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１．研究テーマ

肺癌患者末梢血液リンパ球におけるチトクローム系代謝酵素 mRNA 発現量の解析

２．本年度の研究業績

(1) 学会・研究会等における発表　(有)・無　（学会名・演題）

第10回世界肺癌学会
肺癌患者末梢血液リンパ球におけるチトクローム系代謝酵素 mRNA 発現量の解析
第44回日本肺癌学会総会
肺癌患者末梢血液リンパ球におけるチトクローム系代謝酵素 mRNA 発現量の解析
第10回日本癌病態研究会
肺癌患者末梢血液リンパ球におけるチトクローム系代謝酵素 mRNA 発現量の解析

(2) 学会誌等に発表した論文　(有)・無　（雑誌名・論文名）

1. Annals of Cancer Research and Therapy 2003.11. Vol 11
   The mRNA Expression of Cytochrome P450 Enzymes in Peripheral Blood Lymphocytes in Patients with Lung Cancer.
2. Clinical Cancer Research 2003.6. Vol 2294(9)
   Chromosomal Instability Detected by Fluorescence *in Site* Hybridization in Surgical Specimens of Non- Small Cell Lung Cancer is Associated with Poor Survival.
3. Oncology Reports 2003.11. Vol 11
   An increase in the percentage of HLA-DR-positive peripheral leukocytes predicts a poor prognosis in patients with squamous cell carcinoma of the lung.
4. Oncology Reports 2003.10. Vol 10
   Comparison of immunohistochemistry and real-time reverse transcription-polymerase chain reaction to detect expression of carcinoembryonic antigen in lung cancer.
5. Annals of Thoracic and Cardiovascular Surgery 2003.10.Vol 9(5)
   Significance of expression of TGF-β in pulmonary metastasis in non-small cell lung cancer tissues.

3．今後の研究計画

博士生課程終了後、日本の大学、或いは他の研究機関で生物遺伝子学的、蛋白の分析など分野で研究を続けたいです。 新疆ウイグル自治区は中国の内陸地方に位置し、経済的、技術的にも大変遅れている地域でありますから新分野で研究する条件はございません。 上記の希望が得られなかった場合は日本で学んだ肺癌に対する早期診断，早期治療の新しい方法を身につけ、将来、帰国してから本国の医療事業に貢献したいと思います。

4．指導責任者の意見

阿吾提　伊地力斯君は学術面で肺癌の遺伝子素因の最新的研究を精力的に行なうとともに、人格面でも極めて円満且つ真面目で協調性に富んだ生活態度で日常をすごしております。基礎研究の面においては「肺癌患者末梢血液リンパ球におけるチトクローム系代謝酵素 mRNA 発現量の解析」を研究テーマとして、肺癌患者及び対象とした健常者の末梢血有核細胞のRNAを抽出し、検討した。本研究の目的は肺癌の易罹患性に関与する遺伝的背景の解析で、発癌物質の代謝に関与する多種類のチトクローム系酵素の遺伝子発現量の定量から個人の肺癌感受性を解析しようとするものであります。すでに多くのデータを蓄積し、第１０回日本癌病態治療研究会、第４２回日本肺癌学会総会，第10回世界肺癌学会で発表行い、英文の論文をAnnals of Cancer Research and Therapy雑誌で発表した。このように、立派な業績を残してきており、更に熱心に勉学と技術修得、新しい分野の研究に励んでおります。彼は日本文化へ順応も優れており、本学で学んだ医療技術を生かして日中医学交流の発展に貢献し、良い指導者をなりうることを確信しております。

指導責任者氏名　加藤治文　 

5．研 究 報 告 書

別紙「研究報告書の作成について」に倣い、指定の用紙で作成して下さい。

研究発表または研究状況を記録した写真を添付して下さい。

※研究成果を発表する場合は、発表原稿・抄録集等も添付して下さい。

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— 日中医学協会助成事業 —

The mRNA Expression of Cytochrome P450 Enzymes in Peripheral Blood Lymphocytes in Patients with Lung Cancer

(肺癌患者末梢血液リンパ球におけるチトクローム系代謝酵素 mRNA 発現量の解析)

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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 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.

Key Words: Cytochrome P450 enzymes, quantitative competitive reverse transcription polymerase chain reaction, lung cancer, smoking, carcinogenesis.

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 microphages 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 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 Chilean lung cancer patients. 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 × 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 1. 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 glycerol- dehydes 3-phosphate dehyrogenase (GAPDH) genes. RNA competitors (1.0×10^8 copies/μl) 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 μl of competitor RNA (CYPs; ranging from 1.0×10 to 1.0×10^4 copies/μl, GAPDH; ranging from 1.0×10^4 to 2.5×10^6 copies/μl), 4 μl of 10×RNA PCR buffer, 8 μl of 25 mM $MgCl_2$, 4 μl of 10mM dNTPmix, 1 μl of 40 U/μl Rnase inhibitor, 2 μl of 5 U/μl avian myeloblastosis virus (AMV) reverse transcriptase, 2 μl of 2.5 pmol/μl oligo dT-adaptor primer and finally diethyl-procarbonate (DEPC)-treated water up to total volume of 40 μl. 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 μl of reaction mixture containing 4 μl of cDNA, 1.2 μl of 25 mM $MgCl_2$, 1.6 μl of 10×RNA PCR buffer, 0.4 μl of 10 pmol/μl CYPs primers, 0.1 μl of 5 U/μl Taq polymerase and 12.7 μl 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 measurement 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 x^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 2. The transcription products of CYPs 1A1, 2B6, 4B1, 2C, 2D6 and 2E1 were detectable in both group 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 3). 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 4).

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 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 has 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 is 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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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 ± S.E.)**	60.9±0.9	63.1±0.3	NS
Smoking index (mean ± S.E.)**	1291.9±12.8	914.3±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.*x^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±14.7*	264.0±3.9	< 0.001
CYP2B6	19q13.2	26.4±0.7	19.6±0.5	NS
CYP4B1	1p34-p12	27.2±0.4	6.5±0.2	< 0.001
CYP2C	10cen-q24.1	2970.8±70.4	491.0±4.2	< 0.001
CYP2D6	22q11.2-qter	1830.0±34.0	6438.0±70.9	< 0.0001
CYP2E1	10q24.3qter	1604.0±34.1	5634.0±77.1	< 0.0001

NS: not significant. *Mean ± 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].

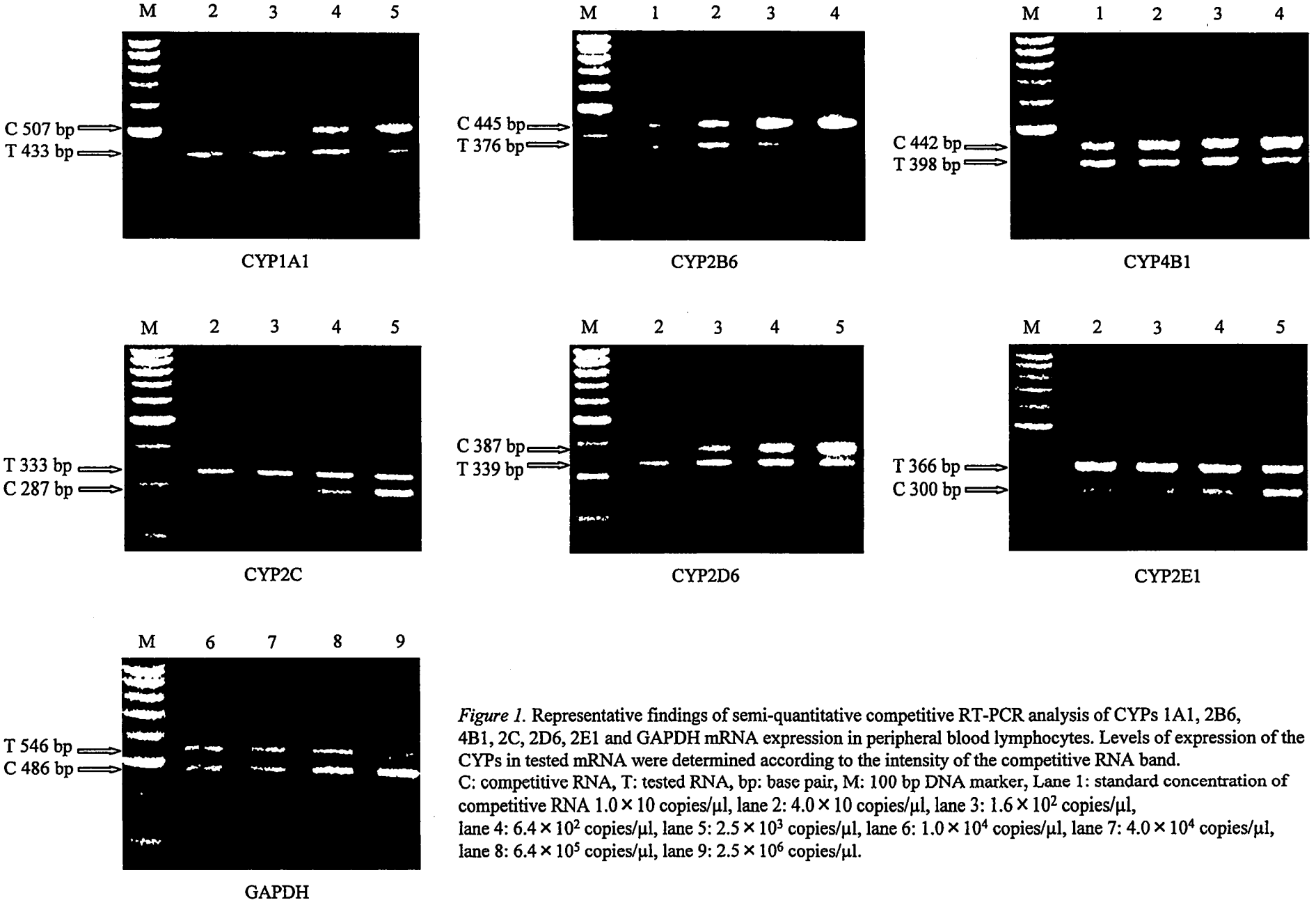

Figure 1. Representative findings of semi-quantitative competitive RT-PCR analysis of CYPs 1A1, 2B6, 4B1, 2C, 2D6, 2E1 and GAPDH mRNA expression in peripheral blood lymphocytes. Levels of expression of the CYPs in tested mRNA were determined according to the intensity of the competitive RNA band.
C: competitive RNA, T: tested RNA, bp: base pair, M: 100 bp DNA marker, Lane 1: standard concentration of competitive RNA 1.0×10 copies/μl, lane 2: 4.0×10 copies/μl, lane 3: 1.6×10^2 copies/μl, lane 4: 6.4×10^2 copies/μl, lane 5: 2.5×10^3 copies/μl, lane 6: 1.0×10^4 copies/μl, lane 7: 4.0×10^4 copies/μl, lane 8: 6.4×10^5 copies/μl, lane 9: 2.5×10^6 copies/μl.