科技部補助專題研究計畫成果報告
期末報告

插入素（integrin）、生長因子受體（EGFR）、Wnt 及特定 microRNA 對 n-3 和 n-6 多元不飽和脂肪酸調控乳癌細胞轉移機制之探討

計 畫 類 別 ：個別型計畫
計 畫 編 號 ：NSC 102-2313-B-040-001-
執 行 期 間 ：102 年 08 月 01 日至 103 年 07 月 31 日
執 行 單 位 ：中山醫學大學營養學系（所）

計畫主持人：李健群

計畫參與人員：此計畫無其他參與人員

處 理 方 式 ：
1.公開資訊：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢
2.「本研究」是否已有嚴重損及公共利益之發現：否
3.「本報告」是否建議提供政府單位施政參考：否

中 華 民 國 103 年 11 月 03 日

中 文 摘 要 ：

乳癌患者高致死率主要來自癌細胞的高轉移力。部分研究顯示 n-3、n-6 多不飽和脂肪酸雖具抗癌作用，但其對預後再復發的乳癌細胞轉移調控機制仍不清楚。研究證實，S100A4、細胞基質金屬結合蛋白酶-1/-9、尿激酶纖維蛋白溶酶原激活劑 (uPA)和 fascin-1 基因表現與早期癌細胞發生轉移有關。本研究將探討 n-3 PUFAs-DHA, EPA 和 n-6 PUFAs-arachidonic acid (AA), inoleic acid (LA)
對各種不同型的乳癌細胞，包括 Hs578T、MCF-7、即可大量表達 ErbB2 受體的 SKBR3 乳癌細胞基因之影響。綜合結果顯示，DHA 可透過負向調控 integrin 脈6β4-mediated Src and Akt 訊號路徑，抑制 S100A4 基因表現，而降低 Hs578T 癌細胞轉移。DHA 也可藉由抑制 EGFR 和 ErbB2 的表現與活化，降低 EGF 所誘發 SKBR3 乳癌細胞 MMP-1/-9 和 uPA 表現。此外，本研究顯示 DHA 可透過減少 microRNA-21(miR21)的表現及負向調控 PKC/Wnt-1/β-catenin 訊號路徑抑制 fascin-1 蛋白表現，降低 MCF-7 乳癌細胞的生長與轉移。

中文關鍵詞：

表皮生長因子、插入素、Wnt,、微 RNA、多元不飽和脂肪酸、乳癌、轉移

英 文 摘 要 ：

Metastasis is the leading cause of death from breast cancer. Previous studies showed
that polyunsaturated fatty acids (PUFAs) exhibit an anti-cancer effect, but the effect of n-3 and n-6 PUFAs on metastasis of breast cancer cells is not fully clarified. S100A4, a calcium-binding protein, matrix metalloproteinase-1 (MMP-1), MMP-9, urokinase plasminogen activator (uPA), and fascin-1 play crucial roles in tumor metastasis. We studied the anti-metastasis potential of n-3 PUFAs-DHA, EPA and n-6 PUFAs-arachidonic acid (AA), inoleic acid (LA) in Hs578T, MCF-7, and ErbB2-overexpressing SKBR3 breast cancer cells. We found that DHA inhibits cell migration and invasion at least in part via attenuation of activation of integrin 脈6β4-mediated Src and Akt and subsequently down-regulates S100A4 expression in Hs578T breast cancer cells. Suppression of EGFR and ErbB2 expression and their downstream signaling pathways are involved in DHA ‘s down-regulation of EGF-induced MMP-1/-9 and uPA expression in SKBR3 human breast cancer cells.

Moreover, attenuation of microRNA-21 and PKC/Wnt-1/β-catenin expression and their downstream signaling pathways are involved in DHA down-regulation of TPA-induced fascin-1 expression in MCF-7 human breast cancer cells.

英文關鍵詞： EGF, Integrin, Wnt, microRNA, PUFAs, Breast cancer, Metastasis

Introduction

Breast cancer is the most prevalent cancer among women worldwide, and it has remained a heterogeneous disease with tumor-specific characteristics and multiple clinical presentations. Metastasis is the most common cause of poor prognosis and worse survival rates in cancer patients. Clinically, therapy targeting the specific breast cancer subtype is recommended for effective treatment. In addition to estrogen receptor and progesterone receptor, ErbB receptor over-expression or elevated activity is particularly observed in malignant breast cancer. The ErbB receptor family consists of epidermal growth factor receptor (EGFR; ErbB1/HER1), ErbB2/HER2/neu, ErbB3/HER3, and ErbB4/HER4 receptors, all of which have an extracellular ligand-binding region with a single membrane-spanning region and a cytoplasmic tyrosine-kinase-containing domain. Previous study showed that EGF-induced ErbB2 phosphorylation at tyrosine residues stimulates various intracellular signaling pathways, including the mitogen-activated protein kinases (MAPKs) and phosphoinositide-3 kinase (PI3K)/Akt pathways, and plays an important role in the modulation of differentiation, apoptosis, and tumor metastasis.

Urokinase-type plasminogen activator (uPA) and MMP-9 have been recognized as biomarkers in the invasive stages of breast cancer. Clinical studies have shown that elevated uPA and MMP-9 levels are associated with tumor metastasis and poor prognosis in breast cancer patients.S100A4, a calcium-binding protein, has an established role in tumor metastasis. Several studies indicate that elevated S100A4 expression was involved in integrin $\alpha6\beta4$-mediated cell motility in many cancers, including breast cancer. A meta-analysis of immunohistochemical studies revealed that fascin, an actin-bundling protein, is associated consistently with increased risk of metastasis and mortality in gastric, colorectal, esophageal and breast carcinomas. Overexpression of fascin-1 significantly increased motility and metastasis, whereas knockdown of fascin-1 results in decreased cell growth and metastasis in a mouse xenograft model of prostate cancer as well as in oral squamous carcinoma cells. The Wnt gene encode a large family of 19 secreted protein ligands involved in modulation of cellular physiologic events, including steroidogenesis, proliferation and differentiation of tissue stem cells. Among members of Wnt gene family, Wnt-1 overexpression has shown to promote tumour progression in non-small cell lung cancer and hepatocellular carcinoma. Recent study indicated that Wnt-1 was detected predominantly in invasive breast carcinomas and the expression of Wnt-1 was required for epithelial-mesenchymal transition (EMT) of breast cancer cells.

Dietary n-3 and n-6 polyunsaturated fatty acids (PUFAs) are not only an important source of energy, but also an essential component for maintaining plasma membrane integrity and function. Among PUFAs, especially docosahexaenoic acid

(DHA), is known to have variety of health benefits in anti-angiogenesis, anti-thrombosis, and anti-tumorigenesis.

Material and Methods

Cell Culture

The human cell line SK-BR3 was cultured and maintained in DMEM (pH 7.2) supplemented with 10% FBS, 1.5 g/L $NaHCO_3$, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2 humidified incubator.

Fatty Acid Preparation

DHA samples were prepared and complexed with fatty acid–free BSA at a 3:1 molar ratio before addition to the culture medium. At the same time, 0.1% butylated hydroxytoluene and α-tocopheryl succinate were added to the culture medium to prevent lipid peroxidation.

Western Blotting

Cells were washed twice with cold phosphate-buffered saline (PBS) and were harvested in 200 μL of 20 mM potassium phosphate buffer. Cell homogenates were centrifuged for 30 min at 4℃. The protein content of the supernatant was measured by using the Coomassie Plus Protein Assay Reagent Kit (Pierce Chemical Company, Rockford, IL). Sodium dodecyl sulfate polyacrylamide gels made with 7.5% or 10% polyacrylamide. After electrophoresis, the separated proteins were transferred to polyvinylidene difluoride membranes. The nonspecific binding sites in the membranes were blockedwith 5% nonfat dry milk in 15 mM Tris–150 mM NaCl buffer (pH 7.4) at room temperature for 1 h. After blocking, the membrane was incubated with antibodies at 4℃ overnight. Thereafter, the membrane was incubated with the secondary peroxidase-conjugated anti-rabbit or anti-mouse IgG at 37℃ for 1 h, and the immunoreactive bands were developed by use of the Western Lightning™ Plus-ECL kit

RNA Isolation and Real-Time PCR

Total RNA was isolated from cancer cells by using TRIzol reagent according to the manufacturer's protocol. Amounts of 1 μg of total RNA were used to synthesize complementary DNA by Reverse Transcriptase. Real-time RT-PCR was performed on an ABI PRISM 7000 Sequence Detection System using the KAPATM SYBR FAST qPCR kit. The PCR reaction consisted of 5 μL of SYBR Green PCR Master Mix, 2 μM of forward and reverse primers, and 2 μL of 1:5-diluted template cDNA in a total volume of 10 μL. Cycling was performed by using the default conditions of the

ABI Prism 7000 SDS Software 1.0 as follows: 10 min at 95℃, followed by 40 rounds of 15 s at 95°C, and 1 min at 60°C. β-Actin was used as an internal standard gene and the threshold cycle (Ct) of a test sample to a control sample was used for relative quantification of target gene
expressions.

Migration and Invasion Assays

The migration assay was performed by using Transwell and the invasion assay was performed by using the BioCoat Matrigel Invasion Chamber (BD Biosciences, pore size, 8 μm) in 24-well dishes. Before performing the migration or invasion assay, 10^5 cells in 100 μL of serum-free medium were placed in the upper chamber, and 200 μL of medium without 10% FBS was added to the lower chamber. The plates were incubated at 37℃ in a 5% CO2 humidified incubator. After 2 h, the cells were then treated with various concentrations of DHA for 12 h with or without pharmacological inhibitors followed by incubation with EGF (40 ng/mL) for another 24 h. Cells were fixed in 1% paraformaldehyde for 10 min and were then washed with PBS two times. The cells were stained with 1% crystal violet in PBS for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the underside of the filters were examined and counted under a microscope. Migration and invasiveness were quantified by counting cells on the lower surface of the filter, and each experiment was repeated at least three times.

Gelatin Zymography and casein zymography Assay

The conditioned medium was collected and separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels containing 0.18% casein. After electrophoresis, the gels were soaked in 2.5% Triton X-100 in ddH2O twice for a total of 60 min at room temperature and then were incubated in substrate buffer (40 mM Tris-HCl, 10 mM CaCl2, 0.02% NaN3, and 1% Triton X-100, pH 8.0) at 37℃ for 18 h. Bands corresponding to uPA activity were visualized by negative staining using 0.3% Coomassie blue in 50% methanol and 10% acetic acid.

RNA Interference

For the ErbB2 knockdown experiment, a small interfering RNA (siRNA; 5′-GCAGUUACCAGUGCCAAUATT-3′) was tested in this study. A nontargeting control-pool siRNA was also tested and was used as the negative control. SK-BR3 cells were transiently transfected for 24 h with 10 nM ErbB2 siRNA by using DharmaFECT1 transfection reagent (Thermo) according to the manufacturer's instructions. After 24 h of transfection, cells were treated with EGF for another 24 h. Cell samples were collected for Western blotting.

Results

Fig.1. Effect of DHA on the cell viability of Hs578T cells

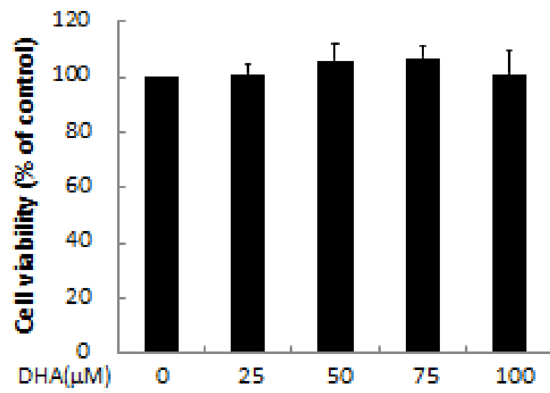


Fig.2. Effect of DHA on migration of Hs578T cells

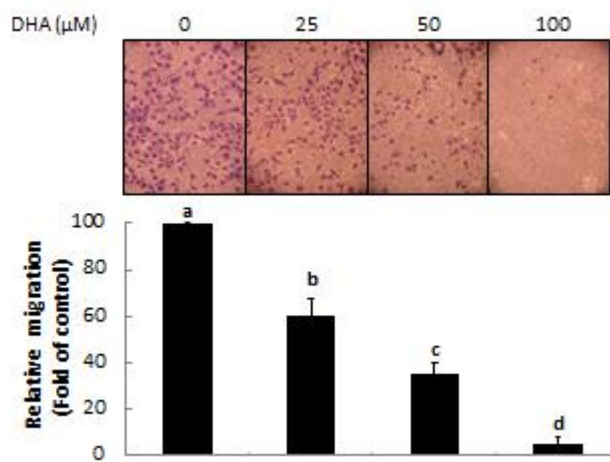


Fig.3. Effect of the DHA on mRNA of integrin β4 of Hs578T cells

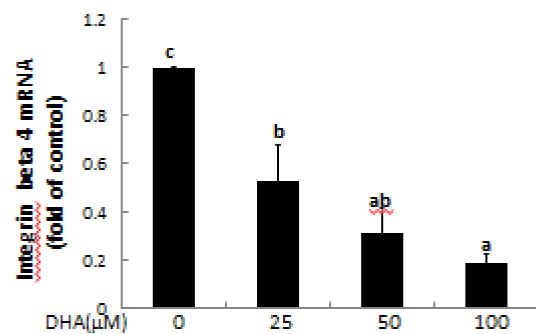
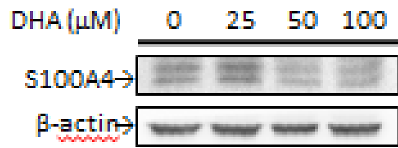


Fig.4. Effect of DHA on S100A4 protein expression in Hs578T cells



PART I conclusion:

These results suggest that DHA inhibits cell migration and invasion at least in part via attenuation of activation of integrin α6β4-mediated Src and Akt and subsequently down-regulates S100A4 expression in Hs578T breast cancer cells.

Fig.5. Effect of DHA on integrin α6β4, p-Src, Src, p-AKT (Ser473), and AKT protein expression

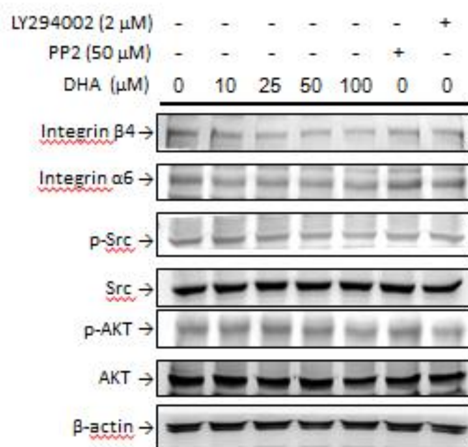


Fig. 6. DHA inhibit EGF-induced uPA and MMP-9 expression and enzyme activity in SK-BR3 cells

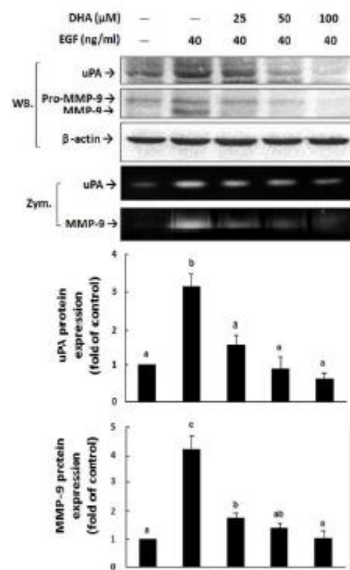


Fig. 7. DHA, AG1478 inhibit EGF-induced uPA and MMP-9 expression and enzyme activity in SK-BR3 cells

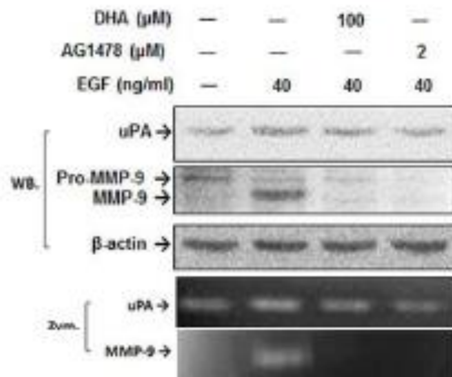


Fig. 8. ErbB2 siRNA inhibit EGF-induced uPA and MMP-9 expression in SK-BR3 cells.

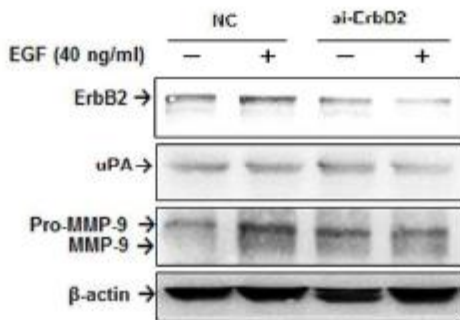


Fig. 9. Effect of DHA or AG1478 on EGF-induced MAPK and Akt activation

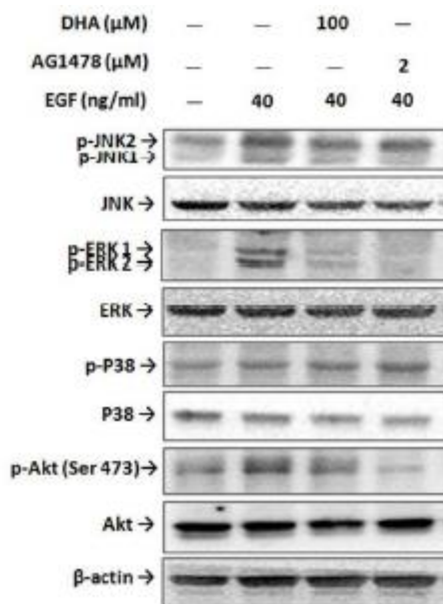
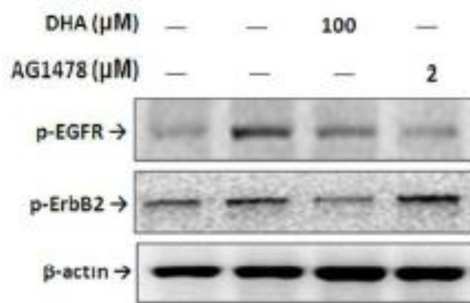


Fig. 10. Effect of DHA or AG1478 on EGF-induced EGFR and ErbB2 expression and activation



PART II conclusion:

DHA down-regulates EGF-induced uPA and MMP-9 gene expression, enzyme activity, cell migration, and invasion at least in part through the inhibition of the EGFR- and ErbB2-mediated JNK2, ERK1/2, and PI3K/Akt signaling.

Fig.11. TPA induces fascin-1 expression in MCF-7 cells.

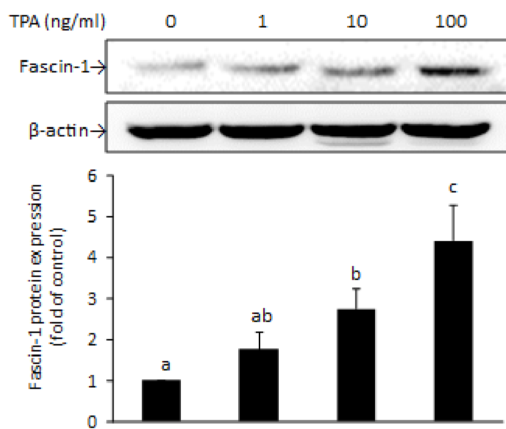


Fig.12. Fascin-1 siRNA abolished TPA-induced fascin-1 expression and cell migration.

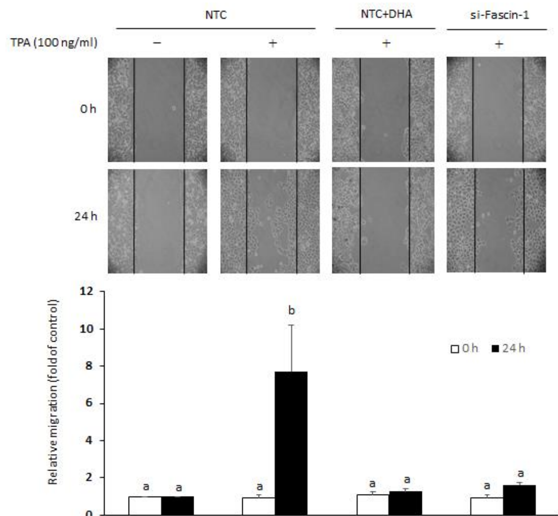


Fig.13. DHA inhibits TPA-induced β-catenin, STAT3α, and fascin-1 expression in MCF-7 cells

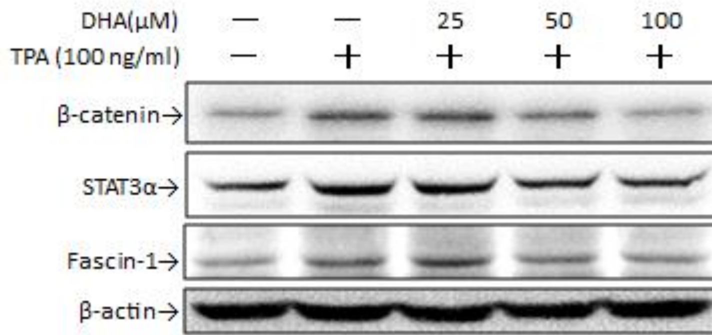


Fig.14. DHA inhibits TPA-induced the phosphorylation of STAT3α in MCF-7 cells

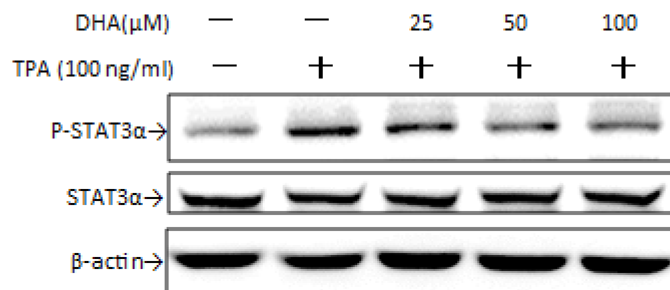
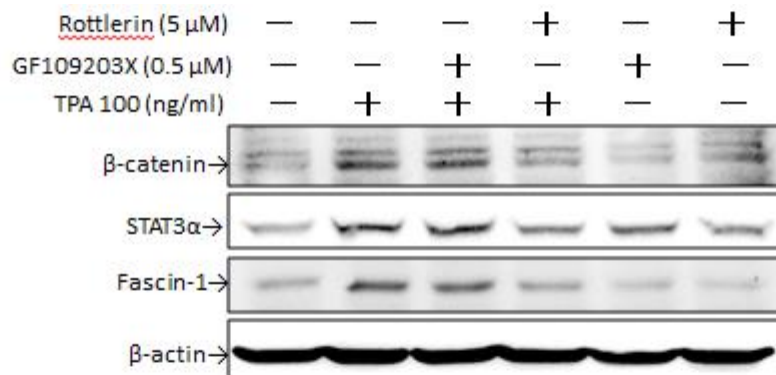


Fig.15. Effect of PKC inhibitors on TPA-induced β-catenin, STAT3α, and fascin-1 expression in MCF-7 cells



PART III conclusion:

DHA inhibits TPA-induced fascin-1 expression, cell migration, at least in part, via the suppression of the PKCδ/β-catenin/STAT3α signaling pathway as well as a reduction of STAT3α activation and DNA binding activity.

科技部補助計畫衍生研發成果推廣資料表

日期:2014/11/03

科技部補助計畫	計畫名稱: 插入素 (integrin)、生長因子受體 (EGFR)、Wnt 及特定 microRNA 對 n-3 和 n-6 多元不飽和脂肪酸調控乳癌細胞轉移機制之探討
	計畫主持人: 李健群
	計畫編號: 102-2313-B-040-001- 學門領域: 食品及農化

無研發成果推廣資料

102 年度專題研究計畫研究成果彙整表

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	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）

目前針對多不飽和脂肪酸調控乳癌細胞轉移之研究已撰寫完成兩篇文章，其中一篇已完成投稿，另一篇即將投稿。

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）

依實驗結果可知魚油中富含的 DHA 具有抑制乳癌細胞轉移之功效，其可能透過抑制 integrin、EGFR 蛋白的表現及降低磷酸化作用，而抑制與轉移相關基因如 uPA、MMP-9、S100A4 表現，降低乳癌細胞轉移。另一研究也證實，DHA 可透過負向調控 PKC/STAT3/beta-catenin 訊號路徑，抑制可促癌細胞轉移的 fascin-1 表現，進而降低癌細胞發生轉移。本研究結果建議 DHA 對於預防或降低乳癌患者術後再復發有益。