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

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一、中文摘要

本計劃利用肺腺癌病人的腫瘤和鄰近肺正常組織建立了兩個精簡雜交基因庫，並利用差異性雜交反應篩選法進一步剔除於肺癌和正常肺組織中表現量接近的基因。由正常肺組織精簡基因庫裡篩選出 174 個 cDNA clones，由肺腺癌組織精簡基因庫裡篩選出 105 個 cDNA clones。經過定序分析將重複出現的基因剔除後，由正常和肺癌基因庫裡分別找出 76 個和 37 個獨立的基因。現在正積極的利用同步定量 PCR 系統進行鑑定這些基因在不同病人的肺腫瘤組織和鄰近的正常組織中是否有差異性的表現。另一方面，有幾個尚未被發現功能的基因正被積極的做進一步的研究。

關鍵詞：肺腺癌，抑制性精簡雜交反應，差異性雜交反應，同步定量系統

Abstract

Two subtracted lung adenocarcinoma cDNA libraries have been established by suppressive subtractive hybridization. Differential screening has been used to identify 174 and 105 differentially expressed cDNA clones in the N-T subtracted cDNA library and T-N subtracted cDNA libraries, respectively. After sequencing, 76 out of 174 cDNA clones and 37 out of 105 cDNA clones were independent clones. Currently, realtime RT-PCR is used to measure the differentially expressed levels of selected clones between lung cancer and adjacent normal tissues among patients. In the meanwhile, two cDNA clones shows repressed expression levels in lung cancers have been selected for further characterization.

Keywords: lung adenocarcinoma, suppressive subtracted hybridization, differential screening, realtime PCR reaction.

二、緣由與目的

Lung cancer has been a leading cause of cancer death in the world including in Taiwan. The high mortality rate of lung cancer is not due to high incidence rate but due to the lack of early detection method and the high level of drug resistant of lung cancer cells. To increase the survival rate of lung cancer requires thorough understanding the biology of lung cancer. Identifying genes involved in lung tumorigenesis is one of the best way to study molecular biology of lung cancer.

To identify genes that may be involved in lung tumorigenesis, two subtracted cDNA libraries were established. One is enriched of genes that are differentially expressed in normal lung tissue, and the other one contains genes that are differentially expressed in lung tumor tissue. The independent clones in these two subtracted cDNA libraries have been sequenced and clones that show novel function have been selected for realtime RT-PCR reaction to further confirm their differential expression levels.

三、結果

Two subtracted cDNA libraries were established from a female lung adenocarcinoma. To generate subtracted cDNA library contains genes that are differentially expressed in lung cancer, an excess molar ratio of normal cDNA were used as driver for suppressive subtractive hybridization to subtract cDNA generated from lung cancer tissue (This subtracted cDNA library is designated as T-N subtracted cDNA library). A reciprocal hybridization also performed for generating a subtracted cDNA library that enriches genes differentially expressed in normal lung tissue (N-T subtracted cDNA library). Since suppressive subtractive hybridization involves many rounds of PCR reaction, subtracted cDNA libraries contain highly repeated clones. To efficiently characterize differentially expressed genes in these two subtracted cDNA libraries, a differential screening method was developed in the lab to identify independent clones and further eliminate common genes that are present in the subtracted cDNA libraries.

Differential screening using dot blot hybridization of PCR products generated from subtracted cDNA libraries. To increase the ability of detecting genes expressed in low abundance, subtracted cDNAs were used to generate radioactive probes. For each round of screening duplicated membranes contain 96 cDNA clones were hybridized with N-T subtraced cDNA probes and T-N subtracted cDNA probes independently. After comparison signals between these two membranes, clones show differential expression levels were pooled together to generate “positive probe”. This positive probe is used in the next round of differential screening hybridization to identify cDNA clones that have been identified in the previous hybridization. For each round of hybridization screening, the newly identified cDNA clones were pooled together with the previous differentially expressed cDNA clones to generate new positive probe. This differential screening continues until no further new differentially expressed cDNA clones can be identified.

After 5- to 6- rounds of differential screening, about 500 to 600 cDNA clones screened, identification of the differentially expressed independent clones was saturated. In N-T subtracted cDNA library, 174 clones were identified, and 105 clones were identified from T-N subtracted cDNA library. Sequenc analysis further eliminated repeated genes. Thus, 76 clones out of 174 clones and 37 clones out of 105 clones encode independent sequences in N-T subtracted cDNA library and T-N subtracted cDNA library, respectively.

Once sequence has been determined, a set of primers was generated for realtime RT-PCR reaction. In the first set of realtime RT-PCR reaction, 6 genes were selected from N-T subtracted cDNA library for further confirmation of their differential expression levels. Two of them are known genes that show high expression levels in normal tissue as controls. Expression level of each gene was normalized with the expression level of GAPDH. The relative expression levels of these 6 genes were15-, 150-, 280-, 440-, 2700-, and 3400- folds higher in normal lung tissue than in tumor tissue. This indicates that the subtractive hybridization and differential screening are successful. Realtime RT-PCR is intensively carried out to confirm the differential expression levels of genes identified from the differential screening.

Among the differentially expressed genes, N6 and N21 show no significant homology at Blast N non-redundant search. To explore whether the expression of these two genes are suppressed in lung cancers, limited sets of lung cancer tissues were used for realtime RT-PCR reaction. The relative expression levels of N6 to GAPDH in twelve patients were 2.1-, 2.7-, 4.8-, 5.5-, 8.5-, 30-, 41.4-, 48.8-, 57.3-, 117-, 2400-, 5000- fold higher in patients’ adjacent normal tissues than in the lung cancer tissues. Furthermore, the expression levels of N6 in lung cancer cell lines are very low. Some of the cell lines have to be amplified for 40 cycles in order to detect the N6 cDNA.

For N21, the relative expression levels

of N21 to GAPDH were tested in thirteen patients. Two patients showed no significant differences of the relative expression levels of N21 between lung cancer and their normal counterpart. The relative expression level of N21 to GAPDH in the rest of eleven patients are 6-, 10-, 12-, 50-, 64-, 121-, 138-, 146-, 168-, 832-, 9742-folds higher in adjacent normal lung tissue than in lung cancer tissue.

The first step toward understanding the function of novels genes is to acquire more sequence information. Databases in the public domain are good sources to access extra sequences. However, we realize it is important to have some cDNA libraries for screening of full-length cDNA. Thus, we had constructed three cDNA libraries from lung cancers, the adjacent normal lung tissues, and selected lung cancer cell lines. With these libraries, N21 has finished screening of the full-length cDNA and under sequencing. Screening full-length of N6 cDNA is in progress.

四、討論

The initial screening of subtracted cDNA library via differential screening method worked quite well. We believe that this screening has identified most of the independent differentially expressed clones in the subtracted cDNA libraries. The most important task now is to confirm the expression patterns of these genes in patients. Currently, realtime RT-PCR is used for confirmation of expression levels in pairs of lung cancer tissue and its normal counterpart. This analysis is very accurate, however it is quite time consuming. Spot these cDNA clones on chip for screening is another choice.

Numbers of genes identified in these subtracted cDNA libraries are much more than one lab can handle. Focusing on few genes for further study would be possible to yield any significant outcome. Since cDNA in these subtracted cDNA libraries are only partial sequence, this information limits further functional studies. However, recent studies show that siRNA can efficiently blocks gene expression in mammalian cells. We are currently developing siRNA system to knock down the expression levels of the identified differentially expressed genes in lung cancer. This functional assay would help us to select genes for further study.

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執行單位：中山醫學大學

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