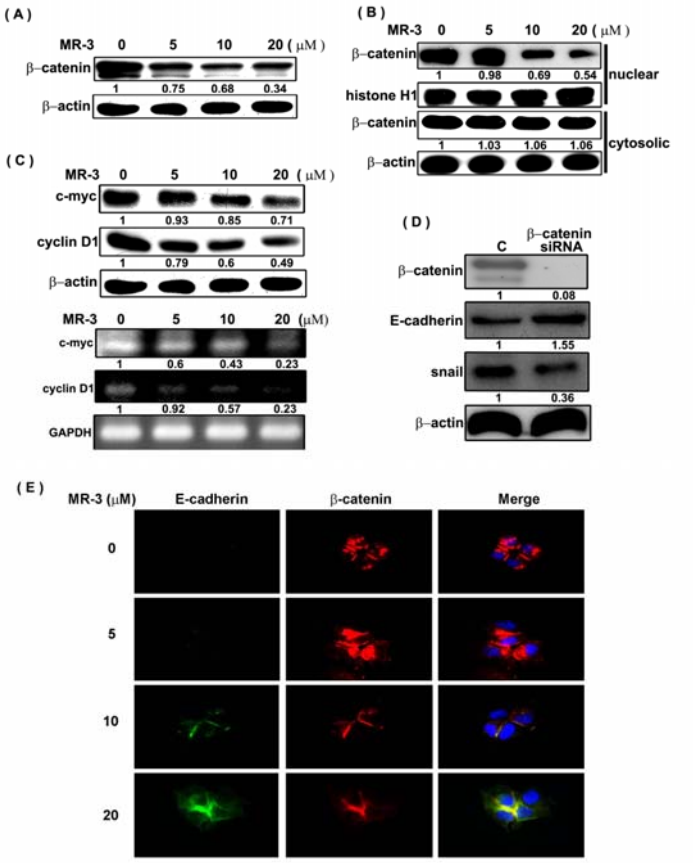


**Fig. 5.** Modulation of expression levels, cellular distribution, and function of β-catenin by MR-3 in MCF-7 cells. (A) Western blot was performed for β-catenin expression after 24 h of exposure to different concentrations of MR-3, as indicated. (B) Western blots for β-catenin in the nuclear and cytosolic fractions. The cells were treated with different concentrations of MR-3 for 24 h. Subsequently, cytosolic and nuclear fractions were prepared and analyzed by Western blotting. Histone H1 and β-actin were used as the internal control for the nuclear and cytosolic extracts, respectively. (C) Protein and mRNA expression levels of β-catenin/TCF-targeted genes, c-Myc and cyclin D1, in MCF-7 cells were determined by Western blotting and RT-PCR, respectively, after 24 h of treatment with the indicated concentrations of MR-3. (D) Effect of β-catenin knockdown on the protein expression of E-cadherin and snail. After transfection with β-catenin siRNA, Western blotting was performed to determine the protein expression levels of β-catenin, E-cadherin, and snail. (E) Immunostaining of β-catenin and E-cadherin. Cells

were cultured on a cover glass and then treated with the indicated concentrations of MR-3 for 24 h. Cells were also immunostained using specific antibodies against β-catenin and E-cadherin. In all the panels, the results provided are representative of at least three independent experiments. Quantification of the Western blot and RT-PCR results was normalized over β-actin, histone H1, or GAPDH levels.

*MR-3 elevates the phosphorylation and ubiquitination of β-catenin by employing the PI3K/Akt/GSK3β-dependent pathway*

An increase in the free cytoplasmic pool of β-catenin is necessary, albeit insufficient, for the induction of Wnt/β-catenin signaling and consequent EMT, unless GSK-3β (one of the components of the destruction complex responsible for β-catenin proteolytic degradation) is inhibited [40, 41]. To determine how MR-3 affects the nuclear import and transactivity of β-catenin, as well as the subsequent conversion of EMT phenotypes, the role of GSK-3β in an MR-3-triggered EMT reversal was explored. As shown in Fig. 6A, MR-3 markedly decreased the levels of GSK-3β phosphorylation, with a corresponding increase in its protein levels. Similarly, the phosphorylated status of Akt, an upstream kinase directly responsible for the phosphorylation and subsequent inhibition of GSK-3β, was blocked by MR-3 in a dose-dependent manner. Significantly, the phosphorylation and ubiquitination of β-catenin, which is necessary for its proteolytic degradation, was enhanced by MR-3 under the same conditions (Figs. 6A, 6B). These findings provide strong evidence that MR-3 adversely affects the nuclear translocation and transcriptional activity of β-catenin, as well as the main events of Wnt/β-catenin signaling activation by inhibiting PI3K/Akt signaling and liberating GSK-3β activity. To verify whether the activation of GSK-3β and the suppression of PI3K/Akt signaling can alter EMT phenotypes, snail and E-cadherin expressions were examined in response to treatment with GSK-3β inhibitor SB216763 and PI3K inhibitor LY294002, respectively. In contrast to the reversal of expression of EMT makers by MR-3, SB216763 enhances snail expression but reduces E-cadherin expression (Fig. 6C). Similar to MR-3 treatment, LP294002 treatment suppressed snail expression and elevated E-cadherin expression (Fig. 6D). Resveratrol inhibits the proliferation of estrogen-responsive MCF-7cells through by modulating the estrogen receptor alpha (ERα)-associated PI3K pathway [42]. Thus, to explore whether ERα is essential for the regulation of the PI3K-Akt-GSK3 signaling pathway and the subsequent EMT process, we selected Tamoxifen, an antagonist of ERα, to block ERα activity. Tamoxifen effectively inhibited PI3K/Akt signaling activation and liberated GSK-3β stability, similar to the decrease in β-catenin and snail protein levels, as well as the concomitant increase in E-cadherin protein levels (Fig. 6E, left and middle panels). Augmented efficacy of Tamoxifen can be observed in the altered expression of EMT-related markers when MCF-7 cells were co-treated with MR-3 (Fig. 6E, right panel). The results suggest that MR-3 reverses EMT program through the modulation of the ERα-PI3K-Akt pathway in estrogen-responsive breast cancer cells. Altogether, the findings highlight the hypothesis that the regulation in the PI3K-Akt-GSK3 signaling axis and the resultant β-catenin proteasome degradation should be involved in MR-3-induced EMT reversal.

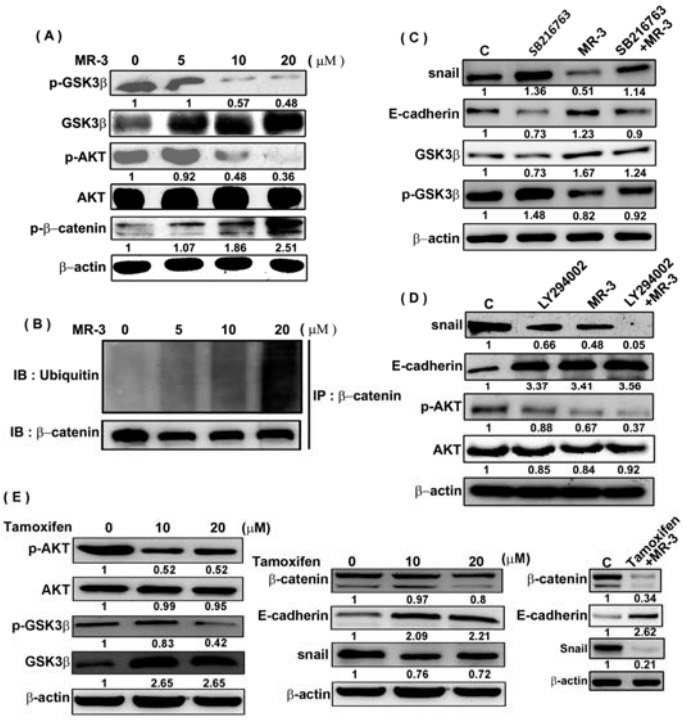


**Fig. 6.** Upstream role of the PI3K/Akt/GSK-3β signaling pathway in MR-3-mediated downregulation of GSK-3β-catenin stability. (A) MCF-7 cells were treated with various concentrations of MR-3. Subsequently, Western blot analysis was performed using specific antibodies against phospho-GSK-3β (Ser9), GSK-3β, phospho-AKT (Ser473), AKT, and phospho-β-catenin (Ser33/37/Thr41). (B) Ubiquitination of β-catenin in response to MR-3. Cell lysates treated with MR-3 were immunoprecipitated with agarose-conjugated β-catenin antibody. The resultant immunoprecipitated samples were monitored by Western blotting for the presence of ubiquitin. IP: immunoprecipitation; IB: Western blotting. (C) MCF-7 cells were treated with 20 μM of GSK-3β inhibitor SB-216763 or (D) 20 μM of PI3K/AKT inhibitor LY294002 as a single treatment or in combination with 20 μM of MR-3 for 24 h, followed by a Western blot analysis of the indicated proteins. (E) MCF-7 cells were treated with 10 and 20 μM of estrogen receptor antagonist Tamoxifen for 24h or with 20μM of Tamoxifen plus 20 μM of MR-3, followed by Western blot analysis of the indicated proteins. Relative GSK-3β, AKT, and β-catenin phosphorylation levels were determined through the densitometric measurement of the phospho-form bands normalized against the total bands, as shown below the blots. The relative protein levels of snail and E-cadherin were normalized to the β-actin level. Each value was calculated on the basis of the data obtained from three independent experiments.

In conclusion, our results constitute the first evidence that MR-3 reverses the EMT process and restores epithelial-like characteristics, such as the emergence of E-cadherin, the depolarization of the cell shape in MCF-7 breast cancer cells, and with the downregulation of Wnt/β-catenin signaling, as well as invasive and migratory phenotypes. Our explorations of the underlying mechanisms reveal that MR-3 may modulate the activation of PI3K/Akt signaling axis, consequently stabilizingGSK-3β activity. Stabilized GSK-3β activity indirectly maintains E-cadherin expression through the limitation of β-catenin nuclear localization and accelerated degradation of β-catenin and snail. The study results and data also reveal that MR-3 serves as the most potent inducer of EMT reversal among resveratrol and the methoxylated analogues in MCF-7 cells. The findings imply that methoxylation enhances the anti-invasive activity of resveratrol, thereby restoring basal-like phenotypes and attenuating the malignant potential and spread of breast cancer.

## 6. 致謝

This work was supported by grants from the National Science Council, Republic of China (NSC 97-2320-B-040-015-MY3 and NSC 100-2320-B-040-009-MY3). We are thankful for the Instrument Center of Chung Shan Medical University, which provides a fluorescence microscope supported by National Science Council,

Ministry of Education and Chung Shan Medical University.

## 7. 參考文獻


1. Engel, J., et al., *The process of metastasisation for breast cancer.* Eur J Cancer, 2003. **39**(12): p. 1794-806.
2. Braun, S., et al., *A pooled analysis of bone marrow micrometastasis in breast cancer.* N Engl J Med, 2005. **353**(8): p. 793-802.
3. Husemann, Y., et al., *Systemic spread is an early step in breast cancer.* Cancer Cell, 2008. **13**(1): p. 58-68.
4. Klein, C.A., *Cancer. The metastasis cascade.* Science, 2008. **321**(5897): p. 1785-7.
5. Barriere, G., et al., *Mesenchymal and stemness circulating tumor cells in early breast cancer diagnosis.* BMC Cancer, 2012. **12**(1): p. 114.
6. Kasimir-Bauer, S., et al., *Expression of stem cell and epithelial-mesenchymal transition markers in primary breast cancer patients with circulating tumor cells.* Breast Cancer Res, 2012. **14**(1): p. R15.
7. Kalluri, R. and R.A. Weinberg, *The basics of epithelial-mesenchymal transition.* J Clin Invest, 2009. **119**(6): p. 1420-8.
8. Acloque, H., et al., *Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease.* J Clin Invest, 2009. **119**(6): p. 1438-49.
9. Morel, A.P., et al., *Generation of breast cancer stem cells through epithelial-mesenchymal transition.* PLoS One, 2008. **3**(8): p. e2888.
10. Halbleib, J.M. and W.J. Nelson, *Cadherins in development: cell adhesion, sorting, and tissue morphogenesis.* Genes Dev, 2006. **20**(23): p. 3199-214.
11. Aberle, H., H. Schwartz, and R. Kemler, *Cadherin-catenin complex: protein interactions and their implications for cadherin function.* J Cell Biochem, 1996. **61**(4): p. 514-23.
12. Palacios, J., et al., *Anomalous expression of P-cadherin in breast carcinoma. Correlation with E-cadherin expression and pathological features.* Am J Pathol, 1995. **146**(3): p. 605-12.
13. Rasbridge, S.A., et al., *Epithelial (E-) and placental (P-) cadherin cell adhesion molecule expression in breast carcinoma.* J Pathol, 1993. **169**(2): p. 245-50.
14. Li, Y.M., et al., *Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis.* Cancer Cell, 2004. **6**(5): p. 459-69.
15. Batlle, E., et al., *The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells.* Nat Cell Biol, 2000. **2**(2): p. 84-9.
16. Bolos, V., et al., *The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors.* J Cell Sci, 2003. **116**(Pt 3): p. 499-511.
17. Hu, X.-C., W.T.Y. Loo, and L.W.C. Chow, *E-cadherin promoter methylation can regulate its expression in invasive ductal breast cancer tissue in Chinese woman.* Life Sciences, 2002. **71**(12): p. 1397-1404.
18. Huiping, C., et al., *Chromosome alterations and E-cadherin gene mutations in human lobular breast cancer.* Br J Cancer, 1999. **81**(7): p. 1103-10.
19. Cleton-Jansen, A.M., *E-cadherin and loss of heterozygosity at chromosome 16 in breast carcinogenesis: different genetic pathways in ductal and lobular breast cancer?* Breast Cancer Res, 2002. **4**(1): p. 5-8.
20. Vleminckx, K., et al., *Genetic manipulation*

*of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role.* Cell, 1991. **66**(1): p. 107-19.

21. Jeanes, A., C.J. Gottardi, and A.S. Yap, *Cadherins and cancer: how does cadherin dysfunction promote tumor progression?* Oncogene, 2008. **27**(55): p. 6920-9.
22. Burns, J., et al., *Plant foods and herbal sources of resveratrol.* J Agric Food Chem, 2002. **50**(11): p. 3337-40.
23. Jang, M., et al., *Cancer chemopreventive activity of resveratrol, a natural product derived from grapes.* Science, 1997. **275**(5297): p. 218-20.
24. Vergara, D., et al., *Resveratrol inhibits the epidermal growth factor-induced epithelial mesenchymal transition in MCF-7 cells.* Cancer Lett, 2011. **310**(1): p. 1-8.
25. Chen, M.C., et al., *Resveratrol inhibits LPS-induced epithelial-mesenchymal transition in mouse melanoma model.* Innate Immun, 2012.
26. Hu, F.W., et al., *Impairment of tumor-initiating stem-like property and reversal of epithelial-mesenchymal transdifferentiation in head and neck cancer by resveratrol treatment.* Mol Nutr Food Res, 2012. **56**(8): p. 1247-58.
27. Szekeres, T., et al., *Chemopreventive effects of resveratrol and resveratrol derivatives.* Ann N Y Acad Sci, 2011. **1215**: p. 89-95.
28. Walle, T., et al., *High absorption but very low bioavailability of oral resveratrol in humans.* Drug Metab Dispos, 2004. **32**(12): p. 1377-82.
29. Walle, T., *Bioavailability of resveratrol.* Ann N Y Acad Sci, 2011. **1215**: p. 9-15.
30. Tolomeo, M., et al., *Pterostilbene and 3'-hydroxypterostilbene are effective apoptosis-inducing agents in MDR and BCR-ABL-expressing leukemia cells.* Int J Biochem Cell Biol, 2005. **37**(8): p. 1709-26.
31. Pan, M.H., et al., *Antitumor activity of 3,5,4'-trimethoxystilbene in COLO 205 cells and xenografts in SCID mice.* Mol Carcinog, 2008. **47**(3): p. 184-96.
32. Pan, M.H., et al., *3,5,3',4',5'-pentamethoxystilbene (MR-5), a synthetically methoxylated analogue of resveratrol, inhibits growth and induces G1 cell cycle arrest of human breast carcinoma MCF-7 cells.* J Agric Food Chem, 2010. **58**(1): p. 226-34.
33. Lu, J., et al., *Resveratrol analog, 3,4,5,4'-tetrahydroxystilbene, differentially induces pro-apoptotic p53/Bax gene expression and inhibits the growth of transformed cells but not their normal counterparts.* Carcinogenesis, 2001. **22**(2): p. 321-8.
34. Valster, A., et al., *Cell migration and invasion assays.* Methods, 2005. **37**(2): p. 208-15.
35. Gillies, R.J., N. Didier, and M. Denton, *Determination of cell number in monolayer cultures.* Anal Biochem, 1986. **159**(1): p. 109-13.
36. Kueng, W., E. Silber, and U. Eppenberger, *Quantification of cells cultured on 96-well plates.* Anal Biochem, 1989. **182**(1): p. 16-9.
37. Huttenlocher, A. and A.R. Horwitz, *Integrins in cell migration.* Cold Spring Harb Perspect Biol, 2011. **3**(9): p. a005074.
38. Brabletz, T., et al., *Invasion and metastasis in colorectal cancer: epithelial-mesenchymal transition, mesenchymal-epithelial transition, stem cells and beta-catenin.* Cells Tissues Organs, 2005. **179**(1-2): p. 56-65.
39. Muller, T., et al., *Phosphorylation and free pool of beta-catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration.* J Biol Chem,

1999. **274**(15): p. 10173-83.
40. Onder, T.T., et al., *Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways.* Cancer Res, 2008. **68**(10): p. 3645-54.
41. Doble, B.W. and J.R. Woodgett, *Role of glycogen synthase kinase-3 in cell fate and epithelial-mesenchymal transitions.* Cells Tissues Organs, 2007. **185**(1-3): p. 73-84.
42. Pozo-Guisado, E., M.J. Lorenzo-Benayas, and P.M. Fernandez-Salguero, *Resveratrol modulates the phosphoinositide 3-kinase pathway through an estrogen receptor alpha-dependent mechanism: relevance in cell proliferation.* Int J Cancer, 2004. **109**(2): p. 167-73.

**8. 計畫成果自評**

(1) 本研究證明白藜蘆醇 (resveratrol) 的甲氧基 (methoxy group) 取代結構類似物 3,5,4’-trimethoxystilbene (MR-3) 能夠抑制乳癌細胞株 MCF-7 之 EMT、轉移與侵襲性。其機轉可能為 MR-3 藉由保留 GSK-3β 的活性，來抑制 Wnt/β-catenin 訊號路徑的活化並抑制 MCF-7 乳癌細胞株之上皮細胞-間質細胞轉換。

(2) 上述計畫成果將有助於了解乳癌細胞株是如何透過 EMT 轉換而增加乳癌細胞的移動性與侵襲力，藉此機轉可以開發出有效的標靶藥物，來抑制乳癌細胞的 EMT 轉換。而本實驗結果亦證明天然化合物 MR-3，具有抗乳癌細胞 EMT 轉換之能力，可以做為抗 EMT 轉換先導藥物之依據。

(3) 本計劃目前已達預定之成效，此一研究成果已投稿於知名 SCI 國際學術期刊 (Toxicology and Applied Pharmacology. 2013, vol. 272, pp. 746-756)，並由科技部(原國科會)計畫 (NSC 100-2320-B-040-009-MY3) 經費輔助支持。

# 科技部補助計畫衍生研發成果推廣資料表

日期:2014/10/30

| | |
|---|---|
| 科技部補助計畫 | 計畫名稱: 白藜蘆醇（resveratrol）與其甲氧基化結構衍生物抑制癌細胞上皮細胞-間質細胞轉換與侵襲性之分子機制研究 |
| | 計畫主持人: 陳威仁 |
| | 計畫編號: 100-2320-B-040-009-MY3　　學門領域: 保健營養 |

無研發成果推廣資料

# 100 年度專題研究計畫研究成果彙整表

| 計畫主持人：陳威仁 | 計畫編號：100-2320-B-040-009-MY3 |
|---|---|
| 計畫名稱：白藜蘆醇（resveratrol）與其甲氧基化結構衍生物抑制癌細胞上皮細胞-間質細胞轉換與侵襲性之分子機制研究 | |

| 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 | 1 | 1 | 100% | | 本年度共參加一場國內舉辦之研討會:<br>Tsai, J. H., Hsu, L. S. and Chen, W. J.* (2014). HMDB Induces G1 Cell Cycle Arrest and Autophagy in HeLa Cervical Cancer Cells. 2014 The 29th Joint Annual Conference of Biomedical Sciences. 第二十九屆生物醫學聯合學術年會. |
| | | 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 | 0 | 100% | 人次 | |
| | | 博士生 | 1 | 1 | 100% | | 本年度計畫有本校中山醫大生化所博士班研究生(兼任助理人員)蔡杰亨同學一位參與 |
| | | 博士後研究員 | 0 | 0 | 100% | | |
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| 國外 | 論文著作 | 期刊論文 | 1 | 1 | 100% | 篇 | 本計劃目前已達預定之成效，此一研究成果已投稿於知名 SCI 國際學術期刊(Toxicology and Applied Pharmacology. 2013, vol. 272, pp. 746-756)，並由科技部(原國科會)計畫 |

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|---|---|---|---|---|---|---|---|
| | | | | | | | 100-2320-B-040-009-MY3)經費輔助支持 |
| | | 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 | |
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| | 技術移轉 | 件數 | 0 | 0 | 100% | 件 | |
| | | 權利金 | 0 | 0 | 100% | 千元 | |
| | 參與計畫人力（外國籍） | 碩士生 | 0 | 0 | 100% | 人次 | |
| | | 博士生 | 0 | 0 | 100% | | |
| | | 博士後研究員 | 0 | 0 | 100% | | |
| | | 專任助理 | 0 | 0 | 100% | | |
| 其他成果(無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。) | | 無 | | | | | |

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

# 科技部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）

乳癌細胞的轉移是造成女性乳癌最主要的死亡原因，而近期的研究發現乳癌細胞侵襲力的獲得可能與乳癌細胞型態上的上皮細胞-間質細胞轉換 (epithelial-mesenchymal transition，EMT) 有極為密切的關聯，因為 EMT 可以賦予乳癌細胞呈現腫瘤起始細胞與癌症幹細胞的特性，進而促進乳癌細胞的擴散與轉移。因此，若能夠找出適當的癌症化學預防或治療試劑來預防乳癌細胞的轉移，或許是預防或治療女性乳癌的方法之一。本三年期計畫即以此為目標，研究白藜蘆醇 (resveratrol) 的甲氧基 (methoxy group) 取代結構類似物 3,5,4’-trimethoxystilbene (MR-3) 對抗乳癌細胞 EMT、轉移與侵襲性的能力。因為在我們近期的研究已指出 MR-3 能夠藉由誘導細胞凋亡，抑制大腸結腸癌細胞株 COLO 205 在小鼠中所形成異體移殖瘤；但 MR-3 是否能夠藉由抑制乳癌細胞的上皮細胞-間質細胞轉換，進而降低乳癌細胞的擴散與轉移則仍不得而知。因此我們以 MR-3 處理 MCF-7 人類乳癌細胞株，發現 MR-3 確實能夠增加 MCF-7 細胞中 epithelial-like marker E-cadherin 的表現，回復 cobblestone-like 細胞形態，並同時抑制 mesenchymal-like markers 如 snail，slug 與 vimentin 等之表現。此外，MR-3 逆轉 EMT 的同時，亦抑制了 MCF-7 細胞在 transwell assay 下之侵襲與轉移的能力。進一步探討 MR-3 逆轉 MCF-7

細胞 EMT 之作用機轉，發現 MR-3 會減少 β-catenin 在細胞核的堆積，降低 β-catenin 下游基因的表現，並同時增加 membrane-bound β-catenin。另一方面，MR-3 亦透過抑制 PI3K/Akt 的活化，來增加 GSK-3β 穩定性與活性，而 GSK-3β 已知會促成 β-catenin 經由 ubiquitin proteasome pathway 被分解。因此，我們推論 MR-3 可能是先藉由調節 PI3K/Akt/GSK-3β 訊號路徑的活性，進而抑制 β-catenin 進入細胞核與減少其下游基因的表現，來促使 MCF-7 細胞 EMT 的逆轉與降低其侵襲力。由此論文的研究成果，除瞭解 MR-3 對抗乳癌轉移與 EMT 之機制外，配合其低毒性和非致癌性等特性，將有助於評價 MR-3 作為乳癌治療劑與預防劑的可能性，甚至可以作為將來開發抗乳癌新藥的基礎。