行政院國家科學委員會補助專題研究計畫成果報告

※※※※※※※※※※※※※※※※※※※※※※※※※
※　　　　　　　　　　　　　　　　　　　　　　　※
※　　以精簡雜交法和基因矩陣探討　　※
※　　肺腺癌腫瘤化過程的基因　　※
※　　　　　　　　　　　　　　　　　　　　　　　※
※※※※※※※※※※※※※※※※※※※※※※※※※

計畫類別：■個別型計畫　　□整合型計畫
計畫編號：NSC　89－2320－B－040－073－
執行期間：　89 年　11 月　1 日至　90 年　7 月 31 日

計畫主持人：蔡菁華
共同主持人：

本成果報告包括以下應繳交之附件：
- □赴國外出差或研習心得報告一份
- □赴大陸地區出差或研習心得報告一份
- □出席國際學術會議心得報告及發表之論文各一份
- □國際合作研究計畫國外研究報告書一份

執行單位：中山醫學大學，毒理學研究所

中　華　民　國　　90 年　10 月　29 日

行政院國家科學委員會專題研究計畫成果報告

國科會專題研究計畫成果報告撰寫格式說明

Preparation of NSC Project Reports

計畫編號：NSC 89-2320-B-040-073

執行期限：89 年 11 月 1 日至 90 年 7 月 31 日

主持人：蔡菁華　中山醫學大學

計畫參與人員：黃惠足　中山醫學大學

洪惠玲　中山醫學大學

一、中文摘要

經由精簡雜交法建立了不抽煙女性肺腺癌的精簡基因庫。具有不同表現量的基因片段經由 differential screening 的方法挑選出來。從正常組織構成的精簡基因庫中我們找出約 150 個基因片段具有較高的表現量。從癌組織構成的精簡基因庫中我們也找出約 100 個基因片段具有較高的表現量。這 250 個基因片段中約有一半已經片面定序，其中有 30 個片段基因序列與現有資料庫的序列相似性非常低。這 30 個基因中約有三分之一的序列尚未存在於資料庫中。為了證實這些經過 differential hybridization 篩選過的基因在腫瘤組織和正常組織中的表現量確實有差異，我們利用 RT-real time-PCR 檢測幾個正常組織之精簡基因庫中篩選出來的基因。與 GAPDH 和 β-actin 的表現值校正後，這六個基因在正常組織中的表現的確高於其在腫瘤中的表現。高出的表現值分別是 15、150、280、440、2700、3400 倍。

關鍵詞：精簡雜交基因庫、differential screening、RT real time PCR

Abstract

Two subtracted cDNA libraries were constructed from lung adenocarcinoma of a female non-smoker. By differential screening, clones that show differential expression in tumor were collected. About 150 clones show differential expression levels in subtracted normal lung cDNA library, and bout 100 clones show differential expression levels in subtracted tumor cDNA library. Half of the clones have been sequenced manually. After searching in database, 30 clones shows no significant homology to know genes in database, and about half of them do not match to any sequence in the database. To confirm the differential expression levels of these clones, RT-real time-PCR was used to quantitate the relative level of gene expression in the tumor tissue and normal counterpart. After normalization to GAPDH and β-actin housekeeping genes, 6 clones identified from subtracted normal lung cDNA library showed higher expression level in normal tissue than in tumor tissue. The expression levels differ in 15-, 150-, 280, 440-, and 3400-folds, respectively.

Keywords: Subtracted cDNA library、differential screening、RT-real time-PCR

二、緣由與目的

The short and long term goals of this project are to identify novel genes that are related to lung adenocarcinoma, and to study their biological functions in tumorigenesis. Several approaches can be used to identify novel candidate genes, however we chose suppressive subtractive hybridization (SSH) method to generate subtracted cDNA library. The advantage of this method is that it has ability to equalize representation of differentially expressed genes in the subtracted cDNA library.

Tissue specific cDNAs are generated by subtract the target cDNAs from large molar

excess of driver cDNAs. By this method, genes differ in small levels would be likely to be subtracted out as common messages. Therefore, highly differentially expressed genes have greater potential to be present in the subtracted cDNA library.

三、結果

Two subtracted cDNA libraries have been generated form tissue of patient with lung adenocarcinoma via PCR- based suppressive subtractive hybridization (SSH). We collaborated with Dr. Hsu and Dr. Wu (Veteran Hospital, Taipei) to collect fresh tissues from patients with lung adenocarcinoma.

The subtracted cDNA libraries were generated by a set of reciprocal hybridization. The subtracted lung adenocarcinoma cDNA library (N-T subtracted cDNA library) is generated by subtracting tumor cDNAs from 25-fold molar eccess of cDNAs from normal tissue. In a reciprocal hybridization, a subtracted normal lung cDNA library (T-N subtracted cDNA library, generated by subtracting normal lung cDNA from 25-fold molar excess of tumor cDNAs) is generated. After subtractive hybridization, portion of the subtracted cDNA products is cloned into a TA-cloning vector and transformed into *E. coli*. Figure 1 repesents the subtracted cDNA products for making subtracted cDNA libraries. The insert of each clone is PCR amplified by a set of SP6 and T7 promoter primers. Figure 2 shows about 60 to 70% of the clones in the subtracted cDNA libraries contain insert.

1 2

1: N-T subtracted cDNA library
2: T-N subtracted cDNA library
Figure 1 Figure 2

To increase the efficiency of identifying differentially expressed genes in the subtracted cDNA libraries, we performed differential screening on subtracted cDNA libraries with probes generated from subtracted cDNAs. Each PCR product was spotted on the membrane. Duplicated membranes were used for differential screening. One membrane was hybridized with probes generated from N-T subtracted cDNA, and the other membrane was hybridized with probes generated from T-N subtracted cDNAs. The reason for using probes generated from subtracted cDNAs but not from total cellular mRNA is that probes generated from total mRNA are not able to detect the clones that have low expression levels in the cells.

Figure 3 shows the first set of the differential screening. Dots A2 to D12 were blotted with PCR products generated form the N-T subtracted cDNA library (enriched putative oncogenes), and E1 to H11 were blotted with PCR products generated from T-N subtracted cDNA library (enriched putative tumor suppressor genes). A1 and H12 were two negative controls containing only vector sequences and adaptor sequences that are present in every subtracted cDNA clones. About 50% of PCR positive clones showed differential expression levels in the first set of screening. Taking account that only 60% of the clones in the libraries containing insert, only 30% of the clones in the libraries show differential expression levels.

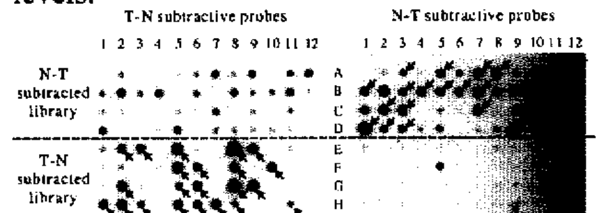

Figure 3

Two problems will be encountered during differential screening: how to eliminate the identification of same genes in the subsequent screenings and when to stop the hybridization screening. Since the subtracted cDNA are PCR amplified, it can produce hundreds of transformants after ligation into a vector. Although PCR-based SSH can normalize the copy number of differentially expressed genes to a certain degree, it is not possible to equalize them completely. In order to eliminate the

possibility of identifying same gene repeatedly, a secondary screening of the same set dot blot membrane with probes generated from previous identified clones was performed.

Figure 4 represents a result of secondary screening. Noted, this was the second set of the membranes with clones generated from T-N subtracted cDNA library, and the control membrane hybridized with N-T subtracted primary probes was not shown in this figure. Figure 4A represents membrane hybridized with probes generated form subtracted T-N cDNAs, and figure 4B shows membrane hybridized with secondary probes generated form the positive clones of the first set of membranes in figure 3. As expected, many clones as shown in secondary screening (Compare solid arrows in figure 5A with the signal in figure 4B). In this set experiment, in a total of 57 positive clones, 30 clones were previously identified (solid arrows) and 27 clones (open arrows) were newly identified genes after differential screening.

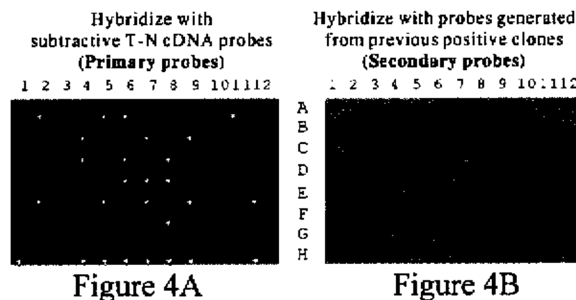

Figure 4A Figure 4B

By this screening strategy, we finished screening of both subtracted cDNA libraries. About 150 clones were identified from normal lung specific cDNA library, and 100 clones were identified from tumor specific cDNA library. About half of clones were sequenced manually. After homology search in the database, 30 clones shows no significant homology to know genes in the database, and about half of them do not match to any sequence in the database. To confirm the differential expression levels of these clones, RT-real time-PCR was used to quantitate the relative levels of gene expression in the tumor tissue and normal counterpart. Figure 5 represents a typical result of RT-realtime-PCR. Two clones N8 and N21 were used to compare the expression levels in lung tumor (T) and its normal counterpart (N). GAPDH and β-actin were used as controls. After normalization to GAPDH and β-actin housekeeping genes, 6 clones identified from subtracted normal lung cDNA library showed higher expression level in normal tissue than in tumor tissue. The expression levels differ in 15-, 150-, 280, 440-, and 3400-folds, respectively.

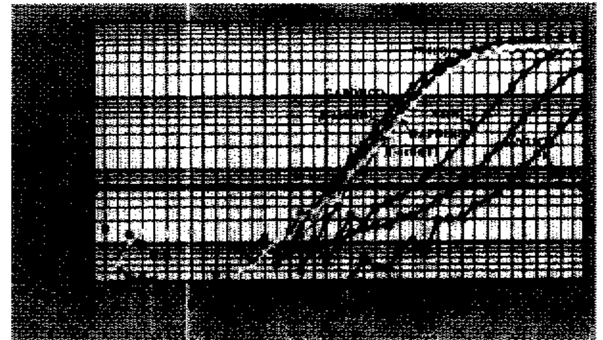

Figure 5

四、討論

The first half year of this project executed quite smoothly. Due to the limit financial support, the step of sequencing clones from subtracted cDNA libraries became quite slow. However, we have finished the proposed first year project. Results of the first year project showed that the subtracted cDNA libraries and the screening strategies were quite effective. We are continuing sequencing the rest of clones and reconfirming their differential expression levels by RT-realtime-PCR. Other than RT-realtime-PCR, we are developing in situ RT-PCR technique to examine the expression level of some novel genes. In addition, we are in the process of cloning the full-length cDNA for some novel genes by 5'- and 3'- RACE.

五、參考文獻

Diatchenko L, Lau Y-F C, Campbell AP, Chenchik A, Moqadam F, Huang Bm Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, Siebert PD: Suppression subtractive hybridization: A method for generating differentially regulated or

tissue-specific cDNA probes and libraries. Proc. Natl. Acad. Sci. USA 93:6-25-6030, 1996

Siebert PD, Chenchik A, Kellogg DE, Lukyanov KA & Lukyanov SA: An imporved method for walking in uncloned genomic DNA. Nucleic Acids Res 23:1087-1088, 1995