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人類上皮膚脂脂肪酸結合蛋白基因與肺癌細胞轉移能力之相關
性研究

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人類上脂脂肪酸結合蛋白基因與肺癌細胞轉移能力之相關性研究

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計畫中文摘要

癌細胞之轉移為癌症病患的主要死因之一，發現癌細胞轉移相關基因仍為目前癌症研究的一大課題。在先前的研究中，利用微陣列及肺癌轉移模式細胞株 (CL₁ 系列)，已經能成功地篩選出許多與癌轉移相關的基因，其中部份已定性出為新的癌轉移相關基因，甚至發現其具臨床轉移上的相關性。本研究計畫所要研究的標的為人類上脂脂肪酸結合蛋白基因 FABP5。經由微陣列篩選檢驗，發現此轉譯此蛋白質之基因的 mRNA 在肺癌腫瘤細胞株的表現，轉移能力較低的細胞株表現較少，並隨著在轉移能力的增強而有逐步且明顯的增加。另由聚合酶鏈反應之結果，亦顯示此基因之表現與肺癌腫瘤細胞株系列之轉移能力可能有正相關性。本研究計畫中，我們由轉移能力較強的人類肺癌細胞株 CL₁₋₅ 中選殖 FABP5 基因並導入較不具轉移能力的細胞株 CL₁₋₀ 中以增加其 FABP5 的表現量，藉以產生穩定的選殖細胞株，並以此細胞株進行人體外之細胞侵襲能力檢定、細胞生長能力鑑定以及轉移能力檢定，以分析此基因在癌轉移促進的能力。我們預期在較不具轉移能力的細胞株 CL₁₋₀ 中過度增加其 FABP5 的表現量會導致細胞侵襲能力與轉移能力的增加。

關鍵詞：癌細胞轉移，微陣列，人類上脂脂肪酸結合蛋白

計畫英文摘要

Metastasis remains the major cause of death from cancer. It may be of importance that discovering novel genes may elucidate the detailed mechanism of cancer metastasis. In the previous studies, by using cDNA microarray, we have identified several invasion/ metastasis-related genes from established lung cancer cell model of metastasis and some them have been considered as novel genes in invasion/metastasis and correlated closely with clinical metastasis. The target of this study is one of the potential metastasis-related genes, human epidermal fatty acid-binding protein (E-FABP) gene FABP5. The results from microarray confirmed that FABP5 mRNA expression was in a lower level in less metastatic cell line such as CL₁₋₀ and was greatly increased in the highly metastatic cell lines such as CL₁₋₅ and CL_{1-5-F4}. Reverse-transcriptase polymerase chain reaction (RT-PCR) also showed that the expression of FABP5 mRNA increase was correlated with the metastatic ability of human lung cancer cell lines. In this proposed study, we performed molecular cloning of the 437-base paired, full length cDNA of FABP5 from highly metastatic CL₁₋₅ cell line onto a mammalian expression plasmid vector, which was then be transfected into a less metastatic human lung adenocarcinoma cell line, CL₁₋₀. The cell line was analyzed the metastasis-promoting function of FABP5 by in vitro invasion assay,

cell growth assay, and in vitro metastasis assay. We expect that overexpression of FABP5 in the less metastatic human lung adenocarcinoma cell line will enhance their activity of invasiveness and metastasis.

Keywords: metastasis, microarray, human fatty acid-binding protein

Introduction

Lung cancer has become one of the leading causes of cancer deaths in Taiwan[1]. The five-year survival rate was only 13% in all patients regardless of their stages of disease at the time of diagnosis[2]. Metastasis is common in patients with lung cancer, especially for small cell lung cancer and adenocarcinoma[3]. Metastasis is a complex process and consists of a series of linked, sequential and selective steps. Invasion of the parenchyma by cancer cells, usually regarded as the first and probably the most crucial step of metastasis, may involve a series of biological changes concerning the interactions between cancer cells and the surrounding micro-environment[4].

It has been believed that during the development of a malignant tumor, some of the tumor cells may acquire new biologic characteristics, mutations with expression of metastasis-promoting genes or loss of function of metastasis-suppressing genes. A progression toward a more aggressive behavioral pattern may be seen during the progression of tumors, and the ability of invasion may become apparent late in the course of cancer progression.

Several genes that are differentially expressed in the cell lines with different metastatic potential have been identified. One candidate gene was identified as human epidermal fatty acid-binding protein (FABP5).

The intracellular fatty acid-binding proteins (FABPs) are members of a multigene family encoding ~15-kDa proteins, which bind a hydrophobic ligand in a non-covalent, reversible manner[5-7]. The functions of these proteins within cells have remained elusive. Although the in vitro binding of a fatty acid has been analyzed extensively, the in vivo function is less well defined. It has been hypothesized that owing to the low solubility of fatty acids in an aqueous environment the FABPs are required to facilitate their solubilization. Additional proposed functions include facilitating the influx of fatty acids across the plasma membrane, transport of the fatty acids within the cell, determining compartmentalization for storage, modulation of activity of enzymes involved in fatty acid metabolism and protection from detergent-like effects of fatty acids. Currently there are nine different members of FABPs, with strikingly similarly defined functions. Because many of these proteins have been cloned from various organisms, and from different cell or tissue types, each has been named according to the location studied. Several types of cells or tissues have been reported to express the epidermal Fabp5, including lens, adipose, mammary and endothelial cells, stratified epithelia of epidermis and tongue, stomach, heart, brain, liver, spleen, muscle, lung, intestine, bone marrow, renal medulla,

testis, urithelium and retina[8-9]. Binding studies have been used to elucidate ligands for FABP5; these include long-chain fatty acids but do not include retinoid acid[10].

FABP5 has been shown to be upregulated in a number of altered states with disturbed lipid profiles. It is highly upregulated in benign papillomas, carcinomas[11], skin warts, and psoriatic keratinocytes[12]. It is upregulated in bladder transitional-cell carcinomas[13] and peripheral nerve trauma. However, the specific functions for FABP5 in the various cell types remains undetermined. Recently in a studies comparing the mRNA expression of benign and malignant human prostate and breast cell lines, the human cutaneous FABP has been shown to express higher levels of mRNAs, and transfection of a C-FABP expression construct into the benign, non-metastatic rat mammary epithelia line Rama 37 with subsequent inoculation produced a higher significant number of metastases[14]. Further studies showed that expression of the endogenous vascular endothelial growth factor (VEGF) gene was increased by 3.8-5.2 fold in the C-FABP-transfected cells[15].

Reverse transcriptase – polymerase chain reaction (RT-PCR) also showed that the relative expression of FABP5 is increased in the more invasive and metastatic cell line (CL_{1-F4} and CL₁₋₅) compared with less invasive cell lines (CL₁₋₀ and CL₁₋₁). We therefore speculate that FABP5 may be a metastasis/invasion associated gene in human lung adenocarcinoma and may be of prognostic importance.

In this study, we underwent molecular cloning and characterization of FABP5 involving metastasis/invasion in lung adenocarcinoma cell lines. Assay the metastasis and invasion ability of CL cell lines after transfection of sense and anti-sense cDNA of FABP5 was performed subsequently.

Materials and Methods

Cell culture

A panel of human lung adenocarcinoma cell lines, CL₁₋₀, CL₁₋₁, CL₁₋₅, and CL_{1-5-F4}, in ascending order of invasiveness, was established in our laboratory[16]. Cells were grown as previously described¹¹. Before functional assays, only 0.02% EDTA will be used to avoid damaging cell-surface antigens.

Isolation of total RNA

Total cellular RNA was extracted from cultured cells as previously described, using a modified guanidium thiocyanate-phenol- chloroform extraction method by using RNAzol B (Biotech Laboratories, Inc., Texas, USA). After centrifugation at 12000 g for 15 minutes, it will be washed with cold 75% ethanol and solubilized in TE buffer and quantitated spectrophotometrically.

Northern hybridization

After the RNA from tumor cell lines was extracted, it was used for electrophoresis. Each lane on 1% agarose formaldehyde gel was loaded with 20 µg of total RNA, and after electrophoresis the gel was blotted onto a Hybond M membrane (Amersham). The membrane was hybridized with DIG-labeled DNA probes synthesized

using the Rediprime DNA labeling system (Amersham). Hybridization was performed in 5X SSPE, 10X Denhardt's solution and 100g/ml salmon sperm DNA, 50% deionized formamide for 18 hours at 42 °C, and the filters was washed twice in 1X SSC for 15 minutes at room temperature and 0.1X SSC and 0.1% SDS for 30 minutes at 60 °C. The amount of RNA in each lane was visualized with hybridization to GAPDH or Gβ-like protein. Densitometric analysis was performed using a phosphorImager (Molecular Dynamics)

Molecular cloning of full-length cDNA of FABP5

RNA from CL₁₋₅ cells was reversely transcribed with SuperScript II reverse transcriptase (Life Technologies, Inc.) and random hexamer. cDNA encoding the entire human FABP5 coding region was amplified by polymerase chain reaction (PCR). Primer sequences are as follows: 5' primer = 5'-CACCATGGCCAGTTCA-3' (nucleotides 45-62); 3' primer = 5'-CCTGTCCAAAGTGATGATGG-3' (complementary to nucleotides 481-462). The reaction mix was denatured at 94 °C for 30 seconds, annealed at 55 °C for 3 minutes; this cycle was repeated 30 times. The 437-base pair FABP5 cDNA fragment was cloned into a TA vector, according to the manufacturer's instructions (pGEM-T-Easy cloning kit; Promega Corp., Madison, WI), and sequenced with an autosequencer (Model ABI 377; PE Applied Biosystem, Foster City, CA). pCI-neo-FABP5 was created by inserting nucleotides 45-462 of the FABP5 cDNA between the EcoRI and NotI sites of a pCI-neo mammalian expression vector (Promega Corp.) and used for transfection and expression of FABP5 in CL₁₋₀ cells. Nucleotides 45-462 of the FABP5 cDNA was inserted into the PstI and EcoRI sites of a pRSET C prokaryotic expression vector (Invitrogen Corp., Carlsbad, CA) to construct pRSET-FABP5, which was used to produce the protein to immunize mice.

Transfection and selection

PCI-neo plasmids (5µg) was transfected into 70% confluent CL₁₋₀ cells with 20 U of LipofectAMINE reagent (Life Technologies, Inc.) as described previously[17]. Other CL₁₋₀ cells were transfected with the pCI-neo vector containing no insert (mock transfected) as a control. Gentamicin was added to 500 µg/mL for the selection of stable transfectants. Selection medium will be changed every 3 days for a 3-week period. Clones of resistant cells were isolated and allowed to proliferate for further characterization. Integration of transfected plasmid DNA into chromosomal DNA was confirmed by Northern blot analysis. For transient Transfection, 70% confluent cultures of CL₁₋₅ and CL₁₋₀ cells were transfected with the pEGFP-FABP5 plasmid as above. Forty-eight hours later, living cells were examined directly and photographed with a Zeiss Axioptot epifluorescence microscopy equipped with an MRC-1000 laser scanning confocal imaging system (Bio-Rad Laboratories, Rockville Center, NY).

In vitro invasion assay

The invasive activity of transfected clones will be examined by use of a membrane invasion culture system, in which a polycarbonate membrane with 10-µm pores

(Nucleopore Corp., Pleasanton, CA) coated with Matrigel (Collaborative Biomedical, Becton Dickinson Labware) at 5 mg/mL will be placed between the upper and lower well plates of a membrane invasion culture system chamber. Cells will be suspended in RPMI-1640 medium containing 10% NuSerum, and 2.5×10^4 cells will be placed into each upper well of the chamber. After incubation for 48 hours at 37 °C, cell that had invaded through the coated membrane will be removed from the lower wells with 1 mM EDTA in PBS and dot blotted onto a polycarbonate membrane with 3- μ m pores. Blotted cells will be stained with propidium iodide (Sigma Chemical Co., St. Louis, MO), and the number of cells in each blot will be counted under a microscope at a magnification of $\times 50$ by use of Analytical Imaging Station software package (Imaging Research Inc., ON, St. Catharines, Canada). Each experiment will be performed three times, and each sample will be assayed in triplicate.

Cell growth assay

Cell growth will be measured with the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [18]. Four thousand cells and 100 μ L of culture medium per well of a 96-well plate will be incubated for up to 4 days, and 10 μ L of MTT (5mg/mL) will be added to each well and incubated for 4 hours at 37 °C. The reaction will be stopped by adding 100 μ L of 0.04 N HCl in isopropanol to each well, with vigorous mixing to solubilize colored crystals produced by this reaction. The absorbance at 570 nm relative to the absorbance at 630 nm, as the reference wavelength, will be measured by a multiwell scanning spectrophotometer. Cell viability will be examined by trypan blue dye exclusion. Each data point is the average of six determinations, and each experiment will be repeated at least three times.

In vitro experimental metastasis

Intravenous metastasis experiment will be used to characterize the lung cancer cell lines transfected with FABP5 (sense transfectant), anti-FABP5 (antisense transfectant), and control (mock transfectant). Six-week-old SCID mice will be used for experimental IV metastasis. The cells will be cultured in RPMI + 10% FCS with a short treatment with 0.25% trypsin and 5 mM EDTA, washed 3 times in PBS. The cells will be counted, re-suspended and adjusted to 10^7 cells/0.1 mL concentration. Each group consists of 5 SCID mice and for each mouse 10^6 cells/0.2 mL will be injected through the tail vein. Six to 8 weeks after the injections, the mice will be sacrificed and the lungs and livers will be removed and fixed with formalin for histological examination or frozen at -70 °C for further experiment.

RESULTS

We have performed molecular cloning of the full-length DNA fragment of *Fabp5* and have transfected it into a panel of cell lines successfully. The following figure 1 represents one of the stable inducible clone using Tet-off system, containing inducible *Fabp5*. Real-time reverse-transcription polymerase chain reaction (RT-PCR) of the mRNA expressions of the FABP5 was

performed to compare the gene expression between various cell lines transfected with *Fabp5* as well as the parental cell lines. Its expression in this cell line is shown that compared with the parental cell line CL₁₋₀ (the most right curve) and a cell line that was transfected with vector only (the right second curve), the induced expression of FABP5 mRNA in the Tet-Off cell line (the most left curve) showed an about 4-fold increased of expression.

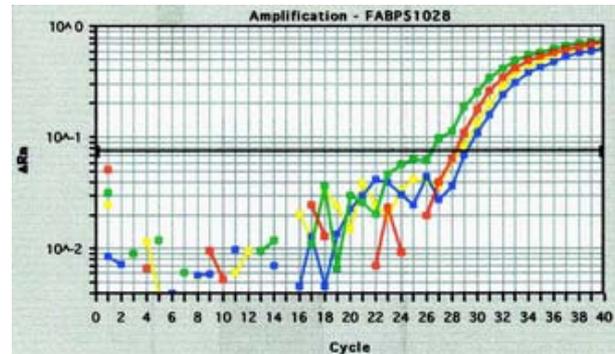


Figure 1. mRNA expression of *Fabp5* detected by real-time RT-PCR.

The protein expression of the transfected *Fabp5* was also detected in the stable clones of the cell lines containing a vector with a Tet-Off system. (Figure 2). The FABP expression in the transfected gene was detected by an antibody targeting on the HA tag as a fusion protein with FABP. It shows that the current dosage of tetracycline used in this study did not completely suppress the expression of FABP-HA, despite apparent difference. He are currently titrating the dosage of tetracycline to achieve more complete suppression of the transfected FABP5 for further investigations. Transfection of *Fabp5* to other cancer cell lines was also started recently to find possible more suitable cell lines that may express higher level of FABP5.

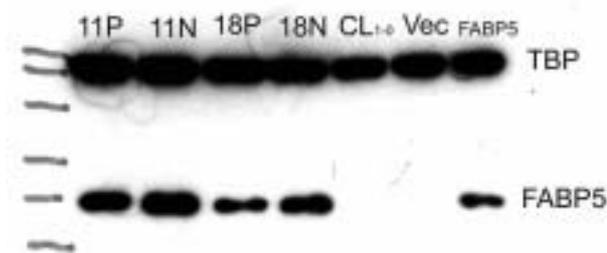


Figure 2. Expression of the FABP5-HA fusion protein detected by Western-blotting.

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