行政院國家科學委員會專題研究計畫成果報告

國人肺腺癌之基礎與臨床研究－建立體外模式研究肺腺癌轉移之機轉（III）

Establishment an in vitro model for study the mechanisms of metastasis in lung adenocarcinoma (III)

計畫編號：NSC88-2314-B-002-079-M39

執行期限：87 **年** 8 **月** 1 **日至** 88 **年** 7 **月** 31 **日**

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一、中文摘要

本子計劃擬做為此整合型計劃之核心計劃，收集病人之臨床資料、血液，建立國人肺腺癌之細胞株及收集肺腺癌組織，包括組織冷凍保存、石蠟包埋及 OCT **冷凍組織，收集正常及肺癌** RNA **及** DNA**，並建立免疫組織染色技術，提供子計劃一、二、三、五、六、七、八應用，我們也擬建立肺腺癌轉移之體外模式，提供其他各子計劃之研究材料，本計畫已建立肺腺癌細胞之高侵襲及轉移傾向和低侵襲及轉移傾向之各類腺癌細胞株（**Am J Respir Cell Mol Biol 1997**），用之研究細胞型態學、細胞表面受體、細胞粘附分子、細胞表面蛋白及分解酵素、細胞移動因子、轉移相關基因等之改變。利用子計畫七建立之多色** cDNA **微陳列技術，及我們已建立之肺癌細胞轉移模式，分析與肺癌轉移之相關基因，初步我們已獲得數十個基因，其分別表現於高侵襲轉移能力的細胞株，或低侵襲轉移能力的細胞株，在北方墨點技術也同時證實其相關性，目前我們正分析及序定這些可能基因轉移機轉,其中我們選植** CRMP-1, **並轉植入** CL1-5, **目前正研究其功能及在肺癌轉移機轉所扮演之可能角色。**

關鍵詞：肺腺癌、轉移機轉

Abstract

Lung cancer is a serious health problem in Taiwan. The epidemiologic characteristics such as: high female to male ratio, high proportion of adenocarcioma, high peripheral type lung cancer which are rather unique and may imply that different etiologic factors involving the pathogenesis of lung cancer in Taiwan. This proposed project will address on the most common cell type of lung cancer: adenocarcinoma, especially female adenocarcinoma and study the molecular epidemiology, genetic alterations and mechanism of metastasis. The subproject 4 will serve partially as a core facilities. We will provide clinical informations, collect tissue samples, for extraction of DNA and RNA, blood, establish cell lines from our own Chinese patients for the studies in other subprojects. We also provide immunohisto-chemical technical support for other collaborated laboratories. We will particularly focus on the establishment an in vitro model for study the mechanisms of metastasis in lung adenocarcinoma, by repeated selection in Transwell Membrane Culture System, we establish high invasive and high metastatic potential sublines. Using these in vitro cell model, we are able to explore the genes involving the regulation of cancer metastasis by the technique of differential display of gene expressions as well as the cDNA microarray technique Several condidate cDNAs have been obtained which are differentially expressed either in high invasive metastatic cell line or in low invasive/metastatic cell line. Northern hybridization also confirmed the results obtained from cDNA microarray. We cloned and transfected one candidate gene-CRMP-1 into CL1-5. Currently we are involved in characterization function of these

genes.

Keywords: Lung adenocarcinoma, metastasis

二、背景與目的

Lung cancer is a serious health problem in Taiwan. It had surpassed hepatoma and became the number 1 cause of cancer death in men and women since 1997. Our previous studies on the epidemiology of lung cancer has revealed several characteristics which are unique in Taiwan: (1) highest rate of increase in the world, (2) high incidence of female lung cancer with male to female ratio of 2:1 for more than 30 years, (3) high histologic subtype of adenocarcinoma, particularly in female, more than 60% of female lung cancers are adenocarcinoma, (4) high prevalence of peripheral type lung cancer. This subproject (subproject 4) will also serve as a core laboratory facility. we will organize a special technician to help to collect clinical information of the patients blood samples, tissues (including tumor and normal lung tissue from patients who are operated or undergoing needle biopsy). The tissues will be preserved in liquid nitrogen as well as paraffin embedding for histologic section, and DNA, RNA extraction. The clinical informations and specimens will then provide for all other subprojects. The core Lab technician will also take care of histologic section of specimen and immunohistochemical staining techniques, and help other subproject for the immunohistochemical staining. We will also use this core facility to establish our own cell lines. We currently have had following cell lines available:

- Adenocarcinoma
  CL1, CL2, CL3 NCL2
  CL1-0, 1, 2, 3, 4, 5, CL1-5F1
  PC9, PC13, PC14
  NC1-H358 (ATCC), NC1-H322 (ATCC)
  Squamous cell carcinoma
  SK-MES-1, Calu-1, NC1-H157, NC1-H520 (ATCC)
- Small cell carcinoma
  NC1-N417 (ATCC)
- BES6 (human bronchial epithelial cells, SV40 transformed)
- Primary culture of human bronchial epithelial cells

The CL series are lung adenocarcinoma established from our own patients. We will try to establish more cell lines for the studies in this program project. The cell lines will provide useful tool to study the genetic alterations, oncogene changes, level of membrane proteinase, metallothionein, glutathione S- transferase, 4 amino-biphnyl DNA adduct and heavy metals, cytokines, growth factors and other biological markers particularly relevent to lung adenocarcinoma in Taiwan. The successful rate of growing a cell line is about 25%.

Tumor cell invasion and metastasis are the most difficult problems faced by the modern researchers and those developing cancer therapy strategies. Unfortunately, no major clinical breakthrough which can efficiently prevent tumor cell invasion has been made. Invasion, which is the most crucial step in the metastatic cascade, contains a series of biological activities involving the interaction of tumor cells with the surrounding environment. It has been sudivided into three steps: (i) attachment of tumor cells to an extracellular matrix (ECM); (ii) production of matrix degrading enzymes; and (iii) migration of tumor cells through the degraded matrix [(10)]. Tumor cells have to successfully complete all these processes in order to enter the circulatory and lymphatic systems to form distant metastases. Many different classes of proteins have been shown to be involved in each step of this cascade. These included cell-matrix and cell-cell adhesion molecules, metalloproteinases, plasminogen activators, motility factor receptor(s) and many more. Several *in vitro* invasion models have been developed to study the mechanisms of tumor cell invasion and the roles these proteins play in this process.

We will use these cell lines to

investigate the genes controlling the metastasis by differential display and gene expression quantitation cDNA microarray system (subproject 7). These cell lines will be also a useful model for other subproject to study the carcinogenesis, genetic alteration, metabolism detoxification and drug resistance in lung adenocarcinoma.

Specific Aim

1. Establish cell lines and *in vitro* model for study mechanisms of metastasis in lung adenocarcinoma
2. Isolation, purification and characterization metastasis related genes lung adeno-cardinoma
3. Study the function of these genes involving the metastasis in lung adenocarcinoma

三、方法與結果

I. Selection of high invasive sublines of lung adenocarcinoma

To obtain high invasive sublines for study the mechanisms of metastasis, we use MICS system to *in vitro* select high invasive sublines from CL1 lung adenocarcinoma cells. The parental CL1 cells were seeded onto Matrigel-coated membrane in Transwell Invasion Chamber. After 72 hour incubation, the cells that invaded Matrigel were collected as CL1-1, signifying one passage through the basement membrane, subsequently, these cells were regrown and repeatedly passed through the invasion selection for four more times, and the cells harvosted from each round of selection were disignated as CL1-2, CL1-3, CL1-4, and CL1-5. Our preliminary results showed that invasive potential increased almost four folds in CL1-1 and six-fold in CL1-5 as compared to the parental CL1 cells. The average invasive potentials of each clones were as follows: CL1-0 (0.40±0.03%); CL1-1 (1.55±0.17%); CL1-2 (1.92±0.27%); CL1-3 (1.62±0.21%); CL1-4 (1.97±0.22%); and CL1-5 (2.44±0.48%).

II. *In vitro* intravenous metastasis experiment

In the experimental metastasis, we intravenously inject 10^6 cells of high and low invasive cell line into tail vein of SCID mice. As shown in Table 1 there were no lung metastasis in SCID injected with parental CL1-0 cells while high metastatic potential were observed in SCID injected with CL1-5 cells. The intermediate invasive subline CL1-2 forms limited tumors in the lung.

Table 1
Metastatic Potentials of CL1 Sublines with Differential Invasive Abilities

Cell line	No. of cell injected	No. of tumors in the lung
CL1-0 (n=5)	10^6/mice	0, 0, 0, 0, 0 (0/5)
CL1-2 (n=5)	10^6/mice	2, 1, 0, 0, 0 (2/5)
CL1-5 (n=6)	10^6/mice	2, 3, 3, 2, 2, 0, (5/6)

* Parethesis indicate number of mice with lung metastasis.

III. cDNA microarray of gene expression involving in the metastatic process

By using the cDNA microarray, we have identified several cDNA clones, which are highly expressed or suppressed in high metastatic potential CL1-5 cells. One of the candidate cDNA clones are subcloned and sequenced. It showed homology with CRMP-1. Northern hybridization and RT-PCR confirmed that the expression of CRMP-1 was decreased in highly metastatic sublines CL1-5 and CL1-F4, as compared with the parent CL1-0 cells (Fig. 1). We are now further characterize CRMP-1 and try to confirm their relevance in the mechanism of lung cancer invasion and metastasis.

IV. In vivo invasion assay using tracheal graft repopulation model

Tracheal graft repopulation model as used to assess the invasion ability of the cell lines selected. The tracheal graft was

isolated from S-D rats. The rat tracheal epithelial cells were denuded by repeated freeze and thaw. The tracheal grafts were then stored at -70°C until use. The highly invasive subline CL1-5 and parent cell line CL1-0 were then used to repopulated in the tracheal graft. CL1-0 and CL1-5 cells were grown to subconfluent, harvested and resuspended in PBS at a concentration of 10^7 cell/ml. Each tracheal graft was injected with 10^6 cells and the tracheal graft was then embeded in the subcutaneous tissue on the back of a nude mice. Each nude mice carried one tracheal graft. After 4 weeks, the tracheal graft was removed for histologic examination.

Figure 2 showed a tumor was formed within a tracheal graft lumen after injection of 10^6 cells of CL1-0 cell line. The basement membrane remains intact and there was no evidence of tumor cell invasion, while tumor cells invade through the basement membrane of the graft was evident in CL1-5 cells. This results indicated that the in vitro selected high invasive subline also demonstrated high invasive ability in the tracheal graft in vivo

四、參考文獻

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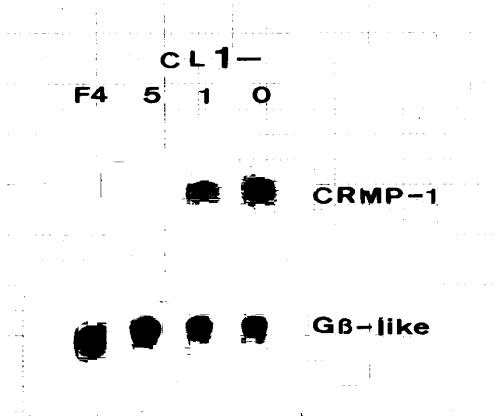

Fig. 1. Northern blot analysis of CRMP-1 in three cell lines with different metastatic potential. The cDNA clones CRMP-1 was obtained from RT-PCR, and the sequence was the same as the data of gene bank. Total RNA were extracted in each cell lines and 20 μg of RNA from each cell lines were electrophoressed on 1.2% agarose and hybridized with ^{32}P-labeled AP4 cDNA clones. The Gβ–like was used as internal control. High expression of CRMp-1 mRNA are observed in low invasive subline CL1-0 and CL1-1.

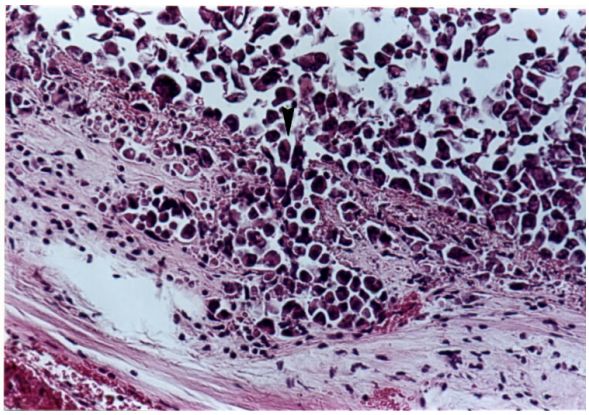

Fig. 2. Tracheal graft tumor repopulation study in nude mice showed tumor grow within the tracheal graft 4 weeks after injection of 10^6 cells of CL1-5 cells, the basement membrane was disrupted and tumor cells

invade into the submucoal area through the basement membrane (arrow head).