行政院國家科學委員會專題研究計畫成果報告

肺癌病人臨床檢體中 p53 基因突變之偵測

Detection of p53 Gene Mutations in Nonsurgical Specimens from Patients with Lung Cancer

計畫編號：NSC 88-2314-B-002-223
執行期限：87 年 8 月 1 日至 89 年 7 月 31 日

主持人：李麗娜　　國立台灣大學醫學院檢驗醫學科

：八十六年度及以前的一般國科會專題計畫（不含產學合作研究計畫）亦可選擇適用，惟較特殊的計畫如國科會規劃案等，請先洽得國科會各學術處同意。

一、中文摘要

p53 抑癌基因的突變是人類腫瘤的基因突變中最常見的一種。p53 基因突變率在小細胞癌約為 70%，在非小細胞癌約為 42 至 52%。本計畫之主持人曾研究過中國人非小細胞癌 p53 基因之突變，發現其突變率亦相當高，在鱗狀上皮癌為 45%，腺癌為 42%，且與縱膈腔淋巴結之轉移有關。然而過去大部分有關 p53 基因之研究，均取材於外科切除之肺癌標本，對於其他檢體，如支氣管鏡切片或胸水內肺癌細胞之 p53 基因之研究卻很少見，使得吾人無法得知那些無法接受外科切除之肺癌的 p53 基因突變狀態。主持人因而開始此項計畫，以 PCR-DGGE（聚合酶鏈鎖反應一變性梯級洋膠電泳法）偵測肺癌病人支氣管鏡切片檢體及胸水之 p53 基因突變。結果顯示，在 20 個原發性非小細胞癌之 20 個檢體（10 個支氣管鏡切片，10 個胸水）中，有 9 個（4 個支氣管鏡切片，5 個胸水）是經組織病理或細胞學檢查証實為惡性者。而在 4 個惡性之支氣管鏡切片中（均為鱗狀細胞癌），有一個（25%）有 p53 基因之突變，其肺癌之分期為 IIIA，突變發生在第 8 顯譯區，codon 273 （CGT→TGT）。在 5 個惡性胸水（均為腺癌）中，有一個（20%）有 p53 基因之突變，其肺癌之分期為 IIIB，突變位置在第 7 顯譯區，codon 250 (CCC→CTC）。與由外科手術切除之肺癌檢體所作之研究比較，本計畫所得到之突變率較低，其可能之原因包括：（1）檢體太小，肺癌細胞太少；（2）檢體數目太少；（3）DGGE 技術之困難。但非外科手術切除之臨床檢體之分子生物學診斷在將來仍可能是腫瘤診斷方面重要的一部分。

關鍵詞：p53 基因，突變，肺癌。

Abstract

Alterations of the p53 tumor suppressor gene are the most common genetic changes in human malignancies, including lung cancer. They have been found in 70% of small cell lung cancer and in 42-52% of the non-small-cell lung cancer. The authors have studied the p53 gene status in non-small-cell lung cancer patients in Taiwan, and has found a high mutation rate, being 45% in squamous cell carcinoma and 42% in adenocarcinoma. The frequency of metastasis in hilar/mediastinal lymph node is significantly higher in tumors with p53 mutation.

Most of the previous studies involving p53 gene status, including ours, were performed on surgically resected tumor samples. Only few studies were done on nonsurgical specimens such as bronchoscopic biopsies or pleural effusion. The diagnosis of lung cancer, however, is often made from nonsurgical specimens, and the majority of lung cancer patients are already in advanced stages at the time of diagnosis, and not fit for surgery. We therefore started this project to explore the possibility of detecting p53 gene mutations in nonsurgical specimens from patients with lung cancer. We used polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) to detect p53 gene mutations in bronchoscopic biopsy specimens and pleural effusion samples. We found that of the 20 specimens (10 from bronchoscopic biopsy and 10 pleural effusions) from 20 patients with untreated primary lung cancer, 9 (4 biopsy specimens, 5 pleural effusions) were proved malignant pathologically or cytologically. Among the 4 malignant bronchoscopic biopsy specimens (all squamous cell carcinoma), one (25%) showed p53 gene mutation. The patient was in stage IIIA, and the mutation occurred in

exon 8, codon 273 (CGT to TGT). Among the 5 malignant pleural effusions (all adenocarcinoma), one (20%) showed p53 gene mutation. The patient was in stage IIIB. The mutation occurred in exon 7, codon 250 (CCC to CTC). Compared with the p53 mutation rate found in surgically resected tumors, the mutation frequencies found in our study were lower. Possible causes for the lower rates include: (1) small specimen volume, (2) small sample size, and (3) technical difficulty associated with DGGE. But we believe molecular diagnosis of the nonsurgical specimens will be an important part in the diagnosis of tumors in the future.

Key words: p53 gene, mutation, lung cancer

INTRODUCTION

Of the more than 10 lung-cancer-related genes discovered in the past 25 years (1,2), the p53 tumor suppressor gene is one of the most important. Alterations of p53 gene are the most common genetic changes in human malignancies, including lung cancer (3-5). They have been found in about 70% of small cell lung cancer (6,7), and in 42 to 52% of non-small-cell lung cancer (8-14). However, these studies, including ours (14), were mainly of surgical specimens. Few papers have been published regarding the p53 status in nonsurgical specimens in inoperable patients who make up the majority of lung cancer patients (15-18). We therefore started to explore the possibility of detecting p53 gene mutations using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) in nonsurgical specimens from patients with lung cancer.

MATERIALS AND METHODS

(1) **Nonsurgical samples**

Using flexible fiberoptic bronchoscopy and thoracentesis, 20 specimens from 20 patients with untreated primary lung cancer were obtained. Bronchofiber-scopic biopsy specimens were obtained from visible tumors. Specimens were frozen at $-70^{\circ}C$ immediately until being processed. Pleural effusions were centrifuged at 7000 g for 5 min. After centrifugation, the cell pellets were stored at $-70^{\circ}C$ until being processed.

(2) DNA extraction

Cell pellets or biopsy specimens were lysed by lysis buffer containing 20 mM Tris HCL, pH 8.0/10 mM EDTA. Genomic DNA was extracted using proteinase K and sodium dodecyl sulfate (SDS) followed by phenol/chloroform extraction and ethanol precipitation.

(3) **PCR-DGGE analysis for p53 mutations in exons 3-9**

The oligonucleotide primers used to amplify the p53 gene were as follows:

Exons 3-4 3F: 5'GGA CTG ACT TTC TGC TCT TG 3' **3R**: 5' TGA AGT CTC ATG GAA GCC AG 3'

Exon 5: 5F: TGT TCA CTT GTG CCC TGA CT 3' **5R**: 5' AGC AAT CAG TGA GGA ATC AG 3'

Exon 6: 6F: 5' TGG TTG CCC AGG GTC CCC AG 3' **6R**: 5' GGA GGG CCA CTG ACA ACC A 3'

Exon 7: **7F**: 5'CTT GCC ACA GGT CTC CCC AA 3" **7R**: 5' AGG GGT CAG CGG CAA GCA GA 3'

Exon 8-9, **8F** 5' TTG GGA GTA GAT GGA GCC T 3' **8R** 5' AGT GTT AGA CTG GGA AAC TTT 3'

The genomic DNA (10-100 ng) was amplified in a 50 ul reaction mixture containing 200 uM each of deoxynucleo-tide triphosphate, 1.5 mM MgCl2, 0.25 uM each primer, and 1 unit of *Taq* DNA polymerase. The PCR protocol was: 60 s at $95^{\circ}C$, 60 s at 60 $^{\circ}C$ (exons 3-4), $63^{\circ}C$ (exons 5-7) or $58^{\circ}C$ (exons 8-9); and 90 s at $74^{\circ}C$ for 40 cycles.

DGGE analysis of the PCR-amplified genomic DNA fragments was carried out as follows: PCR products were electrophoresed at $60^{\circ}C$ on a 12% polyacrylamide gel with a linearly increas-ing denaturant concentration gradient (100% denaturant = 7 M urea and 40% formamide) as follows: exon 6 and 7, 30 to 40% denaturant at 240 V for 3 h; exons 3-4, 5 and 8-9, 20 to 30% denaturant at 150 V for 12 h. The gels

were stained with ethidium bromide and illuminated with ultraviolet light. Every abnormal band revealed by DGGE analysis was cut out from the gels, reamplified by PCR, and sequenced using a automatic double-stranded DNA cycle sequencing system. The primers used to amplify each fragment were the same as the sequencing primers.

Results

A total of 20 specimens from 20 patients with previously untreated primary lung cancer were studied. Of these, 9 specimens (45%) were pathologically or cytologically positive, including 4 bronchoscopic biopsy specimens and 5 pleural effusion samples. We found p53 mutations in 2 (22%) of the 9 patients, including 1 of 5 (20%) pleural effusion samples (all adenocarcinoma), and 1 of 4 (25%) bronchoscopic biopsy specimens (all squamous cell carcinoma) (Table). No p53 gene mutations were detected in any of the 11 pathologically or cytologically negative specimens

Table p53 Mutations in 9 Patients with Non-small-cell Lung Cancer

No	Cell type	Age	Sex	Staging	Specimen
1	Adeno	72	M	IV	P* effusion
2	Adeno	77	M	IIIB	P effusion
3	Adeno	40	M	IIIB	P effusion
4	Adeno	50	F	IIIB	P effusion
5	Adeno	70	M	IV	P effusion
6	Squam	71	M	I	B** biopsy
7	Squam	77	M	IIIA	B biopsy
8	Squam	67	M	IV	B biopsy
9	Squam	70	M	IV	B biopsy

No	p53 mutation exon	Mutation codon
1	-	-
2	7	250 (CCC → CTC)
3	-	-
4	-	-
5	-	-
6	-	-
7	8	273 (CGT → TGT)
8	-	-
9	-	-

*P: pleural; **B: bronchoscopic

DISCUSSION

In this study we have demonstrated the possibility of detecting p53 gene mutations in nonsurgical specimens. In contrast to *ras* mutations, which are usually localized to *ras* codons 12, 13 and 61 (20), p53 mutations showed many different types: missense, non-sense, splicing and deletion(1). These mutations can occur through the open reading frame, though they are most frequent in exons 5 through 8 (1,14). The detection of p53 mutations, therefore, demands more laborious techniques, and could be very difficult in small biopsy specimens or pleural effusions containing few malignant cells. Dowell and coworkers (16) reported their detailed analysis of p53 protein expression in cytologic specimens including sputum, bronchial washings and needle washings of lung biopsy samples, using a murine monoclonal antibody. p53 overexpression was detected in 21% of the cytologically positive samples. Murakami and colleagues (18) used PCR/DGGE techniques and found p53 gene mutations in 40% of cytologically positive specimens, including bronchoscopic biopsy, percutaneous needle aspiration, brushing, washing and pleural effusion samples.

Although PCR-single-strand conformation polymorphism (SSCP) has been widely used to detect p53 mutations, DGGE analysis has an equal capacity for detecting p53 mutations (21). It is preferred

because there is no need for radioisotopes. There-fore we chose this method for our study. We found that p53 mutation rate was 20% (1/5) for adenocarcinoma and 25% (1/4) for squamous cell carcinoma. In previous studies examining surgical specimens, the mutation rate was 32 to 41% in adenocarcinoma (6-11, 14), 33 to 68% in squamous cell carcinoma (6-11, 14) and 61 to 87% in small cell carcinoma (2-5). The frequencies of p53 mutation observed in this study were lower. Possible causes for the lower rates include: (1) the small volume of the bronchoscopic biopsy specimens and pleural fluids, with scanty cancer cells, (2) the small number of samples, and (3) technical difficulty of the DGGE.

The most important application of molecular analysis using nonsurgical specimens is knowing the relationship between p53 gene mutation and prognosis for the patient at the time of first diagnosis. Due to the small sample size in our study, we could not find such relationship. Murakami and coworkers (18) demonstrated that for patients with untreated, inoperable lung cancer at stage IIIA or higher, those without p53 gene mutations survived longer than those with mutations. We believe that molecular diagnosis for nonsurgical specimens could be valuable in prognosis-telling and even therapy-decision-making, as p53 has been shown to determine the sensitivity of cells to radiation and/or anticancer agents (23, 24).

CONCLUSION

We tested the potential of a PCR-DGGE assay in detecting p53 gene mutations in nonsurgical specimens of patients with primary lung cancer, and found it could be used for the molecular diagnosis of nonsurgical specimens.

REFERENCES

1. Vialett J, Minna JD. Dominant oncogenes and tumor suppressor genes in the pathogenesis of lung cancer. Am J Respir Cell Mol Biol 1990; 2: 225-32.
2. Anderson MLM, Spandidos DA. Oncogenes and onco-suppressor genes in lung cancer. Respir Med 1993; 87: 413-20.
3. Levine AJ, Momand J, Finlay CA. The p53 tumor suppressor gene. Nature 1991; 351: 453-6.
4. Hollstein M, Sidransky D, Vogelstein B, Harris CC. P53 mutations in human cancers. Science 1991; 253: 49-53.
5. Harris C, Hollstein M. Clinical implication of the p53 tumor-suppressor gene. N Engl J Med 1993;329:1318-27.
6. Takahashi T, Takahashi T, Suzuki H, Hida T, Sekido Y, Ariyoshi Y, Ueda R. The p53 gene is very frequently mutated in small-cell lung cancer with a distinct nucleotide substitution pattern. Oncogene 1991;6:1775-8.
7. Miller CW, Simon K, Aslo A, Kok K, Yokota J, Buys CHCM, Terada M, Koeffler HP. p53 mutations in human lung cancer. Cancer Res 1992; 52: 1695-8.
8. Chiba T, Takahashi T, Nau MM, D'Amico D, Curiel DT, Mitsudomi T, Buchhagen DL Carbone D, Piantadosi S, Koga H, Reissman PT, Slamon DJ, Holmes EC, Minna JD. Mutations in the p53 gene are frequent in the primary, resected non-small cell lung cancer. Oncogene 1990; 5: 1603-10.
9. Suzuki H, Takahashi T, Kuroishi T, Suyama M, Ariyoshi Y, Takahashi T, Ueda R. p53 mutations in non-small cell lung cancer in Japan: association between mutations and smoking. Cancer Res 1992; 52: 734-6.
10. Kishimoto Y, Murakami Y, Shiraishi M, Hayashi K, Sekiya T. Aberrations of the p53 tumor suppressor gene in human non-small-cell carcinoma of the lung. Cancer Res 1992; 52: 4799-804.
11. Mitsudomi T, Oyama T, Kusano T, Osaki T, Nakanishi R, Sirakusa T. Mutations of the p53 gene as a predictor of poor prognosis in patients with non-small-cell lung cancer. J Natl Cancer Inst 1993; 85: 2018-23.
12. Horio Y, Takahashi T, Kuroishi T, Hibi

K, Suyama M, Niimi T, Shimokata K, Yamakawa K, Nakamura Y, Ueda R, Takahashi T. Prognostic significance of p53 mutations and 3p deletions in primary resected non-small cell lung cancer. Cancer Res 1993; 53: 1-4.
13. Takeshima Y, Seyama T, Bennett WP, Akiyama M, Tokuoka S, Inai K, Mabuchi K, Land CE, Harris CC. p53 mutations in lung cancers from non-smoking atomic bomb survivors. Lancet 1993;342:1520-1.
14. Lee L-N, Shew JY, Sheu JC, Lee YC, Fang MT, Chang HF, Yu CJ, Hang PC, Luh KT. Exon 8 mutation of p53 gene associated with nodal metastasis in non-small-cell lung cancer. Am J Respir Crit Care Med 1994; 150: 1667-71.
15. Hirano T, Franzen B, Kato H, Ebihara Y, Auer G. Genesis of squamous cell lung carcinoma: sequential changes of proliferation, DNA ploidy, and p53 expression. Am J Pathol 1994; 144: 296-302.
16. Dowell SP, Wilson POG, Derias NW, Lane DP, Hall PA. Clinical utility of the immunocytochemical detection of p53 protein in cytological specimens. Cancer Res 1994; 54: 2914-8.
17. Gazdar AF. Detection and sequencing of the p53 gene mutations in bronchial biopsy samples in patients with lung cancer. Chest 1993; 104: 362-5.
18. Murakami I, Fujiwara Y, Yamaoka N, Hiyama K, Ishioka S, Yamakido M. Detection of p53 gene mutations in cytopathology and biopsy specimens from patients with lung cancer. Am J Respir Crit Care Med 1996; 154: 1117-23.
19. Hiyama K, Ishioka S, Shirotani Y, Inai K, Hiyama E, Mucarami I, Isobe T, Inamizu T, Yamakido M. Alterations in telomeric repeat length in lung cancer are associated with loss of heterozygosity in p53 and Rb. Oncogene 1995; 10: 937-44.
20. Boss JL. *ras* oncogenes in human cancer: a review. Cancer Res 1989; 9: 4682-9.
21. Moyret C, Theillet C, Puig PL, Moles J-P, Thomas G, Hamelin R. Relative efficiency of denaturing gradient gel electrophoresis and single strand conformation polymorphism in the detection of mutations in exon 5 to 8 of the p53 gene. Oncogene 1994; 9: 1739-43.
22. Lee JM, Berstein A. p53 mutations increase resistance to ionizing radiation. Proc Natl Acad Sci USA. 1993; 90: 5742-6.
23. Fujiwara T, Grimm EA, Mukhopadhyay T, Zhang WW, Owen-Schaub LB, Roth JA. Induction of chemosensitivity in human lung cancer cells in vivo by adenovirus-mediated transfer of the wild-type p53 gene. Cancer Res 1994; 54: 2287-91.