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**THE mRNA EXPRESSION OF CYTOCHROME P450 ENZYMES IN
PERIPHERAL BLOOD LYMPHOCYTES IN PATIENTS WITH LUNG CANCER**

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Key Words: benzo(a)pyrene, carcinogenesis, cytochrome P450 enzymes, lung cancer, mRNA expression, mutagens, quantitative competitive reverse transcription polymerase chain reaction, smoking, tobacco.

Abstract

Several mutagens including benzo(a)pyrene (B[a]P) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in tobacco smoke are known to be metabolized by cytochrome P450 enzymes (CYPs). These mutagens may induce airway carcinogenesis. We measured the mRNA expression of CYPs 1A1, 1A2, 2A6/7, 2B6, 4B1, 2C, 2D6 and 2E1 the peripheral blood lymphocytes from 30 patients with lung cancer and 30 healthy controls by semi-quantitative competitive reverse transcription polymerase chain reaction (QC-RT-PCR) in order to determine the expression levels of these CYPs in patients with lung cancer. The mRNA levels of CYPs 1A1 ($p < 0.001$), 4B1 ($p < 0.001$) and 2C ($p < 0.001$) were significantly higher and CYPs 2D6 ($p < 0.0001$) and 2E1 ($p < 0.0001$) were significantly lower in patients with lung cancer than in healthy controls. The same results

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were obtained when only smokers in both groups were compared. Our study revealed that the mRNA expression levels of CYPs in peripheral blood lymphocytes were different in patients with lung cancer and healthy controls. The elevated level of expression of some CYPs in the patients may reflect the risk of carcinogenesis of lung cancer.

Introduction

Since lung cancer is the most common cancer worldwide, it will probably remain the major cause of cancer deaths among both men and women at least in the initial period of the 21st century. The etiological factors are still unclear. However, it is clear that long-time cigarette smoking increases the risk of lung cancer. Benzo[a]pyrene (B[a]P) is one of the polycyclic aromatic hydrocarbons (PAH) found in tobacco smoke, and its metabolite benzo[a]pyrene diol epoxide (BPDE) is considered to be a classic DNA-damaging carcinogen (Denissenko, Pao *et al.*, 1996; Li, Wang *et al.*, 1996). In several recent studies have demonstrated that the cytochrome P450 enzyme (CYPs) family plays an important role in the metabolism of pro-carcinogens, for example B[a]P and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) to carcinogens (Hukkanen, Lassila *et al.*, 2000; Vanden Heuvel, Clark *et al.*, 1993; Hecht 1996; Rojas, Camus *et al.*, 1992). Several CYPs 1A1, 4B1, 2C, 2D6 and 2E1 are expressed in normal organs including the liver (Andersen, Farin *et al.*, 1998), lung (Li, Wang *et al.*, 1996; Hukkanen, Hakkola *et al.*, 1997), bronchoalveolar macrophages (Hukkanen, Hakkola *et al.*, 1997) and peripheral blood cells (Hukkanen, Hakkola *et al.*, 1997; Krovat, Tracy *et al.*, 2000). They are also expressed in malignant tissues, such as lung (Hukkanen, Lassila *et al.*, 2000; Goto, Yoneda *et al.*, 1996), esophagus (Itoga, Nomura *et al.*, 2002), larynx (Agundez, Gallardo *et al.*, 2001), liver (Sumida, Fukuen *et al.*, 2000), and breast cancer (Xiong, Bondy *et al.*, 2001). Several previous studies demonstrated that B[a]P is metabolized in the lung microsomes and pulmonary alveolar macrophages by CYPs 1A1, 4B1, and 2C (Hukkanen, Hakkola *et al.*, 1997; Thompson, McCoy *et al.*, 1989; McLemore, Martin *et al.*, 1977). The suitability of using CYPs expression in human lymphocytes as a biomarker of metabolism of pro-carcinogens was demonstrated in other reports (Li, Wang *et al.*, 1996; Rojas, Camus *et al.*, 1992). Li, Wang *et al.*, (1996) demonstrated both a high degree of interindividual variability in CYP1A1 inducibility,

and a correlation between the level of its inducibility in lymphocytes and the risk of smoking-related lung cancer. Other *in vitro* studies have suggested that B[a]P is metabolized in lymphocytes (Okano, Miller *et al.*, 1979; Gurtoo, Vaught *et al.*, 1980). Hukkanen, Hakkola *et al.*, (1997) used reverse transcription polymerase chain reaction (RT-PCR) to evaluate the expression of CYPs in human bronchoalveolar and peripheral lymphocytes to assess the potential utility of peripheral lymphocytes as a surrogate for lung tissue. Law (1990) reported that peripheral lymphocytes can be used as a surrogate for lung tissue to study susceptibility to lung cancer. He found aryl hydrocarbon hydroxylase (AHH) activity was significantly higher in lung cancer cases in 13 (65%) out of 20 studies examining peripheral lymphocytes in case-control studies. Those reports indicate the CYP mRNA expression in peripheral lymphocytes can be used as an indicator of activating or inactivating pro-carcinogens. The metabolism of B(a)P is known very well (Li, Wang *et al.*, 1996; Vanden Heuvel, Clark *et al.*, 1993). B(a)P is metabolized to DNA-binding diol epoxides by phase I CYPs and detoxified by phase II enzymes including glutathione S-transferase. The greater the activation of the phase I enzymes and the lower the detoxification of the phase II enzymes, the more they might be involved in the development of lung cancer (Goto, Yoneda *et al.*, 1996; Rojas, Cascorbi *et al.*, 2000; Quinones, Lucas *et al.*, 2001; Hayashi, Watanabe *et al.*, 1992). Previous studies (Hayashi, Watanabe *et al.*, 1992; Okada, Kawashima *et al.*, 1994) demonstrated that the polymorphisms in CYP1A1, m1 and m2 mutations are risk factors in Japanese smoking-related lung cancer. In addition, Quinones, Lucas *et al.*, (2001) reported that the activated type polymorphism in CYP1A1 enzyme is frequently seen in lung cancer patients in Chile. Thus, we hypothesized that CYPs enzyme mRNA level of peripheral lymphocytes could be used as a biomarker for the risk of lung cancer. The present study is the first report on mRNA expression profiles for a number of targeted CYPs in peripheral lymphocytes from patients with lung cancer in comparison with healthy controls.

The semi-quantitative competitive reverse transcription polymerase chain reaction (QC-RT-PCR) (Andersen, Farin *et al.*, 1998; Krovat, Tracy *et al.*, 2000; Sumida,

Fukuen *et al.*, 2000) employed in the present study is a sensitive tool for semi-quantifying the amount of CYPs mRNA.

Materials and Methods

Subjects

From February to October 2001, a consecutive series 30 previously untreated lung cancer patients with an average age of 60.9 years (range, 34-76 years) and an average smoking index (cigarettes/day $\times$ years) of 1291.9 (range, 660-3040) were examined. Thirty healthy controls with an average age of 63.1 years (range, 31-83 years) with an average smoking index of 914.3 (range, 400-2500) were studied in the same period. All smokers in this study were current smokers and all non-smokers were defined as individuals who had never smoked. Age, gender and smoking states of lung cancer patients and healthy controls are listed in Table I. All patients were given an explanation of the nature of the study and their fully informed consent was obtained prior to obtaining blood samples.

Isolation of total RNA from peripheral blood

Total RNA was extracted from 5 ml of peripheral blood from pretreated patients by the QIAamp® reagent (QIAGEN, Hilden, Germany) following the manufacturer's protocol. To remove contaminating genomic DNA, extracted mRNA preparations were treated with RNase-free DNase I (QIAGEN). The isolated total RNA was stored at -85°C until use.

Primer design and generation of RNA competitor

Gene-specific primers were designed to amplify the specific regions of CYPs 1A1, 1A2, 2A6/7, 2B6, 4B1, 2C, 2D6, 2E1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes. RNA competitors (1.0×10^8 copies/ μ l) were generated using reagents supplied in a commercial kit, Human Cytochrome P450 RT-PCR® (TaKaRa Biomedicals, Tokyo, Japan). The primer sequence of each gene is considered proprietary information by the TaKaRa Company and is not made public.

Quantitative competitive RT-PCR

We first confirmed the presence of CYPs mRNA. In the second step, we assessed the levels of mRNA expression by QC-RT-PCR. Each cDNA was synthesized in a reaction mixture containing 200 ng of total RNA, 2 μ l of competitor RNA (CYPs; ranging from 1.0×10 to 1.0×10^4 copies/ μ l, GAPDH; ranging from 1.0×10^4 to 2.5×10^6 copies/ μ l), 4 μ l of $10 \times$ RNA PCR buffer, 8 μ l of 25 mM $MgCl_2$, 4 μ l of 10mM dNTPmix, 1 μ l of 40 U/ μ l RNase inhibitor, 2 μ l of 5 U/ μ l avian myeloblastosis virus (AMV) reverse transcriptase, 2 μ l of 2.5 pmol/ μ l oligo dT-adaptor primer and finally diethyl-procarbonate (DEPC)-treated water up to total volume of 40 μ l. All reagents were included in a Human Cytochrome P450 Competitive RT-PCR® Set (TaKaRa). Reverse transcription reaction was performed for 10 min at 30° C, 30 min at 55° C, 5 min at 95° C and 5 min at 5° C. The competitive PCR was performed in a total volume of 20 μ l of reaction mixture containing 4 μ l of cDNA, 1.2 μ l of 25 mM $MgCl_2$, 1.6 μ l of $10 \times$ RNA PCR buffer, 0.4 μ l of 10 pmol/ μ l CYPs primers, 0.1 μ l of 5 U/ μ l Taq polymerase and 12.7 μ l of DEPC-treated water. The reaction mixture was heated to 95° C for 2 min and then cycled, denatured at 94° C for 30 sec, and annealed at 60° C (CYPs 1A1, 2C and 2E1), 62° C (CYP2B6 and GAPDH), 64° C (CYP4B1), and 65° C (CYP2D6) for 1 min and extended at 72° C for 1 min. Optimized competitive PCR comprised 35 cycles for CYPs 1A1, 2D6 and 4B1, 31 cycles for CYP2C, 30 cycles for CYP2B6 and GAPDH, and 28 cycles for CYP2E1, respectively. All samples were finally incubated at 72° C for 7 min. QC-RT-PCR products were separated by electrophoresis on 2.5% agarose gels and were stained with ethidium bromide. DNA bands were visualized with UV light (302 nm) and the gel images were photographed. We chose the equivalent or similar intensity bands of target mRNA and competitor RNA to determine the number of target mRNA copies. The good reproducibility of this method was confirmed in duplicate measurements of the first 10 samples.

Statistical analysis

The non-parametric Mann-Whitney U-test was used to test the difference in

clinical factors between patients and healthy controls, and distribution of the mRNA expression of the enzymes between the classified two groups. The χ^2 test was used to test the distribution of gender between patients and healthy controls. A p-value of less than 0.05 was considered to indicate a statistically significant difference.

Results

The representative findings of QC-RT-PCR are shown in Figure 1. The average values of mRNA expression of CYPs in the lung cancer group and the healthy control group are shown in Table II. The transcription products of CYPs 1A1, 2B6, 4B1, 2C, 2D6 and 2E1 were detectable in both groups all objects. However, since CYPs 1A2 and 2A6/7 were not expressed in peripheral blood lymphocytes in either patients or healthy control groups, these enzymes were excluded from the analyses. The mRNA levels of CYPs 1A1 ($p < 0.001$), 4B1 ($p < 0.001$) and 2C ($p < 0.001$) in the lung cancer group were higher than those in the healthy control group. However, CYPs 2D6 ($p < 0.0001$) and 2E1 ($p < 0.0001$) mRNA levels in the lung cancer group were lower than those in the healthy control group.

In smokers, the mRNA levels of CYPs 1A1 ($p < 0.01$), 4B1 ($p < 0.001$), and 2C ($p < 0.001$) were also higher, and CYPs 2D6 ($p < 0.0001$) and 2E1 ($p < 0.001$) were also lower in the lung cancer group than healthy controls (Table III). There were no differences in CYPs mRNA expression between adenocarcinomas and squamous cell carcinomas (data not shown) or between all studied smokers and non-smokers (Table IV).

Discussion

Individual differences in susceptibility to chemically induced cancers are ascribed partly to genetic differences in metabolic balance in the activation and detoxification of environmental pro-carcinogens. Human smoking-related lung cancer, especially squamous cell carcinoma and small cell carcinoma, requires exposure to the pro-carcinogens contained mainly in cigarette smoke. A number of investigators have examined the correlation between CYPs activity and induction of many carcinogens in

Figure 1. Representative findings of semi-quantitative competitive RT-PCR analysis of CYPs 1A1, 2B6, 4B1, 2C, 2D6, 2E1 and GAPDH mRNA expression in peripheral blood lymphocytes. Levels of expression of the CYPs in tested mRNA were determined according to the intensity of the competitive RNA band.

C: competitive RNA, T: tested RNA, bp: base pair, M: 100 bp DNA marker, Lane 1: standard concentration of competitive RNA 1.0×10^4 copies/ μ l, lane 2: 4.0×10^4 copies/ μ l, lane 3: 1.6×10^2 copies/ μ l, lane 4: 6.4×10^2 copies/ μ l, lane 5: 2.5×10^3 copies/ μ l, lane 6: 1.0×10^4 copies/ μ l, lane 7: 4.0×10^4 copies/ μ l, lane 8: 6.4×10^5 copies/ μ l, lane 9: 2.5×10^6 copies/ μ l.

TABLE I
Clinical features of lung cancer patients and healthy controls.

	Lung cancer patients	Healthy controls	p-value
Object numbers	30	30	
Gender*			
Male	25	20	
Female	5	10	NS
Age (mean $\pm$ S.E.)**	60.9 $\pm$ 0.9	63.1 $\pm$ 0.3	NS
Smoking index (mean $\pm$ S.E.)**	1291.9 $\pm$ 12.8	914.3 $\pm$ 9.7	< 0.03
Histologic type			
Ad	14		
Sq	14		
Sm	2		
Stage	I-IV (I/7, II/2, III/18, IV/3)		

Ad: adenocarcinoma, Sq: squamous cell carcinoma, Sm: small cell carcinoma, S.E.: standard error. NS: not significant. * χ^2 -test. **Mann Whitney U-test.

TABLE II
Expression of CYPs mRNA of lung cancer patients and healthy controls.

Gene	Chromosomal location	Patients (n=30)	Healthy controls (n=30)	p-value
CYP1A1	15q22-q24	802.0 $\pm$ 14.7*	264.0 $\pm$ 3.9	< 0.001
CYP2B6	19q13.2	26.4 $\pm$ 0.7	19.6 $\pm$ 0.5	NS
CYP4B1	1p34-p12	27.2 $\pm$ 0.4	6.5 $\pm$ 0.2	< 0.001
CYP2C	10cen-q24.1	2970.8 $\pm$ 70.4	491.0 $\pm$ 4.2	< 0.001
CYP2D6	22q11.2-qter	1830.0 $\pm$ 34.0	6438.0 $\pm$ 70.9	< 0.0001
CYP2E1	10q24.3qter	1604.0 $\pm$ 34.1	5634.0 $\pm$ 77.1	< 0.0001

NS: not significant. *Mean $\pm$ standard error [copies/ng].

TABLE III
Expression of CYPs mRNA in smoking lung cancer patients and smoking healthy controls.

Gene	Smoking patients (n=22)	Smoking healthy controls (n=23)	p-value
CYP1A1	863.6±1.7*	253.4±4.4	< 0.01
CYP2B6	28.9±0.8	16.0±0.5	NS
CYP4B1	25.1±0.4	9.5±0.4	< 0.001
CYP2C	3103.5±83.1	535.7±4.2	< 0.001
CYP2D6	1548.2±20.9	5760.9±79.3	< 0.0001
CYP2E1	1270.0±22.8	4712.2±84.2	< 0.001

NS: not significant. *Mean ± standard error [copies/ng].

TABLE IV
Expression of CYPs mRNA in all studied smokers and non-smokers.

Gene	Smokers (n=45)	Non-smokers (n=15)	p-value
CYP1A1	562.7±9.8*	476.1±1.5	NS
CYP2B6	21.6±0.4	18.4±1.2	NS
CYP4B1	18.2±0.6	16.1±0.3	NS
CYP2C	1652.1±4.1	946.7±3.0	NS
CYP2D6	4368.0±58.2	4808.0±115.4	NS
CYP2E1	3042.7±50.1	4724.0±116.7	NS

NS: not significant. *Mean ± standard error [copies/ng].

mitogen-stimulated lymphocytes and lung tissue, especially the relationship between CYP1A1 induction by exposure to PAH in cigarette smoke and susceptibility to lung cancer (Denissenko, Pao *et al.*, 1996; Hecht 1996; Goto, Yoneda *et al.*, 1996). Recent genetic polymorphism studies demonstrated that CYP1A1 affects BPDE-DNA binding in human leukocytes, and exposure to PAH have a synergistic effect. High levels of BPDE-DNA adducts in leukocytes by specific phase I, phase II enzyme genotype combinations may relate to the increased risk for lung cancer (Rojas, Cascorbi *et al.*, 2000).

Specific polymorphism within the CYP1A1 gene was found to be associated with increased risk of lung cancer in Japanese smokers (Goto, Yoneda *et al.*, 1996; Hayashi, Watanabe *et al.*, 1992; Okada, Kawashima *et al.*, 1994), and similar results were obtained by other ethnic polymorphism studies (Rojas, Cascorbi *et al.*, 2000; Quinones, Lucas *et al.*, 2001; Alexandrov, Cascorbi *et al.*, 2002). Expression of CYP1A1 levels were significantly related to the hydrophobic DNA adduct levels, although a large interindividual variation was observed for both CYP1A1 expression and DNA adducts (Mollerup, Ryberg *et al.*, 1999). Several studies demonstrated a significant correlation between CYP1A1 expression and DNA adducts level (Vanden Heuvel, Clark *et al.*, 1993; Krovat, Tracy *et al.*, 2000; Mollerup, Ryberg *et al.*, 1999). Imaoka, Yoneda *et al.*, (2000) and Fujitaka, Oguri *et al.*, (2001) demonstrated that CYPs 4B1, 2C mRNA expression in bladder and lung cancer tissue was significantly higher than in normal tissue. Their results resemble ours, although we used peripheral blood lymphocytes from patients, instead of bladder or lung cancer tissues. Our study suggests that high levels of mRNA expression of CYPs 1A1, 4B1 and 2C in peripheral blood lymphocytes might be potential biomarkers of the lung cancer risk. Of course, there are some other potential risk factors associated with development of primary lung cancer, as well as factor influencing the expression of these enzymes in lung cancer patients, such as chronic obstructive pulmonary disease, nutritional status, occupational and environmental pollution, and phase II enzyme activity.

In the present study, CYPs 2D6 and 2E1 were more highly expressed in healthy controls than in patients with lung cancer. Krovat, Tracy *et al.*, (2000) reported that these

enzymes were typically expressed at the highest levels in peripheral lymphocytes from healthy donors. Genetic polymorphism of the human CYPs 2D6 and 2E1 genes are known to be associated with lung cancer risk in some reports (Agundez, Gallardo *et al.*, 2001; Quinones, Lucas *et al.*, 2001). Conversely, in other reports these polymorphisms are related to low risk for lung cancer (Ayesh, Idle *et al.*, 1984). Kiyohara, Otsu *et al.*, (2002) mentioned that the relationship between CYP2D6 gene and lung cancer remains inconclusive and that no definitive link between the polymorphism of CYP2E1 and the risk of lung cancer has been identified. Expression levels of these two enzymes might relate to activation of carcinogens, but it is unclear whether carcinogens, such as B[a]P and NNK are activated by CYPs 2D6 and 2E1 in peripheral blood lymphocytes. Further analyses are needed on this point, because we could not find other reports suggesting that CYPs 2D6 and 2E1 may promote the anti-carcinogenetic processes. Interestingly, expression of all CYPs did not significantly differ between smokers and non-smokers. This suggests that these enzymes are not induced in peripheral blood lymphocytes by smoking. We believe, therefore, CYPs expression in peripheral lymphocytes can be used as biomarkers for the risk of lung cancer. This finding was previously confirmed by other reports. Thompson, McCoy *et al.*, (1989) found that smoking does not influence metabolism or DNA adduct formation in lymphocytes. Rojas, Camus *et al.*, (1992) found that metabolic activities for B[a]P-7,8-diol to tetrol conversion are similar in smokers and non-smokers.

In conclusion, increased expression of CYPs 1A1, 4B1 and 2C enzymes in peripheral blood lymphocytes was found in lung cancer patients in comparison with healthy controls. Therefore, increased expression of these enzymes may possibly be susceptibility biomarkers for human lung cancer.

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