行政院國家科學委員會補助專題研究計畫成果報告

※※※※※※※※※※※※※※※※※※※※※※※※※
※ RNA/DNA 短核酸組應用於肺癌之基因治療 ※
※ Gene Therapy of Lung Cancer Using RNA/DNA ※
※ Oligonucleotides ※
※※※※※※※※※※※※※※※※※※※※※※※※※

計畫類別：■個別型計畫　□整合型計畫
計畫編號：NSC90－2314－B－002－287－
執行期間：90 年 8 月 1 日至 91 年 7 月 31 日

計畫主持人：楊泮池
共同主持人：
計畫參與人員：

本成果報告包括以下應繳交之附件：

★出席國際學術會議經費未使用。

執行單位：國立台灣大學醫學院內科

中 華 民 國 九十一 年 十一 月 二十五 日

行政院國家科學委員會專題研究計畫成果報告

RNA/DNA短核酸組應用於肺癌之基因治療
Gene Therapy of Lung Cancer Using RNA/DNA Oligonucleotides

計畫編號: NSC 90-2314-B-002-287

執行期間: 90年8月1日至91年7月31日

主持人: 楊泮池　國立台灣大學醫學院內科

一、中文摘要

肺癌是國人一嚴重之健康問題，治療效果及預後均非常不好。基因治療是近年肺癌治療的新方向. 本研究擬製造嵌合RNA/DNA短核酸組來置換p53的突變點，我們以自己建立的國人肺癌胞株(CL1-5)做為研究模式，p53突變在exon 7的codon 248 CGG→TGG，利用RNA/DNA短核酸組，我們在細胞株用lipofection方式，試圖將p53突變矯正回來，而codon 248 T→G可產生一新的Msp I的新限制酵素作用區，利用此限制酵素來確定細胞株是否p53基因已被矯正。延續上年度之研究目標，我們使用新上市的liposome (lipofectamine 2000)檢測RNA/DNA短核酸組基因置換的效率，分離矯正p53之癌細胞株. 並測定其基因植入的效率. 發現可提升CL1-5肺癌細胞轉殖成功的比率至30%，然而 對細胞p53的突變點仍然無法達成有效的矯正效果。 使用chimeric RNA/DNA oligonucleotide 來矯正p53基因突變在本實驗中無法顯示其效果。今後應研究其他種類之短核酸組來作為肺癌治療之可行性。

關鍵詞: 基因治療、肺癌、RNA/DNA嵌合短核酸組

Abstract

Lung cancer has become the major cause of cancer-related mortality. Gene therapy is a new therapeutic modality for treatment of lung cancer. The current strategies for gene therapy of lung cancer include: 1) the introduction of suicide genes; 2) the inactivation of oncogenes; 3) the replacement of defective tumor suppressor genes; 4) immuno-gene therapy. In this proposed 3-year project, a novel approach using chimeric RNA/DNA oligonucleotides will be applied to correct p53 mutation in lung cancer. A high invasive lung cancer cell line CL1-5 with p53 mutation at codon 248 CGG→TGG was used as an *in vitro* model. We used chimeric RNA/DNA oligonucleotides to correct p53 mutation of CL1-5 cells by lipofection (lipofectamine-mediated) gene transfer. The conversion of mutant T→wild-type C at codon 248 will generate a new Msp I restriction enzyme digestion site (c/cgg). Thus the gene conversion can be identified by Msp I restriction enzyme digestion of PCR product of p53 exon 7. The *in vitro* transfection methods for lung cancer cell were tested for transfection efficiency. This year, we use a new liposome (lipofectamine 2000, Life tech.) found that the transfection efficiency of liposome-oligonucleotide complex for lung cancer cell is about 30%. However, that still demonstrated no obvious MSP-1 enzyme digestion. The PCR-Msp-I restriction enzyme analysis showed no evidence of gene correction. Using chimeric RNA/DNA oligonucleotide to correct p53 mutation failed to be an effective method for cancer therapy in our study. Further study for new oligonucleotide to achieve the correction of p53 mutation is warranted.

Keywords: Lung cancer, gene therapy, RNA/DNA oligonuclecotides

二、Instroduction

Lung cancer has become a serious health problem in Taiwan [1]. Based on the statistics from Department of Health in 1998, lung cancer is the leading cause of cancer death both in men and women. Gene therapy is one of the new therapeutic modalities for treatment of lung cancer [2]. About 80% of gene delivery methods using viral vectors such as retrovirus, adenovirus, adeno-associated virus and herpes simplex virus. The non-viral vectors include direct DNA delivery, liposome and DNA-protein complex [3]. Most of current strategies have been focused on the delivery of therapeutic genes using viral or plasmid vectors *in vivo* and *ex vivo*. In 1996, Kimec and coworkers reported exciting results of homologous gene targeting in mammalian cells using chimeric RNA/DNA oligonucleotides [4]. They found that RNA/DNA hybrids were more active in homologous pairing reactions than corresponding DNA duplexes. Hence, they designed a chimeric oligonucleotide containing a contiguous stretch of RNA and DNA bases, constructed in a double-hairpin configuration. The RNA sequence was

complementary to the DNA bases of the other strand with a single mismatched nucleotide located at the center of the oligonucleotides. The alignment of the chimeric molecule with the genomic site can trigger endogenous recombinases that facilitate homologous recombination. The feasibility of this oligonucleotide-based gene therapy was tested in an *in vitro* system of sickle cell anemia [5] and human hepatoma cell line HuH-7 [4]. The overall frequency of gene targeting in hepatoma cells was up to 11.9%, and when corrected for transfection efficiency, approached 43% [4]. Using similar approach, Lai and Lien have tested homologous gene targeting in correcting the point mutation in CA II deficient cell lines. The RNA/DNA chimeric oligonucleotides were introduced into CA II deficient cells by electroporation. The homologous gene-targeting rate was estimated to be about 1-5% by semi-quantitative PCR analysis [6,7]. In this proposed project, we collaborated with Professor LW Lai and YH Lien in University of Arizona Health Science Center to focus on the application of chimeric RNA/DNA oligonucleotide to correct p53 mutation in lung cancer. A high invasive lung cancer cell line CL1-5 with p53 mutation at codon 248 CGG→TGG will be used as an *in vitro* model. Chimeric RNA/DNA oligonucleotide with corrected wild-type sequence will be transfected into cultured CL1-5 cells using lipofection. The p53 converted CL1-5 cells will be analyzed and characterized their phenotypic changes, cell growth, apoptosis, tumorigenicity and invasive ability.

三、Materials and Method

1) Cultured cells preparation. The human lung adenocarcinoma cells CL1 was established from a 64-year-old man with poor differentiated lung adenocarcinoma [8,9]. The cells were maintained in PRMI-1640 supplemented with 10% fetal bovine serum. 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 CL1-5 cells were maintained in RPMI 1640 medium supplemented with 10 % fetal bovine serum on plastic and were incubated in 5% CO2/95% air at 37 ℃. The cells were diluted to 10^0/ul and cultured in 24-well culture dish for single cell cloning. The DNA sequence of p53 codon 248 from single cell clone was then examined for C→T mutaion.
2) Design and synthesize of chimeric oligonucleotides. The structure of chimeric oligonucleotides is illustrated in Fig. 1. Chimeric oligonucleotides will be synthesized by Cruachem (Dulles, VA) on a PS-250 oligonucleotide synthesizer in 15 μmole scale. The product will be cleaved from the support with 1:3 ethanol-33% ammonia solution with subsequent deprotection at 55 C ^{O} overnight. The ion exchange HPLC purification will be performed on Danamax SD-200 system (Ranin) using Mono Q 10/10 column (Pharmacia Biotech).
3) Liposome mediated gene transfer. After single cell cloning and population expansion, $5x10^4$ cells were seeded into 6-well plate 24 hours before transfection. For test the transfection efficiency, A plasmid with luciferase as reporter gene was transfected using a new liposome with two different ratio of DNA: liposome (1:3/1:6) for 6 hours (Lipofectamine 2000 Gibco, BRL, Gaithersburg, MD, USA). Cells were washed and in complete medium for 24 hours. The cells were lysed and the protein was extracted. The luciferase activity was measured by chemoiluminator as the index of efficiency. For test the efficiency of DNA/RNA oligonucleotides, DNA-liposome complex was added to the washed cell and incubated in serum-free medium for 6 hours. Cells were washed and in complete medium for 24 hours. The cells were lysed and the genomic DNA was extracted.
4) PCR-Msp I restriction enzyme analyses. The genomic DNA isolated from the treated and untreated cells will be subjected to PCR to amplify the 110-bp exon 7 fragments using forward primer, 5'-GGCTCTGACTGTACCACC-3' and a reverse primer, 5'-GGAGTCTTCCAGTGTGATG -3'. The 110-bp PCR product will be subjected to Msp I restriction enzyme digestion and the digest resolved by 7.5% polyacrylamid gel electrophoresis. The intensity of each band will be measured by Molecular Imager (Bio-Rad, Hercules, CA, USA).

四、Results

Transfection efficiency of plasmid/liposome complex in CL1-5 cells

The signal of luciferase activity was evaluated as the index of efficiency. We test a new product of cationic liposome (Lipofectamine 2000) with plasmid. The signals of chemoliumiator were around 30-40% on luciferase activity.

Transfection of DNA/RNA oligonucleotides/ liposome complex in CL1-5 cells

We choose the relatively effective liposome (Lipofectamine 2000) and perform lipofection in p53 mutant CL1-5 cells. The cells were transfected with (1) DNA/RNA oligonucleotide complex, (2) oligonucleotide, (3) liposome, (4) medium only. The purified PCR product for p53 codon 248 were treated with MSP-1 restriction enzyme. The genomic DNA of

SV40-transformed bronchoepithelium cells, which with wild type p53 gene, was used as positive control for MSP-1 RFLP assay. However, no obvious digestion of 110 bp PCR product was noted in 12.5% polyacrylamide gel.

五、Discussion

Our study demonstrates an improvement of lipofection efficiency using a new product of liposome. However, the DNA/RNA oligonucleotides/liposome complex showed little effect on mutant p53 gene of CL1-5 cells.

The possible reasons for poor effect on mutant p53 gene with DNA/RNA oligonucleotide/liposome complex transfection were (1) low lipofection efficiency (2) low rate of homologous recombination for oligonucleotide and mutant p53 gene (3) inactivation of DNA repairing system of cancer cell which is critical for mutaion correction of DNA/RNA chimeric oligonucleotide [10] (4) inadequate timing for harvest of transfected cell since the corrected cells may undergo apoptosis before harvest. We have improved the efficiency of lipofection. Thus the next object in our study is to improve the correction effeciency. Santana et al [11] demonstrated that transformed epithelial cells (HeLa) exhibited a conversion frequency of 5% by similar RNA/DNA oligonucleotides. In comparison, other imortalized epithelial cells (HaCaT) or human primary ketinocytes did not show any detectable level of gene conversion by the restriction fragment length polymorphism analysis, indicating less than 1% conversion frequency. In their study, The concentration of the oligonucleotide in the nuclei of HeLa cells was similar to that of HaCaT or human primary keratinocytes measured by a radiolabeled or a fluorescein-conjugated oligonucleotide appears to be a limiting factor in gene targeting events. Therefore, the frequency of gene targeting varies among different cells, suggesting that cellular recombination and DNA repair activities may be important.

A more recent study showed that correction of a mutant neomycin phosphotransferase gene in a human cell-free extract could be achieved by a using a series of structurally diverse chimeric oligonucleotides, with the presence of RecA protein [12]. In human cells, Rad51 protein is a member of the universally distributed class of RecA-like protein that play important role in homologous recombination and recombinational repair [13]. Overexpression of wild-type Rad51 was found in 66% of human pancreatic adenocarcinoma [14]. To investigate the factors influence the correction effeciency, it might be necessary to clarify the expression and function of Rad5.

Further studies may be needed to clarified and cope with these difficulties. Nevertheless, for the goal of cancer therapy, more oligonucleotides might be tested through the model we have applied in this study. The method we used in this study might also be used in the research of metabolic disorders due to single point mutation, or single nucleotide polymorphism in cancers.

六、Reference

1. Yang SP, Luh KT, Yang PC: New developments in diagnosis and management of lung cancer. *Respirology* 1996;1:37-46.
2. Dubinett SM, Miller PW, Sharma S, Batra RK: Gene therapy for lung cancer. *Hematol Oncol Clin N* 1998;12:569-94.
3. Wivel NA, Wilson JM: Methods of gene delivery. *Hematol Oncol Clin N* 1998;12:483-501.
4. Kren BT, Cole-Strauss A, Kmiec EB, Steer CJ: Targeted nucleotide exchange in the alkaline phosphatase gene of HuH-7 cells mediated by a chimeric RNA/DNA oligonucleotide. *Hepatol* 1997;25:1462-8.
5. Cole-Strauss A, Yoon K, Xiang Y, Byrne BC, Rice MC, Gryn J, Holloman WK, Kmiec EB: Correction of the mutation responsible for sickle cell anemia by an RNA-DNA Oligonucleotide. *Science* 1996;273:1386-9.
6. Lai L, Lien YH: Homologous recombination-based gene therapy. Mini-review. *Exp Nephrol* 1999;7:11-4.
7. Lai L, O'Connor HM, Inge LJ, Chan DM, Lien YH. Homologous recombination-based gene therapy corrects carbonic anhydrase (CA) II mutation in renal tubular cells. (abstract) *J Am Soc Nephrol* 1997;8:392A.
8. Yang PC, Luh KT, Wu R, Wu CW: Characterization of the mucin differentiation in human lung adenocarcinoma. *Am J Respir Cell Mol Biol* 1992;7:161-7.
9. Chu YW, Yang PC, Yang SC, Shyu YC, Hendrix MJC, Wu R, Wu CW: Selection of invasiive and metastatic subpopulations from a human lung adenocarcinoma cell line. *Am J Respir Cell Mol Biol* 1997;17:353-60.
10. Kren BT. Metz R. Kumar R. Steer CJ. Gene repair using chimeric RNA/DNA oligonucleotides. *Seminars in Liver Disease.* 19(1): 93-104, 1999.
11. Santana E, Peritz AE, Iyer S, Uitto J, Yoon K. Different frequency of gene targeting events by the RNA-DNA oligonucleotide among epithelial cells. J Invest Dermatol 1998, 111:1172-7
12. Gamper HB, Jr, Cole-Strauss A, Metz R, Parekh H, Kumar R, Kmiec EB. A plausible mechanism for gene correction by chimeric oligonucleotides. Biochemistry 2000, 39: 5808-16
13. Shinohara A, Ogawa H, Matsuda Y, Ushino N, Ikeo K, Ogawa T. Cloning of human, mouse and fission yeast recombination

14. gene homologous to Rad51 and RecA. Nat Genet 1993 4, 239-43
15. Maacke H, Jost K, Opitz S, Miska S, Yuan Y, Hasselbach L, Luttges J, Kalthoff H, Sturzbecher HW. DNA repair and recombination factor Rad51 is over-expressed in human pancreatic adenocarcinoma. Oncogene 2000 19:2791-5

5'---GGCGGCATGAACCGGAGGCCCATCC---3' **wild-type p53**
3'---CCGCCGTACTTGGCCTCCGGGTAGG---5'

5'--- GGCGGCATGAACTGGAGGCCCATCC---3' **CLI-5 mutation**
3'--- CCGCCGTACTTGACCTCCGGGTAGG---5'

TGCGCG-ccgccgtactTGGCCtccgggtaggT
T T **Oligo-CLI-5**
T T
TCGCGC$^{3'\,5'}$GGCGGCATGAACCGGAGGCCCATCC T

Fig. 1. Sequence of the wild-type p53 gene, CLI-5 mutation, and chimeric RNA/DNA oligonucleotide (Oligo-CLI-5). The site of mutation is underlined. The chimeric oligonucleotide contains 25 homologous DNA residues identical to wild-type allele, and only one bp different from CLI-5 mutation allele. The antisense strand contains five core DNA residues, which is flanked by ten 2'-*O*-methyl RNA residues at each side. A double-hairpin configuration is also included. DNA residues are capitalized and 2'-*O*-methyl RNA residues are in lowercase.