

行政院國家科學委員會專題研究計畫進度報告

2-O-Methyl 反意核醣核酸應用於肺癌之基因治療

Gene Therapy of Lung Cancer Using Antisense 2-O-Methyl RNA

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中文摘要

肺癌佔目前所有癌症的 12.8%，並且佔所有癌症死因的 17.8%。就台灣而言，肺癌也是主要的癌症死因。每年約有 6000 人死於肺癌，顯示了新而有效的療法之重要性。研究顯示使用外加抑制劑來抑制端粒酶的活性會造成差異相當大的結果，包括細胞的立即死亡，到經過一段時間後細胞生長變慢，到細胞生長不受影響。端粒酶的抑制是否可以作為人類癌症治療的一個模式仍不十分清楚。第一年的研究中，我們已使用 2'-O-Methyl RNA，透過抑制端粒酶的作用，來達到抑制肺癌細胞生長的效果。含有人類端粒酶 RNA 模板互補序列的 2'-O-Methyl RNA 利用陽離子性微脂體作為載體來轉殖進入一相當惡性之肺癌細胞株，CL1-5。轉殖後的細胞與對照組比較起來，細胞生長受到明顯的抑制。而這些轉殖後的細胞的端粒酶活性有降低的情形，細胞凋亡的比例也增加。在本年度，我們使用同樣的轉殖模式來應用到不同的肺癌細胞株，A549 上。我們發現，轉殖後的細胞與對照組比較起來，細胞生長受到明顯的抑制。細胞凋亡的比例也增加。但轉殖後的細胞的端粒酶活性降低的情形並不顯著。在下年度，我們將研究轉殖後的細胞的下游基因表現及侵襲能力的變化。

關鍵詞: 基因治療 肺癌 2-O-Methyl 核糖核酸

Abstract

Lung cancer is the cause of 12.8% of malignancy and leads to 17.8% of cancer deaths worldwide. Lung cancer is also the leading cause of cancer death in Taiwan. Annually, there were 6000 patients died of lung cancer in Taiwan, emphasizing the need for new and effective treatment. Studies of the inhibition results by exogenously added inhibitors have produced contradictory results, ranging from immediate cell death, to decreased cell proliferation after a long delay, to no change in proliferation. The potential of telomerase inhibition as a therapeutic modality for human cancer remains unknown. In the first year of this proposed project, we used 2'-O-methyl RNA

for cell growth arrest of lung cancer through telomerase inhibition. 2'-O-methyl RNA with complementary human telomerase RNA sequence was transfected into a cultured lung cancer cells, CL1-5, using cationic liposome as a vector. The cell growth was inhibited by the 2'-O-methyl RNA in comparison with the control groups. The 2'-O-methyl RNA transfected cells showed decreased telomerase activity and increased proportion of apoptotic cells. In the second year, we applied the same methodology to another lung cancer cell lines, A549. The cell growth of A549 cells was inhibited by the 2'-O-methyl RNA in comparison with the control groups. The 2'-O-methyl RNA transfected cells showed little decreased telomerase activity and increased proportion of apoptotic cells. Next year, we will study the downstream gene expression profile and invasion ability change of the 2'-O-methyl RNA transfected cells.

Keywords : Lung cancer, gene therapy, 2-O-Methyl RNA

Introduction

Lung cancer is the cause of 12.8% of malignancy and 17.8% of cancer deaths worldwide [1]. Lung cancer has become a serious health problem in Taiwan [2]. Human telomerase is a ribonucleoprotein that adds repeated units of TTAGGG to the ends of telomeres [3, 4]. Telomerase activity has been found in 87% of all human tumors [5-7]. In contrast, in normal somatic cells, the telomerase activity is usually undetectable [5]. Telomerase activity was one of the most important prognostic factors in the patients with non-small cell lung cancer [8]. Similar correlation was reported for other types of malignancy [9-11]. Thus, telomerase may virtually be a universal target for anticancer therapy.

Previous study demonstrated that HeLa cell, that had been transfected with an antisense construct directed against the RNA component of telomerase, lost telomeric DNA and died after 23-26 doubling [12]. However, several recent reports have shown that telomerase inhibition by antisense approaches resulted in apoptosis, differentiation, decrease of cell motility, or senescence [13-15]. Studies of the inhibition results by exogenously added inhibitors have produced contradictory results, ranging from immediate cell death, to decreased cell proliferation after a long delay, to no change in proliferation [16]. So far, the most potent inhibitors tested are antisense oligonucleotides. Herbert et al had introduced 2'-O-methyl RNA

complementary to the template region of hTR into the immortalized human cell lines HME50-5E (breast epithelial) and DU145 (prostate tumor). In this proposed project, we will focus on the application of 2'-O-Methyl RNA in gene therapy of lung cancer. The basic strategy is using 2'-O-Methyl RNA for cell growth arrest of lung cancer by telomerase inhibition. Lung cancer cell line will be used as an *in vitro* model. 2'-O-methyl RNA with complementary human telomerase RNA (hTR) sequence [17] will be transfected into cultured lung cancer cells using cationic liposome as a vector. The telomerase activity will be analyzed by Telomeric Repeats Assay Protocol (TRAP). The *in vitro* liposome transfection methods in lung cancer cells will be optimized. Fraction of cell cycle stages will be detected by flow cytometry after transfection. The telomerase-inhibited lung cancer cells will be analyzed and characterized their reduction of telomere length, cell growth, apoptosis, tumorigenicity and downstream gene expression change.

Materials and Method

- 1) Cultured cells. The human lung adenocarcinoma cells CL1 was established from a 64-year-old man with poor differentiated lung adenocarcinoma [18,19]. The CL1-5 cells were subline of CL1 cells by selection of high invasive population using Transwell (Costar, Cambridge, MA) membrane coating with matrigel (Matrigel; Collaborative Research Bedford, MA). The human lung epithelium cell line A549 [20] was obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained in PRMI-1640 supplemented with 10% fetal bovine serum and antibiotics, in a humidified CO₂ incubator at 37°C.
- 2) Design and synthesize of 2-O-Methyl RNA. We purchased 2'-O-Me RNA oligonucleotides from TriLink BioTechnologies (San Diego, CA) which was purified by HPLC and purity was confirmed by gel electrophoresis. The sequence of 2-O-Methyl RNA is 5'-CAGUUAGGGUUAG-3' [17,21]. The underlined nucleotides possess phosphorothioate (PS) linkages. PS linkage confer stability against nuclease degradation and improve pharmacokinetic properties by increasing binding to serum proteins and *in vivo* half-life.

- 3) Uptake of 2-O-Methyl RNA into cells. 3×10^5 cells were plated in a 12-well culture plate, which allowed these cells to adhere overnight in PRMI-1640 supplemented with 10% fetal bovine serum and antibiotics, in a humidified CO₂ incubator at 37°C. Next day, the cells were transfected with 6µl of Lipofectamine 2000 and 2µg of 2'-O-Me RNA oligonucleotides in a 1 ml of total Opti-Mem (Gibco, BRL, Gaithersburg, MD). After 6 h at 37°C, the medium with serum was added to stop the reaction. Cells were then harvested 12-15 h later after washes with PBS and treatment with trypsin and assayed for telomerase activity, or allow to growth for 24, 48, and 96 h. The cells were then trypsinized and viable cells were counted by the trypan blue dye exclusion method using hemocytometer then replated.
- 4) Measurement of telomerase activity. Telomerase activity was measured by telomere repeat amplification protocol by using TeloTAGGG Telomerase PCR ELISA^{plus} (Roche molecular Biomedical, Mannheim, Germany).
- 5) Cell cycle analysis. Trypsinized cells were stained with propidium iodide by using the Cycletest kit (Becton Dickinson, mountain View, CA) and analyzed for DNA content by using the FACScan. Cell cycle distribution was determined. Dead cells were gated out by using pulse processing.
- 6) Apoptosis analysis. Using antihistone antibody, we detect the histone level of medium and cell lysate, which represent the quantities of apoptosis.

Results

The A549 cancer cells were classified as four treatment groups according to the type of treatment.

C: treat with medium only

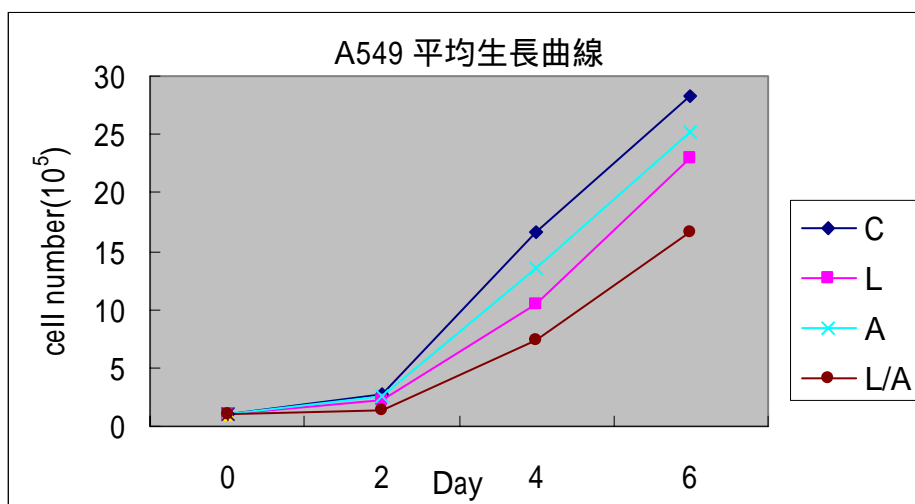
L: treat with liposome only

A: treat with antisense 2-O-methyl RNA only

L/A: treat with liposome + antisense 2-O-methyl RNA

Growth inhibition

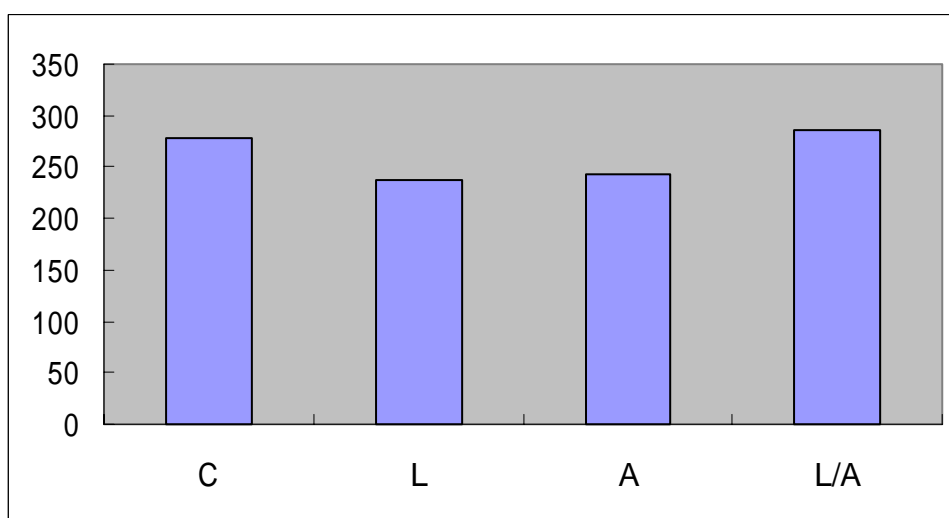
The growth curves of A549 lung cancer cell lines with different treatments, which showed the growth of the cells transfected with liposome+2-O-Methyl RNA was inhibited. The effect of inhibition occurred since the second day.



| | Day 0 | Day 2 | Day 4 | Day 6 |
|-----|-------|-------|-------|-------|
| C | 1 | 2.79 | 16.61 | 28.32 |
| L | 1 | 2.29 | 10.52 | 22.93 |
| A | 1 | 2.61 | 13.48 | 25.14 |
| L/A | 1 | 1.29 | 7.45 | 16.67 |

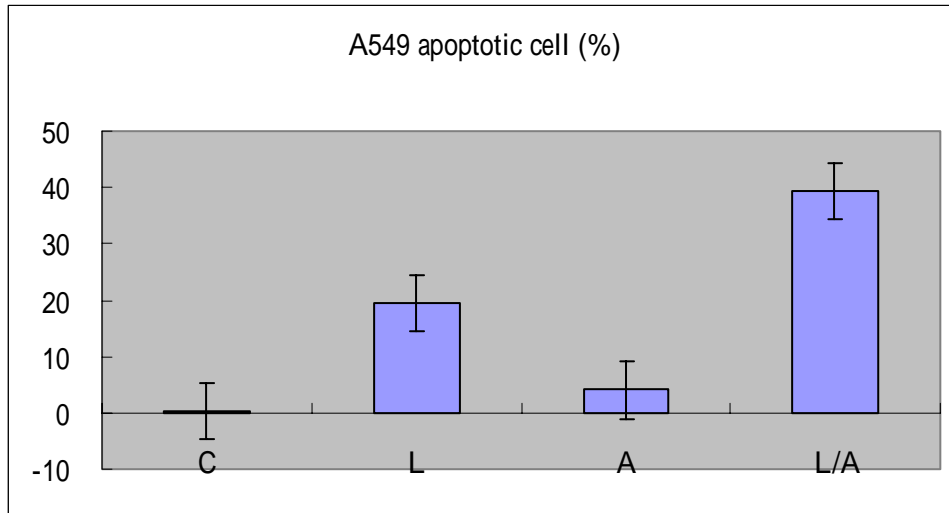
Telomerase inhibition

The relative telomerase activities (RTA) in lung cancer cells of four different treatment showed no significant difference of RTA among the four treatment groups.



Cell cycle analysis

The cell cycle analysis for apoptotic cell fraction showed that the cells treated with liposome only and liposome+antisense 2-O-Methyl RNA had apoptotic cells fraction of more than 10 %. The cells with liposome+antisense 2-O-Methyl RNA treatment showed the highest fraction of apoptotic cells.



Discussion

In this present study, we have applied the protocol of antisense 2-O-methyl RNA transfection for different lung cancer cell lines. We also demonstrated the efficiency of antisense 2-O-Methyl RNA transfection to lung cancer cell line in cell growth, telomerase inhibition, and apoptosis. The results of telomere length measurement are pending.

In this present study, we have demonstrated a 30% growth inhibition in the treatment group of liposome + 2-O-Methyl RNA on day 6th with a single dose for CL1-5 cell line. The inhibition of A549 was even more (58.8%) in the 2nd year study. However, the growth inhibition was present after 60 days in HME50-5E (a human mammary epithelial cell line) cells treated with the same oligonucleotide in previous study [17]. These difference might be due to the different length of telomere in different origin of cancer cell lines. We will perform further experiment for telomere length measurement to clarify this point.

In TRAP assay for telomerase inhibition, we disclose no obvious inhibition in telomerase activity with liposome + antisense 2-O-Methyl RNA, which was less than the previous study [17]. In our preliminary studies have showed that the transfection efficiency of liposome-oligonucleotide complex for lung cancer cell is about 30-40 %. The possible explanation might be that cell death might occurred soon after telomerase was inhibited. Thus we can detect only the telomerase of uninhibit cells, which might be the same level in the four treatment groups.

Previous study [23] demonstrated lectins could improve the lipofection efficiency in lung carcinoma cells. A549, Calu3, and H292 cells were

transfected using lipofectin plus a lectin as the vector. Lipofectin supplemented with Griffonia simplicifolia-I (GS-I) yields largest enhancement of the lipofection efficiency in A549 and Calu3 cells (5.3- and 28-fold, respectively). Maackia amurensis gives the largest enhancement (6.5-fold) of lipofection efficiency in H292 cells. lectin-facilitated lipofection is an efficient gene delivery strategy. Employment of cell type-specific lectins may allow for efficient cell type-specific gene targeting in our study.

A more recent study [24] showed transferrin (TF) and other ligands could improve the efficiency of lipofection in lung carcinoma cells. Confluent A549, Calu3, and H292 cells were transfected using Lipofectin plus TF, insulin, or epidermal growth factor as the vector. Lipofectin supplemented with epidermal growth factor yielded the largest enhancement of lipofection efficiency (< or =23-fold over that by Lipofectin alone) in all three cell lines. Insulin significantly enhanced the lipofection efficiency in A549 and Calu3 cells but not in H292 cells, whereas TF showed significant lipofection efficiency-enhancing effect in Calu3 and H292 cells but not in A549 cells. These results indicate that the gene delivery strategy employing ligand-facilitated lipofection can achieve high transfection efficiency in human lung carcinoma cells.

Our apoptosis analysis using cell cycle analysis and detection of histone showed that liposome also cause high proportion of apoptosis. This effect will make the efficiency of antisense oligonucleotide hard to be evaluated. Next year, we will find some other liposome with less cell toxicity.

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