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Correlation of Elevated mRNA Expression of *p53* Gene and Repair Genes with Lung Cancer Chemoresistance

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ABSTRACT

Lung cancer is the leading cause of cancer death in Taiwan. In the chemotherapeutic modalities used to treat the lung cancer, the great majority of patients will relapse with tumor that is largely resistant to further treatment. In addition, clinical and experimental studies suggest that alteration of the expression level of the *p53* gene and repair genes may be associated with chemoresistance. Therefore, we evaluated the differences in the expression levels of *p53* gene and four repair genes, including *XPCC*, *hMSH2*, *XRCC1*, and *ERCC1*, in 12 chemo-resistant lung cancer patients and 11 chemo-sensitive lung cancer patients using an optimized quantitative multiplex reverse transcriptase-polymerase chain reaction (RT-PCR). The modification experiments of the quantitative multiplex RT-PCR showed that amplification was linear between 26-32 cycles and 30-34 cycles for the analyses of the *p53* gene and repair genes, respectively. We used this optimized quantitative multiplex RT-PCR to analyze the correlation of mRNA expression of the *p53* gene and the repair genes with chemo-resistance of lung cancer. The data indicated that the mean mRNA level of the *p53* gene in chemo-resistant patients (0.65) was 132.1 % higher than that of the chemo-sensitive patients (0.28). The chemo-resistant cases had an almost significantly higher ($P = 0.07$) mean expression level of the *p53* gene than did the effective cases. Our results suggest that an elevated *p53* mRNA level may predict the chemoresistance of lung cancer patients.

Key words: p53 gene, Repair gene, RT-PCR, Lung cancer, Chemoresistance

Introduction

Lung cancer is the leading cause of cancer death in Taiwan (Department of Health, 1998). Lung cancer patients who have undergone potentially curative resection often died due to cancer recurrence within 5 years of resection. In addition, in the radiotherapeutic and chemotherapeutic modalities used to treat the lung cancer, the great majority of patients will relapse with tumor that is largely refractory to further treatment. Therefore, the understanding of the mechanism of resistance of cancer cells is necessary for the development of a powerful treatment regimen.

Clinical and experimental studies suggest that alteration of the expression level of repair genes may be associated with chemoresistance. For example, the mRNA expression level of *ERCC1* and *XPAC* genes is associated with the

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resistance of tumor tissues to chemotherapy in ovarian cancer patients (Dabholkar *et al.*, 1992; 1994), whereas the expression level of *ERCC2* is not (Dabholkar *et al.*, 1992). Increased mRNA expression of *ERCC1* also occurs with chemoresistance in chronic lymphocytic leukemia patients (Geleziunas *et al.*, 1991). In addition, evidences show that exposure of cells to DNA damage and other types of stress causes overexpression of the *p53* tumor suppressor gene (Fritsche *et al.*, 1993; Lowe *et al.*, 1993a). In addition, *p53*-dependent apoptosis mechanisms appear to be involved in the cytotoxicity induced by ionizing radiation and by several anticancer agents (Lowe *et al.*, 1993a; 1993b). *In vitro* and *in vivo* studies have shown that aberrant p53 protein expression is linked to the clinical resistance of several chemotherapeutic drugs (Blandino *et al.*, 1999; Rusch *et al.*, 1995). In addition, the overexpression of p53 protein has been found to positively correlate with an elevated *p53* mRNA expression in human hepatocellular carcinoma (Hsu *et al.*, 1993) and colorectal cancer (El-Mahadani *et al.*, 1997). However, the correlation of the mRNA expression levels of the *p53* gene and the repair genes in lung tumorigenesis as well as in the chemotherapeutic response still remains undefined due to the lack of an appropriate population screening method.

For population screening, such an assay must be simple, rapid, reproducible, and easy to apply to readily accessible tissue specimens such as blood. Blood lymphocytes have been previously shown to be a valid surrogate tissue for estimating the DNA adduct level and repair capacity of the target tissues (Athas *et al.*, 1991; Li *et al.*, 1996). Wei and associates have developed a multiplex reverse transcriptase-polymerase chain reaction (RT-PCR) method to co-amplify the transcripts of multiple genes in a single reaction with ethidium bromide staining in human peripheral blood lymphocytes (Wei *et al.*, 1995). The levels of gene expression determined by this nonradioactive PCR analysis were associated with the levels determined by slot blot analysis (Dabholkar *et al.*, 1992; Chen *et al.*, 1997).

To investigate the association of the mRNA expression levels in the *p53* gene and repair genes in lung chemoresistance, we modified the multiplex RT-PCR method to compare the expression levels of the *p53* gene and the repair genes, including two nucleotide excision repair genes (*XPCC* and *ERCC1*) (Sancar *et al.*, 1996), one base excision repair gene (*XRCC1*) (Cappelli *et al.*, 1997), and one mismatch repair gene (*hMSH2*) (Thibodeau *et al.*, 1996), in the isolated lymphocytes of lung cancer patients with different chemo-responses. We tested the possibility of whether the mRNA expression levels of the *p53* gene and the repair genes correlated with the chemotherapeutic responses of lung cancer patients.

Materials and Methods

Subjects. Twenty-three patients received cisplatin-based chemotherapy. We followed the course of the disease in these patients by physical examinations and by radiographic means. Complete response was defined as the

resolution of all radiographic findings after chemotherapy. Partial response was defined as a greater than 50% reduction in the sum of the products of the perpendicular diameters of all measurable lesions or the appearance of new lesions. Progressive disease was defined as an increase in disease of greater than 25%, as measured for partial response. Stable disease was applied to those patients who did not achieve a partial response but did not develop new lesions and whose symptoms did not worsen for a minimum of 8 weeks. Complete response and partial response are included in the responder category. Patients with progressive disease and stable disease are both included in the non-responder category. Using these criteria, there were 11 responders (chemo-sensitive patients) and 12 non-responders (chemo-resistant patients).

Multiplex RT-PCR analysis. Heparinized venous blood was collected from lung cancer patients. Buffy coats containing approximately 85-90% lymphocytes were isolated. The poly(A)+RNA of blood lymphocytes was prepared by using a TRIZOL reagent (Gibco) from the peripheral blood samples. The RNA was quantified and its purity was checked by measuring the A_{260} and A_{260}/A_{280} ratios (which were 1.8-2.0). cDNA was synthesized from the isolated RNA by RT reaction in 20 *u*l reactions containing 0.5 *u*g of oligo dT primers (New England BioLabs), 50 units of SuperSCRIPT™ RT enzyme (Gibco), 4 *u*l of 5x RT buffer, 100 mM DTT, 0.1 mM of each dNTP, and 2.5 *u*g of total cellular RNA. Each reaction was incubated at 70°C for 10 min and then at 42°C for 50 min, heated to 70°C for 10 min, and then quick-chilled on ice.

The expression levels of the repair genes, including the *XPCC*, *hMSH2*, *XRCC1*, and *ERCC1* genes, were assayed in a multiplex PCR analysis using the *β-actin* gene as an internal control. The oligodeoxynucleotide primers designed to produce DNA fragments of these repair genes have been described previously (Wei *et al.*, 1995) and are shown in Table 1. The thermocycle PCR conditions were as described in Wei *et al.* (1995) with the modifications of 37.5 *u*M *XPCC* primers, 25 *u*M *hMSH2* primers, 2.5 *u*M *XRCC1* primers, 2.5 *u*M *ERCC1* primers, and 0.25 *u*M *β-actin* primers. The cycling profile was an initial denaturation at 95°C for 5 min; 34 cycles at 95°C for 1 min, 58°C for 1.75 min, and 72°C for 1.75 min; and a final elongation step at 72°C for 10 min. The expression level of the *p53* gene was detected by a multiplex PCR assay using the *β-actin* gene as an internal control. The primer nucleotide sequences for the *p53* gene are also shown in Table 1. The primer concentrations were 4 *u*M for *p53* primers and 0.25 *u*M for *β-actin* primers. The cycling profile was an initial denaturation at 95°C for 5 min; 31 cycles at 95°C for 1 min, 56°C for 1.75 min, and 72°C for 1.75 min; and a final elongation step at 72°C for 10 min. Each PCR reaction was performed in a 50 *u*l reaction mixture containing 1 *u*l of RT reaction mixture, 1.25 units of *Taq* polymerase (Takara), 1x PCR buffer, 0.1 mM of each dNTP, and the indicated amount of primers. The samples were assayed in batches including both cases and controls. Each sample was assayed by repeating PCR

Table 1. Sequences of oligonucleotide primers used to amplify four DNA repair genes, the *p53* gene, and the *β-actin* gene.

Target gene	Sense primer	Antisense primer
XPCC	5'AAGCAGGAGAAGGCAACCCAG3'	5'AGCCGTCACTGTCAATGCCCA3'
hMSH2	5'GTCGGCTTCGTGCGCTTCTTT3'	5'TCTCTGGCCATCAACTGCGGA3'
XRCC1	5'GCAAACCCCGAGGAGAAGGCA3'	5'ACTGCTGGAACCTGGCCCTGC3'
ERCC1	5'CCCTGGGAATTTGGCGACGTAA3'	5'CTCCAGGTACCGCCCAGCTTCC3'
p53	5'AAGACCTGCCCTGTG3'	5'TGACGCACACCTATTGCAAG3'
β-actin	5'GGCGGCACCACCATGTACCCT3'	5'AGGGGCCGGACTCGTCATACT3'

analyses at least twice. The PCR products were then electrophoresed in a 3% agarose gel and stained with 0.5 *ug*/ml ethidium bromide.

Densitometric quantification of the mRNA expression. To quantify the relative levels of gene expression in the multiplex RT-PCR assay, the value of the internal standard (*β-actin*) in each test tube was used as the baseline gene expression of that sample, and the relative value was calculated for each of the target genes amplified in the reaction. These values were then used to compare expression across the samples tested.

Statistical analysis. The mRNA expression level of the repair genes was calculated for each individual after having been adjusted by the internal control *β-actin*. The mean value and the standard error were calculated. Differences in the expression level of genes between chemo-resistant and chemo-sensitive subjects were analyzed for significance by the two-tailed Student *t* test.

Results

Modifications and optimization of the multiplex RT-PCR assay. Initial experiments were designed to determine the number of cycles that could provide quantitative amplify-cation during multiplex RT-PCR. For these experiments, the expression level of repair genes, including *XPCC, hMSH2, XRCC1,* and *ERCC1* genes, was analyzed by the multiplex RT-PCR for 30-40 cycles. Amplification was linear between 30-34 cycles for these genes (Figure 1). The expression level of the *p53* genes was analyzed by the multiplex RT-PCR for 26-34 cycles (Figure 2). Amplification was linear be- tween 26-32 cycles. To prevent the plateau effect, the subsequent amplification was performed at 34 cycles for the *XPCC, hMSH2, XRCC1,* and *ERCC1* genes and 31 cycles for the *p53* gene.

mRNA expression of *p53* and repair genes in lung cancer patients with respect to chemotherapeutic response. Table 2 shows the differences in the expression levels of the *XPCC*, *hMHS2*, *XRCC1*, *ERCC1,* and *p53* genes among the 23 lung cancer patients who received the cisplatin-based therapy. In general, lung cancer patients showing a resistance to therapy tended to have higher mean expression levels of the repair genes than did the therapeutically effective patients, though the differences were not statistically significant. However, a nearly significant difference in the

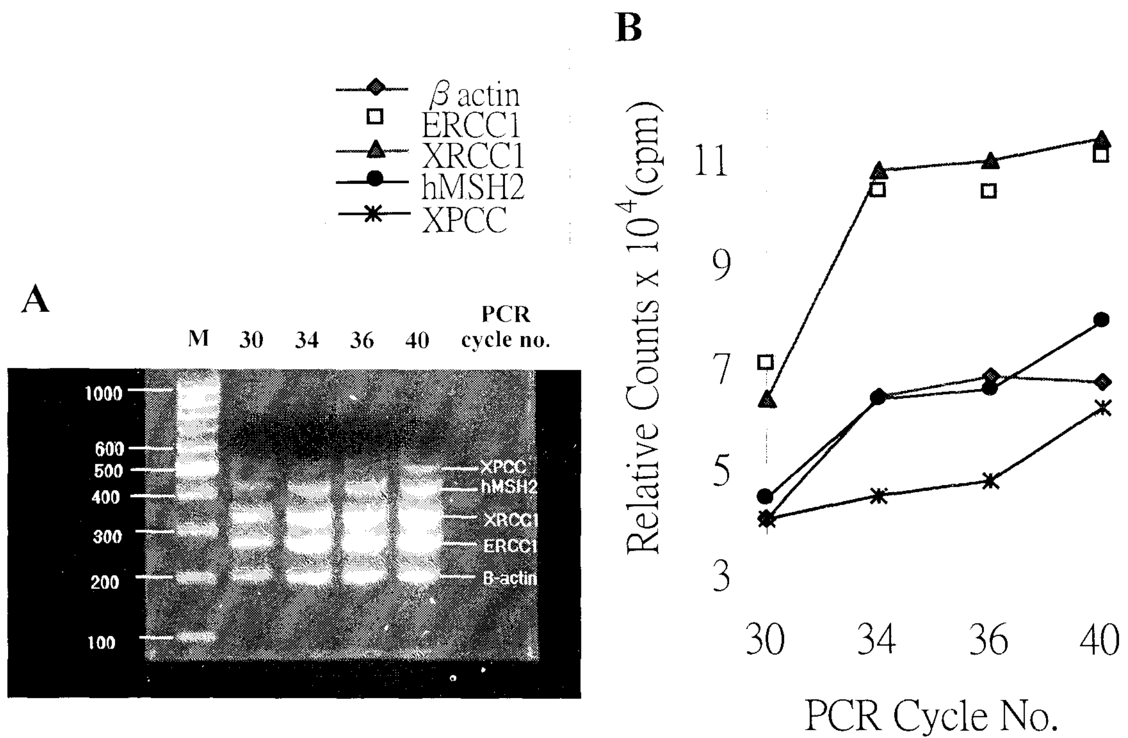

Figure 1. Kinetics of RT-PCR amplification of *XPCC*, *hMSH2*, *XRCC1*, and *ERCC1* genes from cycles 30 to 40. (A) is the photograph of an agarose electrophoresis gel. The amplification of the *β-actin* indicates the initial RNA template used for each reaction. RNA was purified and complementary DNA was made from 5 *ug* RNA and subsequently amplified by PCR as described in Materials and Methods. (B) is the densitometric quantitation of each band in the left panel.

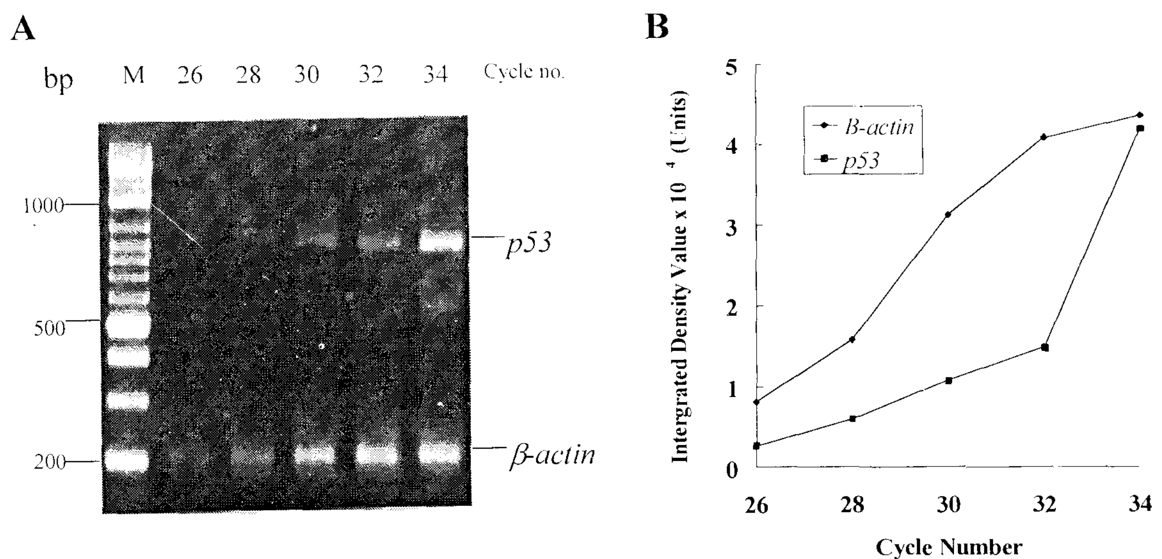

Figure 2. Kinetics of multiplex RT-PCR amplification of the *p53* gene and the *β-actin* gene from cycles 26 to 34. (A) is the photograph of an agarose electrophoresis gel. The PCR and electrophoresis conditions were described in Materials and Methods. (B) is the densitometric quantitation of each band in the upper panel.

Table 2. Differences in the mRNA expression[a] of *XPCC*, *hMSH2*, *XRCC1*, *ERCC1*, and *p53* genes in chemotherapeutic cases with respect to the effectiveness of therapy.

Gene	Chemotherapy effectiveness	Number of Samples	Range	Median	Mean±SD	Percent[b] difference	P[c] values
XPCC	No	12	0.16-1.24	0.59	0.60±0.34	+7.1	0.970
	Yes	11	0.14-1.66	0.32	0.56±0.50		
hMSH2	No	12	0.16-1.34	0.40	0.47±0.34	+11.9	0.720
	Yes	11	0.16-0.80	0.45	0.42±0.21		
XRCC1	No	12	0.22-1.75	0.73	0.74±0.43	0	0.903
	Yes	11	0.29-1.60	0.74	0.74±0.40		
ERCC1	No	12	0.21-1.76	0.68	0.79±0.46	+3.9	0.845
	Yes	11	0.36-1.24	0.89	0.76±0.31		
p53	No	12	0.15-1.61	0.50	0.65±0.53	+132.1	0.073
	Yes	11	0.07-0.99	0.13	0.28±0.29		

[a] The expression level of each gene was calculated by using the internal standard (β-*actin*) in each reaction as the baseline gene expression of that sample to calculate the relative value for each of the target genes amplified in that reaction.
[b] Percent difference = $[(mean_{no} - mean_{yes})/mean_{yes}] \times 100$.
[c] P values were calculated by the two-sided t test analysis for the difference of Mean ± SD between cases and controls.

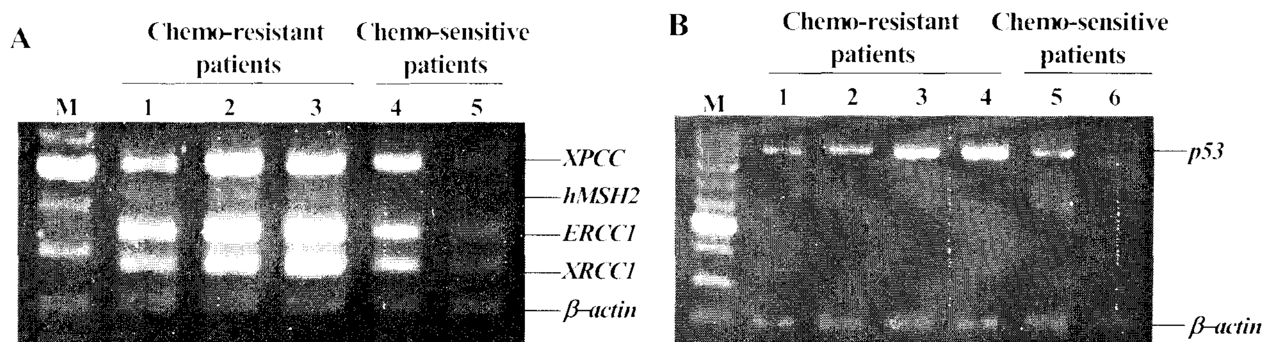

Figure 3. Representative figures of multiplex RT-PCR analysis of (A) *XPCC*, *hMSH2*, *ERCC1*, *XRCC1* and (B) *p53* genes in a batch of blood samples from lung cancer patients. The amplification of the *β-actin* indicated the initial RNA template used for each reaction. M is molecular weigh marker. Numbers are the sample numbers. The chemo-responses of each patient were indicated above the sample numbers.

mean expression level between the chemo-resistant patients and the chemo-sensitive patients was observed for *p53*. The mean level of the chemo-resistant patients (0.65±0.53) was 132.1 % higher than that of the chemo-sensitive patients (0.28±0.29). The chemo-resistant lung cancer patients had an almost significantly higher (P=0.073) mean expression level of the *p53* gene than did the chemo-sensitive patients. Figure 3 shows the representative figure of quantitative multiplex RT-PCR analyses in lung cancer patients with different chemoresponses.

Discussion

Quantitative multiplex RT-PCR used for comparing the relative amount of mRNA in different cells has been shown to be a reliable method for the simultaneous amplification of several genes in laboratory studies (Chen *et al.*, 1997; Wei *et al.*, 1997; 1998). Our study showed that the method is a useful tool for screening the genetic alterations in population studies. To test the reproducibility of the assay procedure, the quantitative multiplex RT-PCR assayed at different times and with different aliquots of RNA from each individual. The samples from chemo-resistant and chemo-sensitive cases were processed together in batches, without knowledge of which were from resistant cases and which from sensitive cases, to eliminate the possibility of information bias. The results of different assays were consistently similar, and the rank order of the expression levels of different genes remained constant across the different assays (data not shown). Therefore, the observed wide range of expression levels should result from inter-individual variation rather than experimental variation. The genetic heterogeneity of the expression levels of the repair gene has been previously suggested (Thibodeau *et al.*, 1996; Wei *et al.*, 1998), and possibly there is an un-measured environmental influence on epi-genetic regulation of gene expression (McLeod *et al.*, 1996).

In this study, we found an overexpression of *p53* mRNA may be linked to chemo-therapeutic resistance in lung cancer patients. Other studies have shown that the over-expression of p53 protein is significantly correlated with unresponsiveness to chemo-therapy in lung cancer patients (Rusch *et al.*, 1995; Kawasaki *et al.*, 1997). However, little clinical data has been reported to support the association of an elevated *p53* mRNA expression with chemoresistance. The quanti-tative multiplex RT-PCR method permitted us to explore the relationship between *p53* mRNA expression and response to chemotherapy in lung cancer patients. The results suggested that the non-responder cases tended to have an elevated level of *p53* mRNA expression compared to the responder cases (Table 2). The comparison of *p53* RNA expression before and after chemotherapy showed that there was no change in the level of *p53* expression (data not shown). This indicated that the observed elevated *p53* mRNA expression in resistant cases is not induced by the chemotherapy and may be determined by intrinsic host character-istics. The elevated *p53* mRNA expression has been found to positively correlate with an overexpression of p53 protein in human hepato-cellular carcinoma (Hsu *et al.*, 1993) and colorectal cancer (El-Mahadani *et al.*, 1997). The elevated level of *p53* mRNA expression in chemoresistant lung cancer patients may be due to the mutation in the *p53* gene. When a *p53* gene mutation occurs, the absence of negative feedback might be responsible for *p53* mRNA overexpression (Hussain *et al.*, 1998). Alterna-tively, overexpression of *p53* mRNA may result from the deregulation of *p53* transactivation controls, such as the Myc/Max (Roy *et al.*, 1994)

or MDM-2 pathway (Wu *et al.*, 1993). In addition, exposure of cells to various genotoxic agents, including anticancer drugs such as mitomycin and 5-fluorouracil, has been found to result in an increase in *p53* mRNA levels and in *p53* promoter activation, indicating that the *p53* genotoxic stress response is partly regulated at the transcriptional level (Sun et al., 1995). The high mRNA expression of the *p53* gene in the chemoresistant patients may cause high expression of the p53 protein, thus trigger elevated expression of damage response genes involved in cell cycle arrest and DNA repair in resistant patients. These possibilities are currently under the investigation. The number of cases available for analysis in this study is too small to allow statistically valid correlation between the mRNA expression levels and chemoresistance of patients. However, our results have important clinical implications. *p53* mRNA expression levels that predict response to chemotherapy could identify those most likely to benefit from treatment while avoiding needless toxic effects in those patients who will not respond. A larger prospective study is needed to confirm the findings of this report. Further work on the correlation between induced transcription or transcript stability of the *p53* gene with p53 protein function should shed light on the underlying molecular mechanisms.

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p53 抑癌基因與四種 DNA 修補基因其 mRNA 表現量增加與肺癌病人化療抗藥性之相關性研究

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摘　要

肺癌是國人最重要的癌症致死原因，大部分接受放射線治療及化學治療的肺癌患者，常常因產生抗性而治療失敗，因此了解癌細胞抗藥性之機制對於發展更有效的治療將有莫大的幫助。已有文獻指出 *p53* 抑癌基因與數種 DNA 修補基因其 mRNA 表現增加與癌症病人化療抗藥性具有正相關，所以本研究以定量同步反轉錄聚合酵素反應（quantitative multiplex reverse transcription-polymerase chain reaction）同時檢測 *p53* 抑癌基因與四種 DNA 修補基因 *XPCC*, *hMSH2*, *XRCC1*, 與 *ERCC1* 的表現量；為了瞭解這些基因的 mRNA 表現是否能作為肺癌病人治療效果的監控指標，此定量同步反轉錄聚合酵素分析，以 12 位具有化療抗藥性的肺癌病人與 11 位治療有效的病人作比較分析。在改良過的定量同步反轉錄聚合酵素反應中，顯示 *p53* 抑癌基因與四種修補基因分別在放大 26-32 與 30-34 個循環數時，會呈現線性關係，因此利用此反應條件，分析肺癌病人之 *p53* 抑癌基因與四種修補基因 mRNA 的表現量與抗藥性的相關性。結果顯示，具化療抗藥性的病人其 *p53* 抑癌基因的平均表現量為 0.65，化療無效的病人為 0.28，此差異有接近顯著的統計分析趨勢（$P=0.07$）。本研究結果推測，*p53* 基因的 mRNA 表現量的提高或許可以成為肺癌病人化療抗藥性的預測指標。

關鍵詞： *p53* 抑癌基因、DNA 修補基因、反轉錄聚合酵素反應、肺癌、抗藥性

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