行政院國家科學委員會補助專題研究計畫成果報告

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犬視網膜細胞凋亡調控基因(sFRP 基因族)之選殖，及在犬正常組織及乳腺腫瘤細胞之表現及細胞凋亡變化之分析 (2/2)

Cloning, expression, and apoptosis analysis of novel retinal apoptosis regulatory genes (secreted frizzled related genes) in normal canine tissues and mammary tumors (2/2)

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執行期間：90 年 8 月 1 日至 91 年 8 月 20 日

計畫主持人：林中天 (email: ctlin@ccms.ntu.edu.tw)

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中 華 民 國 91 年 9 月 1 日

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一、中文摘要

分泌性細胞凋亡蛋白或稱分泌性 frizzled 蛋白(secreted frizzled related protein，sFRP)有調控 Wnt-Frizzled 信號傳遞途徑及細胞凋亡活動之雙重功能。Wnt-Frizzled 信號傳遞途徑在維持正常發育扮演十分重要之角色。此信號傳遞途徑之改變或異常，將導致腫瘤之發生。

本計劃分為三個部份:第一部分進行自外科切除之犬乳腺腫瘤組織之新細胞株建立，以作為 sFRP 基因轉染(gene transfection)之用。這一年我們已成功地建立數個本地病例之犬乳腺腫瘤細胞株。將一部份組織保存於 DMEM 組織培養液(加入 10% 胎牛血清及抗生素)，作為初代細胞培養之用。第二部份分析 sFRP 基因群在犬乳腺腫瘤細胞之 mRNA 及蛋白質表現方面，利用 northern blotting hybridization，RT-PCR，原位雜交法及免疫組織化學染色等分析技術。結果發現 sFRP2 基因之 mRNA 及蛋白質在犬乳腺腫瘤細胞有大量之表現，然而在犬正常乳腺細胞則無表現。

在計劃之第三部份，分析乳腺細胞之細胞凋亡現象，包括受 sFRP 轉染之犬乳腺腫瘤細胞、犬正常乳腺細胞及未受 sFRP 轉染之細胞，以了解在犬腫瘤細胞之細胞凋亡現象是否受到 sFRP 基因調控之影響而產生改變。利用 lipofection 方法把 sFRP 基因傳送(gene delivery)入乳腺細胞並給予紫外光誘發細胞凋亡，結果發現經 sFRP2 基因轉染之犬乳腺腫瘤細胞具有顯著抗紫外光造成之細胞凋亡現象，相較於未經 sFRP2 轉染之犬乳腺腫瘤細胞及犬正常乳腺細胞，sFRP2 基因在犬乳腺腫瘤細胞具有顯著抗細胞凋亡之功能。

本計劃之研究結果，預期將提供重要及創新之學術資訊，以了解 sFRP 基因族在犬乳腺腫瘤細胞之表現及調控細胞凋亡情形。此外，此計劃也為未來進一步研究 sFRP 基因族不同成員之各種功能，及了解犬乳腺腫瘤複雜之病因，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，細胞凋亡，Wnt-Frizzled 信號傳遞途徑，基因表現，基因轉殖，乳腺腫瘤

ABSTRACT

The secreted apoptosis related protein (also named secreted frizzled related protein, sFRP) family is implicated to have dual roles of modulation of Wnt-Frizzled signal transduction pathway and regulation of apoptosis. The Wnt-Frizzled signaling plays an important role in normal development and oncogenesis, particularly in mammary neoplasia. Therefore, members of the sFRP gene family are good candidates to investigate their roles in apoptosis regulation and mammary tumors of canine species.

The project is comprised of three major parts: at the first stage, primary canine mammary gland tumor (MGT) cell lines were established from freshly excised MGT specimens. An aliquot of tumor tissues was rinsed by sterile PBS and kept in DMEM medium supplemented with 10% fetal calf serum for primary cultures. We have successfully established more native primary MGT cell lines from surgically excised MGT specimens.

At the second stage, the primary MGT cell lines were analyzed for mRNA and protein expression of sFRPs. RNA was extracted from MGT cells. extraction. Expression analysis included mRNA *in situ* hybridization, immunohistochemistry, RT-PCR, and northern blotting hybridization. Expression revealed the sFRP2 was abundantly expressed in canine MGT cell lines, but not expressed in normal canine MG cells nor human breast cancer cell line MCF7.

At the third stage, primary MGT cell lines have been prepared for gene transfection. sFRP genes were transfected into MGT cells using a mammalian expression vector by lipofection and UV-induced apoptosis (25 J/m2) was performed. The apoptosis activity was investigated in sFRP-transfected MGT and MG cells, untransfected MGT and MG cells, and human breast cancer cell line MCF7, respectively. Results showed the sFRP2 transfected MGT cells possessed marked anti-apoptotic activity, compared to cells from untransfected MGT cells, and normal MG cells.

The results of the project should offer important and novel scientific information to understand the roles of sFRP gene family in apoptosis control of canine normal and neoplastic cells. 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 canine model of mammary tumors.

Keywords: secreted apoptosis related protein secreted frizzled related protein, apoptosis, Wnt-Frizzled signal transduction pathway, gene expression, gene transfection, mammary neoplasia

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 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.

Canine mammary gland tumor (MGT) is the canine counterpart of human breast cancer that shares significant similarities in several aspects. MGT is the most common tumor type in female dogs comprising of 52% of all neoplasms in the bitches. However, the etiology of MGT is mostly unknown and surprisingly very few advanced molecular studies regarding MGTs have been done to date. 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 canine MGT.

The purposes of the study are 1) to establish native primary MGT cell lines from surgically excised MGT specimens at our hospital; 2) to analyze sFRP2 expression at mRNA and protein levels in canine MGT cell lines by *in situ* hybridization, northern blotting, and immunohistochemistry; 3) transfection of sFRP2 into primary canine MGT cells, normal MG cells, and human breast cancer cell line MCF7; 4) to investigate the effects of sFRPs gene expression on apoptosis activity of normal MG and MGT cells following sFRP gene transfection.

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. Establishment of canine MGT primary cell lines from excised MGT specimens

Freshly excised MGT tumors 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 canine MGT specimens were established by more than 90 to 100 passages of the MGT cells. The cell types were confirmed by morphology and immunostaining of cytokeratin.

2. RNA extraction from canine normal MG and MGT cells

Canine normal MG and MGT 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.

3. mRNA expression analysis of sFRP2 in

normal MG and primary MGT cells

RT-PCR and *in situ* hybridization showed sFRP2 was abundantly expressed in primary MGT cells, but not in normal MG cells. Following northern blotting hybridization, **t**hree 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. sFRP2 gene in the dog was abundantly expressed only in the MGT cells, not in normal MG cells nor human breast cancer cell MCF7.

4. Immunohistochemical analysis of sFRP2 in primary MGT cell lines and normal MG cells

The primary culture of the MGT cell lines and normal MG cells was analyzed by immunohistochemical staining of sFRP2. Again, the sFRP2 protein was abundantly accumulated in the MGT cells, not in normal MG cells nor human breast cancer cell MCF7.

5. Transfection of sFRP2 into primary MGT cells and normal MG cells

Cells was transfected with the pcDNA4 mammalian expression vector (Invitrogen) containing no insert (mock), and canine sFRP2 cDNA, by using Lipofectamine reagent (Gibco) according to manufacturer's protocol.

6. Apoptosis analysis of sFRP2-transfected cells and untransfected MGT/MG cells

Apoptosis analysis was performed in sFRP2-transfected cells and untransfected cells before and after UV light treatment (25J/m2) for 2, 4, 6, 8 minutes, respectively. The methods of apoptosis analysis included TUNEL assay (Tdt-mediated dUTP nick end labeling) and DNA laddering analysis. TUNEL assay was performed using *in situ* cell death detection kit (Boehringer Mannheim). The apoptosis analysis indicated that the apoptosis level following UV treatment was markedly reduced in sFRP2-transfectd MGT cells (less than 5%), compared to cells from untransfected MGT (30%) and normal MG cells.

四、計畫成果自評

During the second year of this 2-year project, we have obtained significant and important research data and progress. Firstly, we have established more primary cell lines from excised canine MGT specimens.

Secondly, the quality of extracted RNA from canine normal MG and MGT cells was good and sufficient for further RNA–based expression studies. Thirdly, RNA and protein expression of sFRP2 showed that the gene was highly expressed in the MGT cells, but not in normal MG cells. It is an important finding of differential expression of sFRP2 between in canine normal cells and neoplastic cells.

Fourthly, we have successfully established an efficient apoptosis induction protocol by UV treatment. Besides, we further prove and characterize the apoptosis modulation property of the sFRP2 gene in MGT cell lines. It is the first finding of sFRP2 regulated apoptosis in canine MGT.

The research results will be helpful to elucidate the relation between the pathogenesis of canine MGT, sFRP gene family, and apoptosis activity. The results of this project obtained to date have been prepared for publication on the journal "*Experimental Cell Research*".

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