行政院國家科學委員會補助專題研究計畫

成果報告

台灣地區早發性乳癌之分子細胞學研究-台灣地區早發性乳癌在細胞凋亡調控基因之研究

計畫類別： 整合型計畫 （一年期計畫）

計畫編號： **NSC 91-2320-B-002-161**

執行期間： 91 年 8 月 1 日至 92 年 7 月 31 日

計畫主持人：林中天

共同主持人：張金堅、張繼堯、郭應誠

計畫參與人員：果伽蘭、研究生 李佳霖、林念儀

成果報告類型(依經費核定清單規定繳交)：精簡報告

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢

執行單位：**國立台灣大學獸醫學系**

中 華 民 國 92 年 8 月 4 日

行政院國家科學委員會專題研究計畫成果報告

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一、中文摘要

乳癌對人類是重要且常見的腫瘤，其產生的病因是複雜為多因子牽涉的結果。Secreted frizzled related proteins (sFRPs)是最近被發現與Wnt-Frizzled訊息傳遞傳導路徑的調節和細胞凋亡(apoptosis)的調節中扮演著雙重角色的蛋白質。我們實驗室最近發現 sFRP2 基因在人類與犬乳癌中有大量的表現及活化，但是在正常乳腺組織中則沒有表現。我們為了有系統地進一步研究隻乳癌中的 sFRP2 在功能上的角色與腫瘤分子生物學上的機制，擬定了下列的幾項研究策略。

這個計畫原預定包括了六個主要的部分，需要 3 年的研究時間，但最終僅獲核給一年計劃經費，在這一年主要的工作在於建立並分析臺灣地區早發性乳癌組織之 sFRP2 基因表現，並建立早發性乳癌的初代培養(primary culture)細胞株並且純化、分析乳腺上皮細胞。這一年我們已成功地分析早發性乳癌組織的 sFRP2 基因之表現。並建立及分析數個本地病例之早發性乳癌的初代培養細胞株。這些細胞利用下列技術分析其特性，包括反轉錄鏈聚合胺反應(RT-PCR)、原位雜交法(*in situ* hybridization)與免疫化學染色(immunohistochemistry)法偵測 sFRP2 的表現。結果發現sFRP2基因之mRNA及蛋白質在乳癌組織大量且顯著高比例之表現(86%mRNA表現陽性)，然而在正常乳腺組織則顯著較低比例之表現(43%mRNA表現陽性)。未來在下一個階段，sFRP2將被轉殖入含有GFP基因與CMV啟動子的哺乳類細胞表現載體，藉由 lipofection 方式將 GFP-sFRP2 穩定地轉染入(transfect) 早發性乳癌的初代培養細胞株，以進行更進一步的sFRP2功能分析。

本階段之研究結果，預期將提供重要之學術資訊，以了解 sFRP 基因族在人早發性乳癌

之表現情形。此外，此計劃也為未來下一階段進一步研究 sFRP 基因族不同成員之各種功能，及了解早發性乳癌複雜之病因，提供進一步研究分析之基礎。

關鍵詞：分泌性細胞凋亡基因，分泌性 frizzled 蛋白基因，基因表現，基因轉染，早發性乳癌

二、計畫英文摘要 (Abstract)

Breast cancers are one of the most important and common tumors in humans. The etiology of breast cancers is complex. The secreted frizzled related proteins (sFRPs) are newly identified proteins and implicated to have dual roles of modulation of Wnt-Frizzled signal transduction pathway and regulation of apoptosis. We have recently found that sFRP2 was expressed abundantly in human breast cancer (mostly late onset) and canine mammary gland tumors (MGT) tissues but was undetectable in normal mammary glands. To systematically investigate the functional roles and molecular mechanisms of sFRP2 in early onset breast cancers, several strategies are to be carried out as described below.

The project was intended to be comprised of six major parts for a period of 3 years. However, only one year of funding was granted. During this year, breast cancer tissues and primary cell cultures from native early onset breast cancer tissues have been established and purified for epithelial cells. We have successfully analyzed the sFRP2 expression in early onset breast cancer tissues. In addition, we have established and analyzed native primary early onset breast cancer cell lines from surgically excised breast cancer specimens. The cells are characterized for their cell origins, expression of sFRP2 by RT-PCR, *in situ* hybridization, and immunohistochemistry. Expression experiments revealed the sFRP2 was abundantly expressed with significantly a higher expression rate in early onset breast cancer tissues (86% mRNA positive), while much lower expression rate in normal breast tissues (43% mRNA positive). In the following step, human sFRP2 is cloned into a mammalian expression vector with GFP reporter gene and CMV promoter. The GFP-sFRP2 is stably transfected into primary early onset breast cancer cell lines by lipofection for further analysis from the next stage of the project although no funding is granted for the next year's study.

The results of this stage of the project should offer important scientific basis and information to understand the roles of sFRP gene family in early onset breast cancers. It also provides a basis for further analysis of functions of different members of the sFRP gene family and elucidation of the complex etiology of early onset breast cancers.

Keywords: secreted apoptosis related protein secreted frizzled related protein, apoptosis, gene expression, gene transfection, early onset breast cancer

二、緣由與目的

The frizzled and secreted frizzled related protein family is thought to modulate Wnt-Frizzled signal transduction pathway which plays an important role in normal development and oncogenesis, particularly in mammary neoplasia. More recently it has been reported that the sARP1 (also named sFRP2) possesses anti-apoptosis activity while sARP2 (also named sFRP1) induces pro-apoptosis in the one type of breast tumor cells. In our previous NSC project (NSC 90-2313-B-002-048), sFRP2 was found to be expressed abundantly in 31 different canine MGT tissues, but not expressed in normal MG tissues. This striking finding stimulated our interest in further investigation of the gene family. The roles of the gene family in tumor tissues remain to be determined.

Breast cancer patients in Taiwan are recently found to be younger than their counterpart in western countries. The etiology of early onset breast cancers in Taiwan has not been studied in details and it is thought to be different from that in late onset ones. According to the statistical data of female cancers in Taiwan by the Department of Health, Taiwan, the incidence of breast cancer is increasing significantly during recent years. In addition to the overall increasing incidence of breast cancer, a unique feature of the cancer in Taiwan is the higher incidence of early onset malignancy. According to a report of analyzing 2,397 breast cancer patients' data by the Department of Surgery, National Taiwan University Hospital during the past 10 years, early onset breast cancer (age < or = 35) composed of 9.18% of all breast cancer patients in this study (unpublished data). Besides, another study by Sun Yat-Sen Cancer Center in Taiwan also revealed that the early onset breast cancer (age < or = 40) composed of 29.3% of total 1,461 patients during the period between 1990 and 1997. The early onset breast cancer has a more aggressive cancer behavior and poorer prognosis than that in the older age group. The etiology and contributing factors of early onset breast cancers in Taiwan have not been determined yet. The characterization of the complex factors involved in early onset breast cancers in Taiwan is in desperate need. In this project, the roles of a group of newly identified apoptosis regulatory proteins, secreted frizzled related protein family (sFRPs), in early onset breast cancers in Taiwan are investigated.

We would like to understand whether the dual roles of secreted frizzled related genes-mediated apoptosis and Wnt-signaling pathway play a part in the pathogenesis of early onset breast cancers.

The purposes of the study at this stage include 1) to analyze sFRP2 expression in early onset breast cancer tissues; 2) to establish native primary early onset breast cancer cell lines from surgically excised specimens in Taiwan; 3) to analyze sFRP2 expression at mRNA and protein levels in early onset breast cancer cell lines.

三、結果與討論

Please be noted that the following is the

"summary" of all experimental results, not detailed data, due to space limit of this report.

1. mRNA extraction of early onset breast cancer tissue specimens

RNA extraction was performed in breast cancer tissues using Trizol reagent. Total RNA from was extracted. The $OD_{260/280}$ of the RNA was ranged between 1.5-1.8 and analyzed through formaldehyde denaturing gel electrophoresis indicating reasonably good purity for RNA-based work.

2. RT-PCR and Northern blotting hybridization analysis of sFRP2 in normal breast and early onset breast cancer tissues

RT-PCR and *in situ* hybridization showed sFRP2 was abundantly expressed in early onset breast cancer tissues with 86% of positive rate. However, only 43% of normal breasts expressed sFRP2. Following northern blotting hybridization, three mRNA species of approximately 1.5 kb, 2.2 kb, and 4.2 kb hybridized to the sFRP2 cDNA probe of which the 2.2 kb mRNA species was the major abundant transcript.

3. Establishment of primary cell cultures of early onset breast cancers from fresh tissue specimens

Freshly excised early onset breast cancers were rinsed by PBS and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with streptomycin (1μg/ml) and 10% fetal calf serum Primary cell culture and cell lines from breast cancer specimens were established by more than 90 to 100 passages of the breast cancer cells. The cell types were confirmed by morphology and immunostaining of cytokeratin.

4. RNA purification from early onset breast cancer and normal breast primary cell cultures

Human normal breast and early onset breast cancer cells were prepared for RNA extraction using Trizol reagent. Total RNA from was extracted. The $OD_{260/280}$ of the RNA was ranged between 1.5-1.8 and analyzed through formaldehyde denaturing gel electrophoresis indicating reasonably good purity for RNA-based work.

5. RT-PCR and in situ hybridization analysis of sFRP2 in normal breast and primary breast cancer cells

RT-PCR and *in situ* hybridization showed sFRP2 was abundantly expressed in primary breast cancer cells, but much less or no expression in normal breast cells.

6. Protein analysis of sFRP2 in primary breast cancer cell lines by immunohistochemistry

The primary culture of the breast cancer cell lines and normal breast cells was analyzed by immunohistochemical staining of sFRP2. Again, the sFRP2 protein was abundantly accumulated in the breast cancer cells, but much less or no expression in normal breast cells.

四、計畫成果自評

During this year, we have obtained initial important research data and progress. Firstly, we have analyzed the sFRP2 expression in early onset breast cancer tissues. Secondly, we have established and characterized primary early onset breast cancer cell lines from excised breast cancer specimens. Thirdly, the quality of extracted RNA from normal breast and breast cancer tissues and cells was good and sufficient for further RNA–based expression studies. Fourthly, RNA and protein expression of sFRP2 showed that the gene was highly expressed in the breast cancer tissues and primary cells, but much less or no expression in normal breast tissues and cells. It is an important finding of differential expression of sFRP2 between in normal breast and breast cancer tissues and cells.

In summary, the research results, produced during limited one year, provide a scientific basis of further elucidation of the relation between the pathogenesis of early onset breast cancers and sFRP gene family from the next stage.

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行政院國家科學委員會補助專題研究計畫

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實驗結果圖表部分

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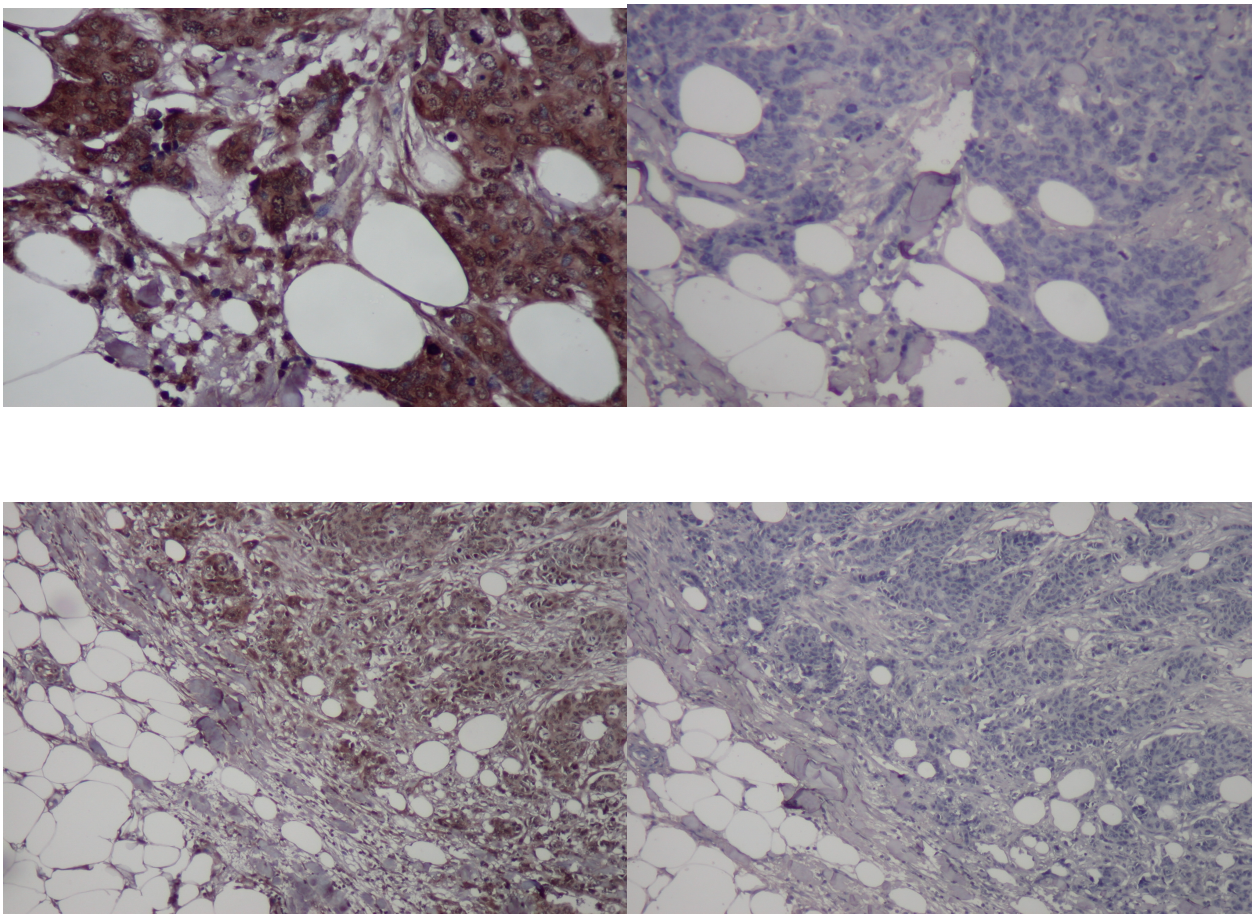
中 華 民 國 92 年 8 月 4 日

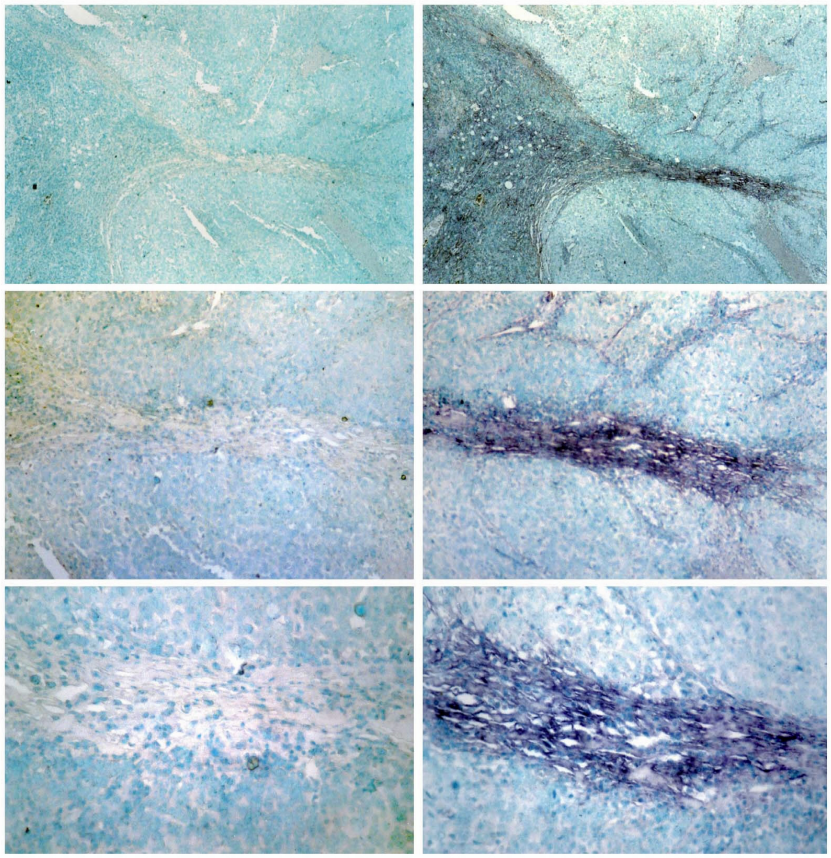
Immunohistochemical analysis of sFRP2 in breast cancer tissues



Left: malignant breast cancer expressed sFRP2

Right: negative control

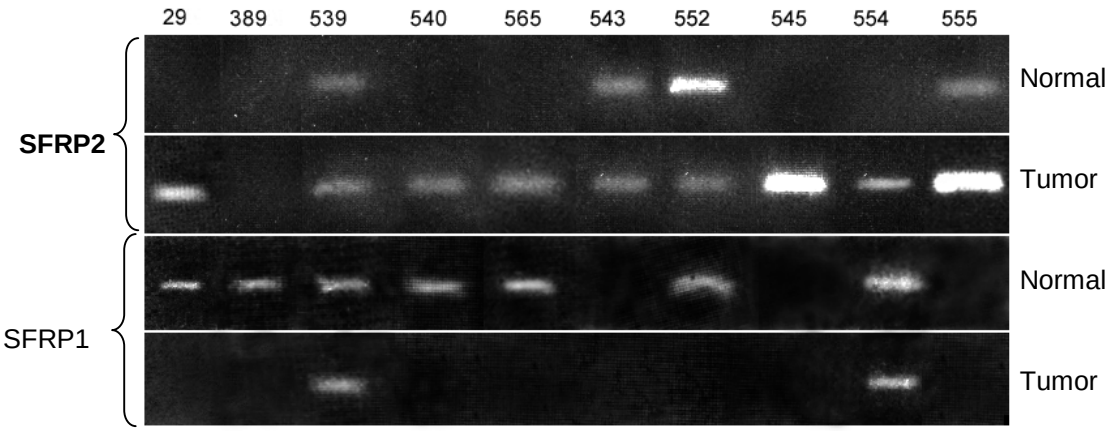
In situ hybridization analysis of sFRP2 in breast adenocarcinoma



Right: malignant breast cancer expressed sFRP2

Left: negative control

RT-PCR analysis of secreted frizzled related protein 2 (sFRP2 or ARP1) in breast cancers

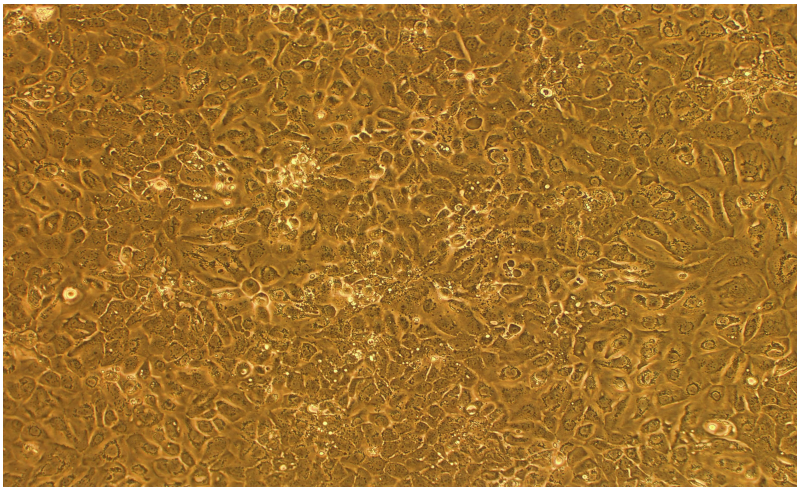


II. Summary of RT-PCR of sARP1/sFRP2 data in 88 specimens of breast cancers

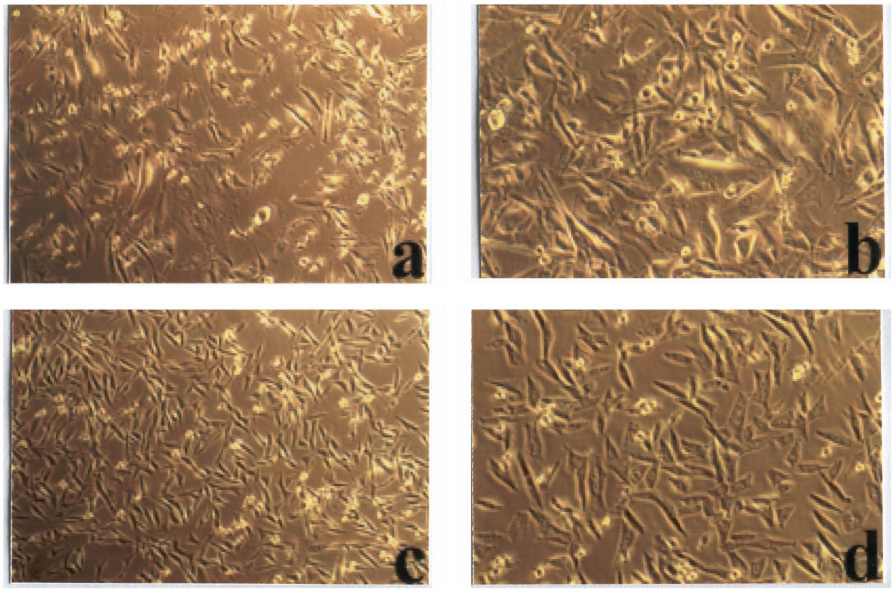
Gene	SARP1 (sFRP2)		SARP2 (sFRP1)	
Tissues	Breast cancers	Normal breast	Breast cancers	Normal breast
RNA expression	86% +	43% +	29% +	31% +

Establishment of cancer primary cultures from early onset breast cancer specimens

Primary cell line 1



Primary cell line 2



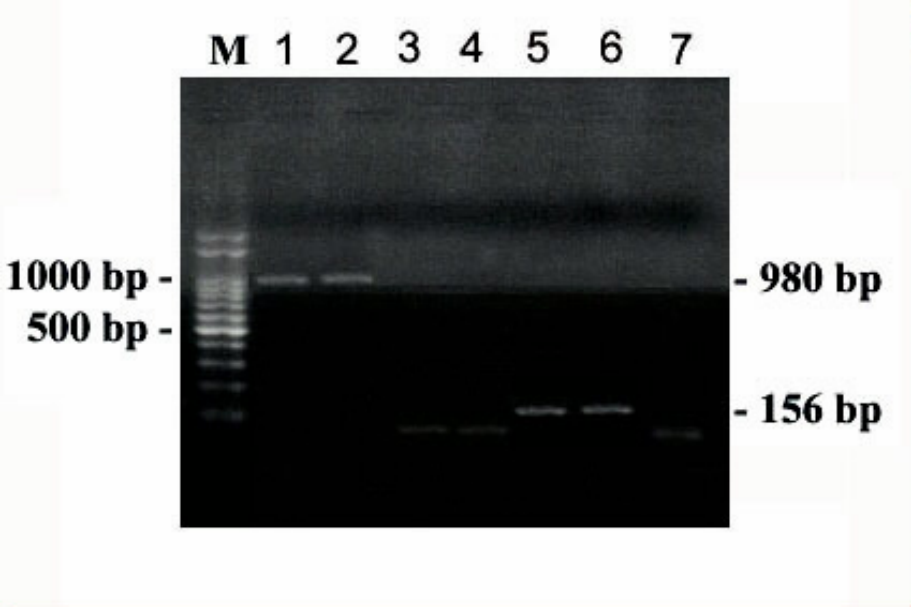
a: Mixed cell population , 100 X

b: Mixed cell population , 200 X

c: Cells following purification, 100 X

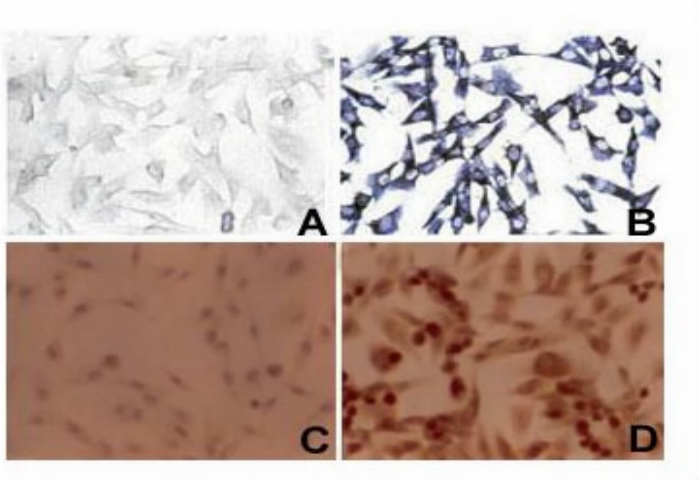
d: Cells following purification, 200 X

RT-PCR analysis of sFRP2 in primary cell cultures of breast cancers



RT-PCR of SFRP2. Transcripts of *SFRP2* were analyzed in breast tissues by RT-PCR. Lane 1, breast cancer tissue; Lane 2, primary cell culture from breast cancer; Lane 3, normal breast tissue; Lane 4, primary culture from normal breast; Lane 5-6, RNA integrity was confirmed by monitoring for β-actin mRNA of normal breast tissue and primary culture from normal breast; M, molecular weight markers; Lane 7, negative control. The expected sizes of *SFRP2* and β-actin PCR products are 980 bp and 156 bp, respectively.

***In situ* hybridization and immunohistochemical analysis of sFRP2 in primary cancer cell lines**



Expression of SFRP2 in primary breast cancer cells. (A, B) *In situ* hybridization of *SFRP2* in primary culture. The cells were hybridized with a digoxigenin-labelled SFRP2 sense (A) and antisense probe (B). (C, D) Immunohistochemical detection of SFRP2 in primary culture. (C) negative control. Original magnification: x200.