[Frontiers in Bioscience 12, 2299-2308, January 1, 2007]

Cytochrome P450 expression (CYP) in non-small cell lung cancer

Tsunehiro Oyama [1,2], Kenji Sugio [1], Hidetaka Uramoto [1], Toshihiro Kawamoto [2], Norio Kagawa [3], Sorena Nadaf [4], David Carbone [4], Kosei Yasumoto [1]

[1] Second Department of Surgery, University of Occupational and Environmental Health, Kitakyushu, 807-8555, Japan, [2] Department of Environmental Health University of Occupational and Environmental Health, Kitakyushu, 807-8555, Japan, [3] Department of Biochemistry, RRB 626, Vanderbilt University School of Medicine, Nashville, TN 37232-0146, USA, [4] Vanderbilt Cancer Center and Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, 37232-6838, USA

TABLE OF CONTENTS

1. ABSTRACT

The cytochrome P450 (CYP) is associated with tumor development and progression as well as activation of anti-cancer prodrugs and their metabolic clearance. In this study, we investigated the expression of aryl-hydrocarbon receptor (AH-R) and four CYPs (CYP1A1, CYP2A6, CYP2E1 and CYP3A) as putative diagnostic markers in 78 non-small cell lung cancers (NSCLC) along with clinical features of the patients. In non-small cell lung cancer, the expression of the five markers was mainly observed in adenocarcinoma but not in the most squamous cell cancers. The expression of them in adenocarcinoma was more frequent in females than in males, suggesting that a higher risk of women for developing lung adenocarcinoma might be associated with the frequent expression of AH-R and the CYPs. These factors were also more frequently expressed in early stage adenocarcinoma and more differentiated adenocarcinoma. Multiple types of CYPs are more frequently expressed in early stage of adenocarcinoma than in advanced stage of adenocarcinoma. There were positive relationships among AH-R, CYP1A1, CYP2E1 and CYP3A expressions in adenocarcinoma, which suggests a metabolite-mediated cross talk in the gene regulation of these markers. However, any of them was unrelated with the expression of CYP2A6, suggesting that the gene regulation of CYP2A6 in adenocarcinoma may be different from the other three CYPs. The expression frequency of CYP1A1 and CYP2E1 in tumors is independent of their genetic polymorphism. The survival of the patients with advanced adenocarcinoma expressing more than one of CYPs was lower rate than the patients with those expressing no CYPs, suggesting that the expression of the CYPs in advanced adenocarcinoma may be associated with poor survival. Our results suggest that AH-R and 4 CYPs may be good markers for the determination of quality of lung cancer. The information could be useful for the better management of lung cancer by molecular targeting therapy and selection of anti-cancer drug based on individual spectrum of the marker proteins. Therefore, the spectrum of CYP proteins in lung cancer could be useful for changing the present "order-made" therapy to the "tailor-made" therapy.

2. INTRODUCTION

Lung cancer is known to be the most frequent cancer in the world, and the size of the lung cancer epidemic is still increasing. The lung constitutes the primary site of entry into the body for a wide variety of inhaled chemicals, such as polycyclic aromatic hydrocarbons (PAH) from cigarette smoke. The relative risk of lung cancer in Japanese smokers compared with that in Japanese nonsmokers is approximately 5 fold in men and 3 fold in women, indicating that smoking contributes the most to susceptibility to lung cancer compared with other risk factors (1). Tobacco smoke contains many of carcinogens and pro-carcinogens, such as benzo(a)pyrene and nitrosamine. These compounds are metabolized by the phase I enzymes including CYP family enzymes and converted to inactive metabolites by the phase II enzymes. Benzo(a)pyrene itself, for instance, does not exhibit carcinogenicity but undergoes metabolic activation by the phase I enzyme CYP1A1 into the diol epoxide, which is mostly converted to an inactive metabolite by the phase II enzyme glutathione S-transferase (GST), particularly the Mu class of GST (GSTM1) (2). However, this epoxide is extremely reactive and can make its way into the nucleus and form DNA adducts which exhibit strong carcinogenicity. The CYP mediated benzo(a)pyrene DNA adducts are capable of causing specific mutations in the p53 gene (3).

CYP is a multi-gene superfamily of constitutive and inducible haem-containing mono-oxygenases (P450) characteristic with their absorption peak of the reduced CO-complex at 450nm. The microsomal enzymes of this family require molecular oxygen and equivalent electrons from NADPH-dependent P450-reductase. The CYP system plays important roles in the metabolism and excretion of endogenous and exogenous compounds as well as varieties of carcinogens, such as polycyclic aromatic hydrocarbons, heterocyclic amines, nitrosamines, azodyes, and alkylating agents (4, 5). Metabolic intermediates of the compounds produced by CYP1A1, CYP2A6, CYP2E1, and CYP3A4/5 in this pathway are often highly active and are associated with both initiation and promotion of tumor development and progression (4-6).

The aryl hydrocarbon receptor (AH-R) binds a variety of environmental pollutants, such as PAH, and mediates their ability to induce enzymes in the CYP pathway (7). The ligand-bound AH-R activates CYP1A1 gene transcription through interaction with specific DNA sequences, termed xenobiotic responsive elements (XREs) (8). AH-R nuclear translocator protein (Arnt) is also shown to be a structural component of the XRE binding form of the AH receptor (8). AH-R expression is observed in normal lung tissues as well as in lung carcinomas, though AH-R immunostaining is more intense in lung adenocarcinoma than in squamous cell carcinomas and normal bronchiolar epithelial cells (9). AH-R might play an important role in the development of in lung adenocarcinoma.

CYP1A1 is the most actively studied human pulmonary CYP enzyme because CYP1A1 is one of major enzymes involved in PAH metabolism, and thus may play a role in the development of lung cancer. CYP1A1 mRNA was expressed in normal lung tissue (10) and was also overexpressed in 23% (10/43) of the lung cancers (11). McLemore *et al*. also showed the CYP1A1 mRNA expression (2.8kB) in normal lung tissue and 10kB CYP1A1 mRNA, which was not detectable in normal lung tissue, was found in 5 of 43 (12%) lung cancers (11). The CYP1A1 protein was detected by immunoblotting in a study of 12 lung cancer patients (12). CYP2E1 is known to actively metabolize some tobacco-specific nitrosamines (13) and mRNA encoding CYP2E1 was expressed in normal lung tissue (14).

Regarding the metabolism of anti-cancer drugs, CYP1, CYP2 and CYP3. CYP3A, CYP2C, CYP1A2 and CYP2E1 have been estimated to contribute to the drug metabolisms up to 30%, 20%, 13% and 7% of total CYPs in human liver samples, respectability (15). Recently, some CYPs, in particular enzymes of the CYP3A subfamily (CYP3A4, CYP3A5, and CYP3A7), have been found to play a role in the metabolism of many anticancer drugs (16). CYP3A not only inactivates major anticancer drugs, such as tamoxifen, taxol and vinca alkaloids, but also activates major anticancer prodrugs, such as cyclophosphamide and ifosphamide (17). The expression of CYP3A including CYP3A4 and CYP3A5 was found in both tumor and normal tissue by immunohistochemistry (14, 18, 19). Kivisto *et al*. showed that CYP3A5 mRNA in four of eight lung cancers and CYP3A7 mRNA in one of eight lung cancers and both CYP3A5 and CYP3A7 mRNA were also expressed in normal lung tissues (18). In addition to CYP3A subfamily, the local expression of other CYPs within the tumor thus has a possibility to alter the local activation or elimination of anticancer drugs.

For the better management of lung cancer, it is essential to understand the roles of CYPs and the relationships between their expression and the clinical feature of individual lung cancers. In this study, we therefore investigate the expression of AH-R and four CYPs (CYP1A1, CYP2A6, CYP2E1 and CYP3A) in 78 non-small cell lung cancer (NSCLC) and their relationships to each other, tumor p53 expression, and clinical features of the patients. In addition, we evaluated the relationship between expression and genomic polymorphisms for two of the CYPs (CYP1A1 and CYP2E1).

3. MATERIALS AND METHODS

3.1. Samples

We examined 78 of 98 (79.6%) consecutive Japanese patients with NSCLC who underwent surgical resection at the Department of Surgery II, School of Medicine, University of Occupational and Environmental Health, in Kitakyushu, Japan, between 1992 and 1993. The criterion for inclusion in the study was the availability of specimens and follow-up data. The median survival time (MST) of this group was eight years. There were 56 men and 22 women ranging in age from 38 to 80 years (mean: 65.7 years). None of these patients received chemotherapy or radiotherapy prior to the operation. Twenty-six patients

Table 1. Antibodies and conditions for immunohistochemistry (IHC) and Western blotting of aromatic hydrocarbon receptor (AH-R) and cytochrome P450 (CYP)

Antigen	Duration of primary antibody	Primary antibody	Polyclonal or monoclonal	Dilution (folds) for IHC
AH-R	Santa Cruz Biotechnology	Goat anti-human	polyclonal	400
CYP1A1	Affiniti Research Products Ltd.	Rabbit anti-human	polyclonal	200
CYP2A6	Gentest Corp.	Mouce anti-human	monoclonal	200
CYP2E1	Gentest Corp.	Mouce anti-human	monoclonal	100
CYP3A (CYP3A4, CYP3A5, CYP3A7)	Gentest Corp.	Mouce anti-human	monoclonal	1000

had stage I disease, 10 stage II, 39 stage III, and 3 stage IV, according to TNM staging (20). Histological typing of the tumors was performed according to the WHO classification (21). Resected specimens were fixed in 10% formalin for three days and were embedded in paraffin. For histological study, sections were stained with hematoxylin and eosin.

3.2. Staining

Immunohistochemical staining for AH-R, CYP1A1, CYP2A6, CYP2E1 and CYP3A was performed in patients with 79 non-small cell lung cancer. Human liver samples were also examined as a positive immunohistochemical staining control. Tumor was stained using the avidin-biotin complex (ABC) method with labeled streptavidine-biotinrelated antibody (LSAB) kit (Dako Corp., Carpinteria, USA) (22). Antibodies and conditions were described in table 1. Briefly, 3 μm sections of each tumor were incubated first with a 1:100 - 1000 dilution of primary antibodies for 40 minutes at room temperature, followed by a 10 minute incubation with secondary antibodies and peroxidase-labeled streptavidin. Staining was completed after 15 minutes incubation with a freshly prepared substrate-chromogen solution. We defined as positive cases when more than 10% of tumor was stained (negative cases; > 10% positivity, positive cases; < 10% positivity).

3.3. Western blotting

Cytoplasm and microsomes from murine liver were separated as previously described (11). Cytoplasm and microsomes from human liver (Gentest Corp., Woburn MA) was used as the control of the Western blotting analysis. The protein concentration was estimated by the Bradford method (Bio-Rad) (23). Cytoplasmic protein (20 μg) was used for AH-R and microsomal protein was used for CYP1A1, CYP2A6, CYP2E1 and CYP3A. Proteins were separated by 10% SDS-PAGE, transferred to polyvinylidene Hybond-P membrane (Amersham, Arlington Heights, IL), and visualized by ECL (Amersham) using the indicated antibodies (Table 1).

3.4. Real-time quantitative polymerase chain

Total RNA was extracted from human lung tumor and normal organs (17.7 ~ 38.2 mg) using the Rneasy Mini Kit and QIAshredder TM (QIAGEN K.K., Tokyo). Human total RNA was diluted with yeast tRNA at 50 mg/ml. The RTPCR assay was performed in 50 mL of Taq-Man One-Step RTPCR Master Mix Reagents containing 300 nM forward primer, 900 nM reverse primer, 200 nM TaqMan probe. Amplication and detection were performed using an ABI PRISM 7000 Sequence Detector system (Applied Biosystems Japan Ltd, Tokyo) with the following profile: 1 cycle of 48° C for 30 min, 1 cycle of 95° C of 10 min, and 40 cycles each of 95° C for 15 s and 60° C for 1 min. The PCR primers were synthesized by Hokkaido System Science according to the published sequence (24).

3.5. Detection of the HincII polymorphism in *CYP1A1*

The HincII polymorphism at codon 462 of CYP1A1 was detected using designed restriction fragment length polymorphisms created by artificially introduced mismatchs as previously described (25). This allele is named as CYP1A1*2C (m2) by CYP1A1 allele nomenclature (see http://www.imm.ki.se/CYPalleles/cyp1a1.htm). The primer sequences used were as follows; 1A1S, 5' - GAACTGCCACTTCAGCTGTCT - 3'; 1A1ASHincII, 5' - GAAAGACCTCCCAGCGGTCA - 3'. After amplification with these primers, the PCR product from the valine (Val) allele is cleaved by the HincII restriction enzyme (Wako Chemical Ltd., Tokyo, Japan) but that from the isoleucine (Ile) allele is not cleaved.

3.6. Detection of the RsaI polymorphism in *CYP2E1*

The identification of the CYP2E1 genotype ascribed to the presence (c1) or absence (c2) of the RsaI (GTAC) site was carried out by the PCR/restriction digest-genotyping method as described previously (26). This allele is named as CYP2E1*5A by CYP2E1 allele nomenclature (see http://www.imm.ki.se/CYPalleles/cyp2e1.htm). The primer sequences used were as follows: CYP2E12S, 5' - CCAGTCGAGTCTACATTGTCA - 3'; CYP2E1AS, 5' - TTCATTCTGTCTTCTAACTGG - 3'. PCR was performed using 35 thermal cycles and the following amplification conditions: 1 min at 95° C for denaturation, 1 min at 55° C for primer annealing, and 1 min at 72° C for primer extension. The PCR products including the polymorphic site were digested with RsaI and subjected to electrophoresis in 2% agarose gels.

3.7. Detection of p53 overexpression and mutation

Immunohistochemical staining for p53 was also performed on 3 μm paraffin-sections using the mouse monoclonal antibody DO-1 (Oncogene Science Inc., Cambridge, MA) against p53 protein, which recognizes the codon regions 37 - 45 of the p53 protein using standard immunohistochemical methods as previously described (22). We determined the p53 expression score according to the proportion of positively stained tumor cell nuclei (0%, negative; < 10%, low expresser; > 10%; high expresser).

Point mutations of the p53 gene occurring in exons from 5 through 8 were detected using the PCR-SSCP method as described (27). The p53 mutations were

sequenced by the dideoxy chain termination method using an ABI 373A DNA Sequencer (Applied Biosystems) (28).

3.8. Statistical Analysis

Statistical differences (Table 2, Table 3, Table 4, Table 5, and Table 6) were evaluated by analysis of variance (ANOVA). Relationships between CYPs expression and *CYPs* polymorphism in adenocarcinoma (Figure 3) were also examined by ANOVA. The survival rate (Figure 4) was calculated using the Kaplan-Meier method and the significance of survival rate was evaluated by the log rank test.

4. RESULTS

4.1. Expression of the five markers in adenocarcinoma and squamous cell carcinoma

The expression of AH-R and four CYPs may play important roles for the development of lung cancer as well as its management. However, the expression of these markers has not been well investigated. Therefore, we systematically investigated the expression of the five markers in lung cancer. Using immunohistochemical analysis, we evaluated the frequency of expression of AH-R and CYPs (CYP1A1, CYP2A6, CYP2E1 and CYP3A4) in patients with adenocarcinoma (n=48) and squamous cell carcinoma (n=30). In the human liver as a control tissue, CYP3A is strongly expressed in hepatocytes around the central vein, with less expression for AH-R and CYP2A6 observed (Figure 1A). In four adenocarcinoma cases from 78 patients with lung cancer, all five markers were detected in tumor cells by immunohistochemistry. Figure 1B showed representative images from a case of lung adenocarcinoma expressing all five markers. As shown in Figure 2A, CYP3A4 was also detected in microsomes from tumor tissues of the four cases upon Western blotting. To evaluate the relative expression levels of the markers in the tumor tissues compared with normal tissues, transcripts were extracted from both normal and tumor lung tissues in the four cases. The expression levels of CYP1A1, CYP2E1 and CYP3A4 mRNA were analyzed by the real-time PCR method using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA for normalization. As seen in Figure 2B, two tumor tissues of the four cases showed greatly enhanced levels of CYP3A4 although the expression of CYP1A1 and CYP2E1 in the tumors was similar or lower than in the normal tissues.

The evaluation of the results from immunohistochemical analysis was summarized in Table 2 as the frequency of expression of AH-R and CYPs (CYP1A1, CYP2A6, CYP2E1 and CYP3A4) in patients with adenocarcinoma and squamous cell carcinoma. The first column shows the number of cases positively stained by more than one of the five tested factors (AH-R, CYP1A1, CYP2A6, CYP2E1 and CYP3A4). The second column shows the incidence of cases positively stained by more than one of the four CYPs (CYP1A1, CYP2A6, CYP2E1 and CYP3A4). Thirty-three of 48 (68.8%) adenocarcinoma patients were positive for 2 or more of the five factors, whereas only one of 30 squamous cell carcinoma patients fell into this category and none were positive for more than one CYP.

4.2. Relationship of the expression frequency of the markers with various clinical parameters

The relationships of the expression frequency of these enzymes with various clinical parameters in adenocarcinoma patients were also analyzed (Table 3). Again, the first column shows the number of positive cases for more than one of the five factors (AH-R or CYPs; AH-R, CYP1A1, CYP2A6, CYP2E1 and CYP3A4). The second and third columns show the number expressing only AH-R or CYP3A. Expression of more than one of these markers and expression of AH-R alone trended higher in female adenocarcinoma patients than in male adenocarcinoma patients ($p = 0.06$). Expression of CYP3A alone in female adenocarcinoma patients (59.7%) was significantly higher than in male adenocarcinoma patients (27.6%, $p = 0.04$). Expression of more than one of the five factors and expression of CYP3A alone tended to be more frequent in adenocarcinoma patients aged 66 years or older. When adenocarcinoma patients were divided into two groups, those with more and those with less than 400 cigarette-years (# of cigarettes per day X # years smoked) of smoking exposure, the frequency of expression of more than one of the five factors was significantly higher (84.0%) in the patients with a low smoking exposure than in those with more smoking exposure (52.1%, $p = 0.01$). The frequency of AH-R positive tumors in adenocarcinoma patients with a low smoking exposure was higher (56.0%) than in tumors of high-level smokers (17.4%, $p < 0.01$).

Related to clinical factors, expression of CYP3A in adenocarcinoma patients with T1 disease was significantly higher (61.1%) than in patients with T3 and T4 disease (20.0%, $p = 0.03$). Expression of either AH-R and CYP3A in adenocarcinoma patients with N0 disease was significantly more frequent (53.8% and 57.7%, respectively) than in adenocarcinoma patients with N1-3 disease (18.2%, $p = 0.01$ and 18.2%, $p < 0.01$, respectively). Expression of more than one of these five factors in adenocarcinoma patients with stage I disease was significantly more frequent (85.5%) than in those with stage II – IV disease (57.1%, $p = 0.04$). Expression of either AH-R or CYP3A in adenocarcinoma patients with stage I disease was significantly higher (60.0% and 70.0%, respectively) than in patients with stage II – IV disease (21.4% and 17.9%, respectively, $p < 0.01$). The level of differentiation is also associated with cases of more than one of these five factors, AH-R alone, and CYP3A alone. The cases of more poorly differentiated tumors showed much lower frequency of the three categories.

4.3. The expression of the markers and pathological stages

Pathological stages were compared with the number of factors expressed, including or excluding AH-R (either AH-R, CYP1A1, CYP2A6, CYP2E1 and CYP3A, or CYP1A1, CYP2A6, CYP2E1 and CYP3A4) in adenocarcinoma patients (Table 4). The frequency of more than three of the five factors in adenocarcinoma with stage I disease (75.0%) was significantly higher than that with stage II – IV disease (17.8%, $p < 0.01$). The frequency of expression of more than three of the four CYPs in adenocarcinoma with stage I disease (75.0%) was also significantly higher than that with stage II – IV disease (14.3%, $p < 0.01$).

Table 2. The frequency of AH-R and CYPs (CYP1A1, CYP2A6, CYP2E1 and CYP3A4) expression in patients with adenocarcinoma and squamous cell carcinoma

		Positive for >1 AH-R or CYPs [1] (%)			**Positive for >1 CYPs [2] (%)**		
Histology	n	+	-	p	+	-	p
Adenocarcinoma	48	33 (68.8)	15		33 (68.8)	15	
Squamous cell carcinoma	30	1 (3.3)	29	<0.01	0 (0.0)	30	<0.01
Total	78	34 (43.6)	44		33 (42.3)	45	

First column [1]: The number of cases in which the tumor was positive for more than one of the five factors (AH-R, CYP1A1, CYP2A6, CYP2E1 and CYP3A4). Second column [2]: The number of cases in which the tumor was positive for more than one of the four CYPs (CYP1A1, CYP2A6, CYP2E1 and CYP3A4).

Table 3. Relationships between the immunopositivity of AH-R and CYPs (CYP1A1, CYP2A6, CYP2E1 and CYP3A4) and various clinical or pathological parameters in adenocarcinoma patients

Parameter		**AH-R or CYPs [1] (%)**			**AH-R [2] (%)**			**CYP3A [3] (%)**		
	n	+	-	**p**	+	-	**p**	+	-	**p**
Sex										
Female	19	16 (84.2)	3		10 (52.6)	9		11 (57.9)	8	
Male	29	17 (58.6)	12	0.06	8 (27.6)	21	0.08	8 (27.6)	21	0.04
Age										
< 65	23	13 (56.5)	10		7 (30.4)	16		6 (26.1)	17	
> 65	25	20 (80.0)	5	0.08	11 (22.0)	14	0.33	13 (52.0)	12	0.07
Smoking [4]										
< 400	25	21 (84.0)	4		14 (56.0)	11		13 (52.0)	12	
> 400	23	12 (52.1)	11	0.02	4 (17.4)	19	<0.01	6 (26.1)	17	0.07
T [5]										
1	18	15 (83.3)	3		9 (50.0)	9		11 (61.1)	7	
2	10	6 (60.0)	4		4 (40.0)	6		4 (40.0)	6	
3, 4	20	12 (60.0)	8	0.24	5 (25.0)	15	0.28	4 (20.0)	16	0.03
N [6]										
0	26	20 (76.9)	6		14 (53.8)	12		15 (57.7)	11	
1, 2, 3	22	13 (59.1)	9	0.18	4 (18.2)	18	0.01	4 (18.2)	18	<0.01
Stage										
I	20	17 (85.5)	3		12 (60.0)	8		14 (70.0)	6	
II - IV	28	16 (57.1)	12	0.04	6 (21.4)	22	<0.01	5 (17.9)	23	<0.01
Differentiation										
• Well differentiated	3	3 (100)	0		3 (100)	0		3 (100)	0	
• Moderately differentiated	31	26 (83.9)	5		13 (41.9)	18		15 (48.4)	16	
• Poorly differentiated	14	4 (28.6)	10	<0.01	2 (14.3)	12	0.01	1 (7.1)	13	<0.01
Adenocarcinoma	48	33 (68.8)	15		18 (37.5)	30		19 (39.6)	29	

AH-R or CYPs [1]; The cases with more than one of five factors (AH-R, CYP1A1, CYP2A6, CYP2E1 and CYP3A4) expressed. AH-R [2]; Only AH-R is expressed. CYP3A [3]; Only CYP3A is expressed. Smoking [4]; The amount of lifetime cigarette consumption (cigarette/day X year). <400; low level smoker, >400; high level smoker.
T [5]; Tumor stage. N [6]; Nodal stage.

Table 4. Relationships between pathological stage and the number of expressed factors by RT PCR, including or excluding AH-R (AH-R, CYP1A1, CYP2A6, CYP2E1 and CYP3A vs. CYP1A1, CYP2A6, CYP2E1 and CYP3A) in adenocarcinoma patients

Frequency of tumors simultaneously expressing different numbers of the five tested factors, AH-R, CYP1A1, CYP2A6, CYP2E1 and CYP3A								
	Total	0	1	2	3	4	5	p
n (%)	48 (100)	15 (31.3)	7 (14.6)	6 (12.5)	5 (10.4)	10 (20.8)	5 (10.4)	
Stage I (%)	20 (100)	3 (15.0)	1 (5.0)	1 (5.0)	3 (15.0)	7 (35.0)	5 (25.0)	
Stage II-IV (%)	28 (100)	12 (42.9)	6 (21.4)	5 (17.9)	2 (7.1)	3 (10.7)	0 (0.0)	<0.01
Frequency of tumors simultaneously expressing different numbers of the four CYPs, CYP1A1, CYP2A6, CYP2E1 and CYP3A								
	Total	0	1	2	3	4	p	
n (%)	48 (100)	15 (31.3)	10 (20.8)	4 (8.3)	13 (27.1)	6 (12.5)		
Stage I (%)	20 (100)	3 (15.0)	1 (5.0)	1 (5.0)	10 (50.0)	5 (25.0)		
Stage II-IV (%)	28 (100)	12 (42.9)	9 (32.1)	3 (10.7)	3 (10.7)	1 (3.6)	<0.01	

Table 5. Relationships among AH-R, CYP1A1, CYP2A6, CYP2E1 and Cyp3A RNA expression in adenocarcinoma

	No of positive cases	**AH-R**	**CYP1A1**	**CYP2A6**	**CYP2E1**	**CYP3A**
AH-R	18		<0.01	0.10	<0.01	<0.01
CYP1A1	21			<0.05	<0.01	<0.01
CYP2A6	22				0.17	0.17
CYP2E1	19					<0.01
CYP3A	19					

Upper right; p-values for co-expression of AH-R, CYP1A1, CYP2A6, CYP2E1 and Cyp3A.

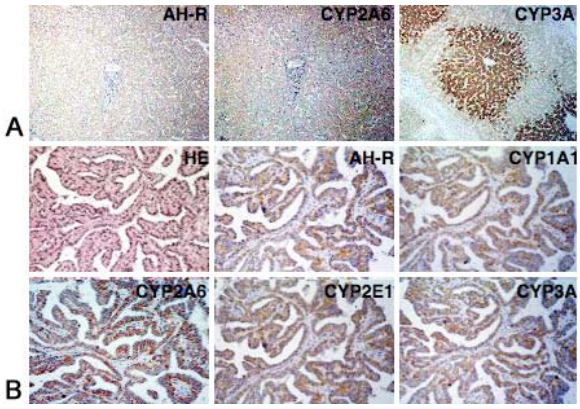

Figure 1. Immunohistochemical staining of the markers A. Immunostaining for AH-R (Aromatic hydrocarbon receptor), CYP2A6, CYP3A was carried out using human liver as a control. B. HE staining and immunohistochemistry represent typical images for AH-R, CYP1A1, CYP2A6, CYP2E1, and CYP3A in adenocarcinoma.

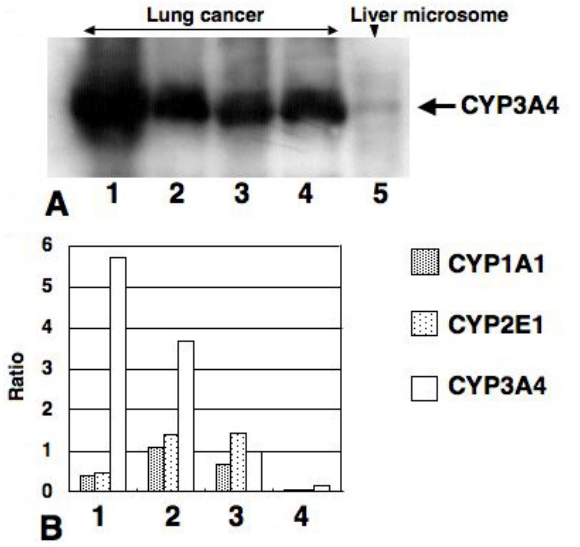

Figure 2. Expression of CYPs in lung adenocarcinoma. A. Western blot analysis of CYP3A in these four cases (lane from 1 to 4) and in liver microsomes (lane 5). CYP3A4 was detected in the tumor microsomes of four adenocarcinoma cases. AH-R in these four cases was detected in the tumor cytoplasm and CYP1A1, CYP2A6 and CYP2E1 were also detected in the tumor microsomes (data not shown). B. Normal and tumor lung tissue mRNA were extracted from the four cases shown in Figure 2A. Real-time polymerase chain reaction was carried out for quantitative determination of CYP1A1, CYP2E1 and CYP3A4 mRNA expression (CYPs expression levels) with normalization using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA. The ratio of CYP1A1, CYP2E1 and CYP3A4 expression levels in tumor tissue to those in normal tissue are shown in Figure 2B.

4.4. Correlation among the five markers for the expression in lung adenocarcinoma

Ah-R is known to regulate the expression of CYP1A1. As expected, the expressions of AH-R and CYP1A1 in adenocarcinoma were well correlated (Table 5). Intriguingly, the expressions of AH-R, CYP1A1, CYP2E1, and CYP3A were also found correlated each other while the expression of CYP2A6 was not correlated with those of other makers, suggesting that CYP2A6 in lung adenocarcinoma may be independently regulated from the other four markers.

4.5. Polymorphisms and the expression of CYP1A1 and CYP2E1

Since polymorphisms of CYP1A1 and CYP2E1 may be associated with the expression of the genes and susceptibility of lung cancer (2, 29), we examined whether these polymorphisms alter the expression frequency of CYP1A1 and CYP2E1 in lung adenocarcinoma. As seen in Figure 3A, Ile-Val polymorphism in the *CYP1A1* gene determined by the *Hinc II* digestion method was not correlated to the frequency of the expression of CYP1A1 protein in lung adenocarcinoma. Similarly, c1-c2 polymorphism in the *CYP2E1* gene determined by the *Rsa I* digestion also did not affect the frequency of the CYP2E1 expression (Figure 3B).

4.6. Mutations in p53 and the frequency of the marker expression

Upon immunohistochemical analysis, the expression of p53 protein was observed in 24 adenocarcinoma from 48 cases. In the 48 cases, mutations of p53 determined by PCR-SSCP method were found in 13 adenocarcinorma. However, we found no relationships between p53 alterations and the frequency of AH-R, CYP1A1, CYP2A6, CYP2E1 and CYP3A expression (Table 6). Therefore, we concluded that mutations of p53 and the expression frequency of the five markers are not correlated.

4.7. Poor survival and the expression of the CYPs

We divided 24 adenocarcinoma patients with stage III and IV disease into two groups, according to the status of CYPs expression. One is CYP (CYP1A1, CYP2A6, CYP2E1 and CYP3A) positive cases (n=14); the cases with more than one of four CYP expressed. Another is CYP negative cases (n=10); the cases with none of four CYP expressed. Figure 4 shows the survival curves for 24 adenocarcinoma patients with stage III and IV disease, according to the status of CYPs expression. There was a significant trend ($p = 0.03$) indicating that the expression of CYPs in advanced adenocarcinoma is associated with poor survival. This result suggests that better understanding of the CYP expression and its role may be important for the management of lung adenocarcinoma.

5. DISCUSSION

In this study, expression of the five tested factors (AH-R, CYP1A1, CYP2A6, CYP2E1 and CYP3A) was readily detected by immunohistochemistry. Protein and mRNA expression were demonstrated by Western blot

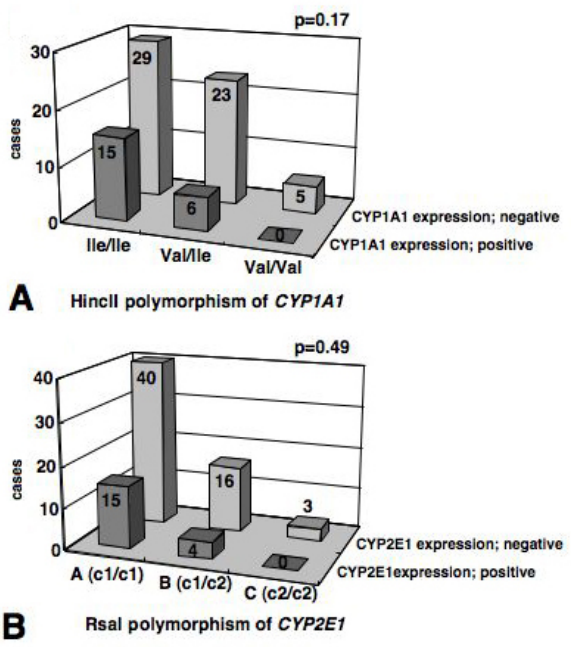

Figure 3. Polymorphisms and expression of CYP1A1 and CYP2E1. A. Relationship between HincII polymorphism of CYP1A1 and CYP1A1 expression. B. Relationship between RsaI polymorphism of CYP2E1 and CYP2E1 expression.

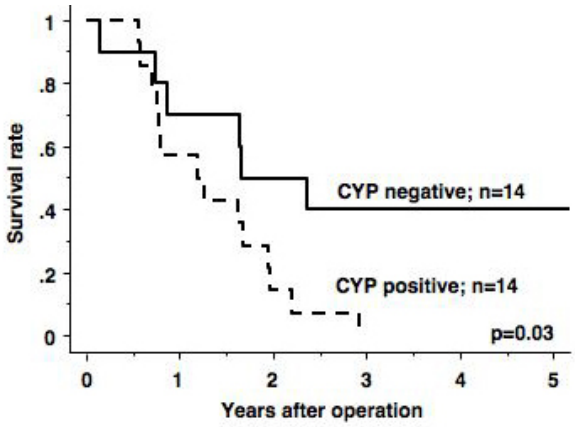

Figure 4. Survivals of adenocarcinoma patients with stage III and IV versus frequency of CYPs expression. CYP positive cases (n=14); cases with more than one CYP were expressed. CYP negative cases (n=10); cases with none of CYP were expressed.

analysis and real-time quantitative PCR analysis, respectively. We showed that the CYPs are frequently expressed in adenocarcinomas but not in squamous cell carcinomas in lung cancer (Table 2). AH-R immunostaining was also more intense in adenocarcinomas than in squamous cell carcinomas (9), although there was no association between CYP1A1 mRNA expression and histologic cell type of NSCLC (11).

In the human liver, Wolbold *et al*. reported that CYP3A4 is expressed at a higher level in women than in men (30). In the small intestine, however, the sex difference in the expression of CYP3A4 was not observed (31). Therefore, the sex difference of the expression may be tissue-dependent. In the adenocarcinoma, we observed that CYP3A RNA expression as well as the frequency of more than one factors were significantly greater in female than in male (Table 3). suggesting a sex difference in the expression of CYP3A in adenocarcinoma patients. In women, both smokers and non-smokers are at a 2-2.5-fold greater risk than in men for developing lung adenocarcinoma (32, 33). Therefore, our results suggest that the sex-difference in the expression of the factors might be associated with a higher risk of lung adenocarcinoma in women. Since CYP3A4 is involved in the metabolism of >50% of drugs on the market (34), the sexual difference of CYP3A expression in adenocarcinoma is an important factor for the management of lung cancer.

We demonstrated that the less smoking group had a higher frequency of the expression of five factors in adenocarcinoma (Table 3) as the poor differentiation and early cancer stage groups showed a higher frequency (Table 3 and 4). The stage-dependence of the expression of the factors also showed a tendency that more numbers of CYPs in a specimen was observed more frequently in early stage adenocarcinoma compared with in stage II-IV tumors (Table 4). These results suggest that CYPs might play important roles in tumor development and progression in early stage adenocarcinoma.

The expression of AH-R, CYP1A1, CYP2E1 and CYP3A in adenocarcinoma was correlated with each other (Table 5). Although the ligand-bound AH-R activates *CYP1A1* and *CYP1B1* gene transcription through interaction with xenobiotic responsive elements (XREs) (35), the induction of CYP3A and CYP2E1 mediated by AH-R has not been well known. The pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) are putative factors that regulate the transcription of *CYP3A* genes. Sp1, a constitutive transcription factor, may activate the transcription of *CYP2E1* gene (36). Therefore, the positive correlation among the four factors does not indicate the transcriptional activation of the three *CYP* genes by AH-R. Recently, Gerbal-Chaloin *et al*. reported that a metabolite of omeprazole (an atiulcer drug) by CYP3A4 activates AH-R and results in the induction of a series of AH-R dependent gene transcription (37). Therefore, the positive correlation among the expression of AH-R, CYP1A1, and CYP3A found in lung cancer might be due to the CYP3A-dependent metabolism of exogenous compounds. It is also important to note that CYP2A6 is not in a metabolite-mediated cross-talk that may involve AH-R, CYP1A1, CYP2E1, and CYP3A.

There are many single-nucleotide polymorphisms (SNPs) in the cytochrome P450 (CYP) genes (38). Individuals with Val/Val type of CYP1A1 (*CYP1A1; Val/Val*) have been shown to be increased susceptibility to lung cancer (2) although the incidence of the *p53* mutations in lung cancer did not seem to be significantly affected by

CYP1A1 and *GSTM1* polymorphisms in NSCLC patients (25). On the other hand, the cytochrome P450 2E1 gene (*CYP2E1*) is reported to have genetic and racial variations that are caused by *Pst*I, *Rsa*I, and *Dra*I RFLPs (29, 39, 40). The *Pst*I and *Rsa*I polymorphisms in the 5' flanking (promoter) region of the gene are reported to affect the transcriptional activity of the gene (increased inducibility) (29), and are completely linked with each other in the Japanese population (41). The predominant homozygous allele, the heterozygous allele, and the homozygous rare allele of the *Rsa*I polymorphism are named *type A* (*c1/c1*), *type B* (*c1/c2*), and *type C* (*c2/c2*), respectively, and the enhancer activity of *c2* allele was seen to be about ten times that of *c1* allele using a CAT analysis (41). There is positive relationship between the *Rsa*I polymorphism of CYP2E1 and the risk of squamous cell carcinoma (26). Since we found no relationship between their genetic polymorphism and tumor expression in both CYP1A1 and CYP2E1 (Figure 3), expression levels of the CYPs in tumor may be independent of their genetic polymorphism.

The investigation of alternations in the p53 gene is not only useful to evaluate tumor malignancy and tumor drug sensitivity but also useful for early diagnosis, molecular staging, molecular targeting therapy and gene therapy (42, 43). Many chemical carcinogens, such as benzo(a)pyrene, suffer from metabolic activation by CYPs (44). To elevate concentration of activated carcinogens, such as epoxide, by CYPs in tumor may cause increased incidence of gene mutations although there are no relationship between p53 alterations and CYPs expression in adenocarcinoma in this study. We observed a significant difference between positive and negative patients with CYPs expression statistically in adenocarcinoma patients with stage III and IV disease (Figure 4). Six of twenty-eight adenocarcinoma patients with stage III and IV disease were treated by anticancer drugs (cisplatin, carboplatin, etoposide and vindesine sulfate) during their perioperative period. The metabolism of anticancer drugs may be changed by CYPs in tumor. For instance, vinca alkaloids, such as vindesine sulfate, might be inactivated by CYP3A in tumor although survival effects of relationship between anticancer drugs and CYPs expression is still unclear in this study. Further study will be needed for selection of anti-cancer drug to adenocarcinoma.

Our results strongly suggest that better understanding of CYP expression in tumors should be useful and essential, 1) to investigate the mechanism of carcinogenesis, 2) to apply CYP expression as tumor marker, 3) to use the knowledge of CYP expression for molecular targeting therapy, 4) for selection of anti-cancer drug. The study of CYP expression may be one of aspects to change the order-made therapy to the tailor-made therapy.

6. ACKNOWLEDGMENT

This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sport, Science and Technology of Japan (16590492 to T.O.) This work was also obtained the patent (2003-270583) from Japan Patent Office.

7. REFERENCES

1. Sobue, T., S. Yamamoto, M. Hara, S. Sasazuki, S. Sasaki & S. Tsugane: Cigarette smoking and subsequent risk of lung cancer by histologic type in middle-aged Japanese men and women: the JPHC study. *Int J Cancer*, 99, 245-51 (2002)

2. Kawajiri, K., H. Eguchi, K. Nakachi, T. Sekiya & M. Yamamoto: Association of CYP1A1 germ line polymorphisms with mutations of the p53 gene in lung cancer. *Cancer Res*, 56, 72-6 (1996)

3. Denissenko, M. F., A. Pao, M. Tang & G. P. Pfeifer: Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in P53. *Science*, 274, 430-2 (1996)

4. Gonzalez, F. J. & H. V. Gelboin: Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins. *Drug Metab Rev*, 26, 165 -83 (1994)

5. Windmill, K. F., R. A. McKinnon, X. Zhu, A. Gaedigk, D. M. Grant & M. E. McManus: The role of xenobiotic metabolizing enzymes in arylamine toxicity and carcinogenesis: functional and localization studies. *Mutat Res*, 376, 153-60 (1997)

6. Oyama, T., T. Isse, N. Kagawa, T. Kinaga, Y. D. Kim, M. Morita, K. Sugio, H. Weiner, K. Yasumoto & T. Kawamoto: Tissue-distribution of aldehyde dehydrogenase 2 and effects of the ALDH2 gene-disruption on the expression of enzymes involved in alcohol metabolism. *Front Biosci*, 10, 951-60 (2005)

7. Kobayashi, A., K. Sogawa & Y. Fujii-Kuriyama: Cooperative interaction between AhR.Arnt and Sp1 for the drug-inducible expression of CYP1A1 gene. *J Biol Chem*, 271, 1210-6 (1996)

8. Reyes, H., S. Reisz-Porszasz & O. Hankinson: Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. *Science*, 256, 1193-5 (1992)

9. Lin, P., H. Chang, W. T. Tsai, M. H. Wu, Y. S. Liao, J. T. Chen & J. M. Su: Overexpression of aryl hydrocarbon receptor in human lung carcinomas. *Toxicol Pathol*, 31, 2-30 (2003)

10. Mollerup, S., D. Ryberg, A. Hewer, D. H. Phillips & A. Haugen: Sex differences in lung CYP1A1 expression and DNA adduct levels among lung cancer patients. *Cancer Res*, 59, 317-20 (1999)

11. McLemore, T. L., S. Adelberg, M. C. Liu, N. A. McMahon, S. J. Yu, W. C. Hubbard, M. Czerwinski, T. G. Wood, R. Storeng, R. A. Lubet & et al.: Expression of

CYP1A1 gene in patients with lung cancer: evidence for cigarette smoke-induced gene expression in normal lung tissue and for altered gene regulation in primary pulmonary carcinomas. *J Natl Cancer Inst*, 82, 1333-9 (1990)

12. Toussaint, C., N. Albin, L. Massaad, D. Grunenwald, O. Parise, Jr., J. Morizet, A. Gouyette & G. G. Chabot: Main drug- and carcinogen-metabolizing enzyme systems in human non-small cell lung cancer and peritumoral tissues. *Cancer Res*, 53, 4608-12 (1993)

13. Kushida, H., K. Fujita, A. Suzuki, M. Yamada, T. Endo, T. Nohmi & T. Kamataki: Metabolic activation of N-alkylnitrosamines in genetically engineered Salmonella typhimurium expressing CYP2E1 or CYP2A6 together with human NADPH-cytochrome P450 reductase. *Carcinogenesis*, 21, 1227-32 (2000)

14. Raunio, H., J. Hakkola, J. Hukkanen, O. Pelkonen, R. Edwards, A. Boobis & S. Anttila: Expression of xenobiotic-metabolizing cytochrome P450s in human pulmonary tissues. *Arch Toxicol Suppl*, 20, 465-9 (1998)

15. Shimada, T., H. Yamazaki, M. Mimura, Y. Inui & F. P. Guengerich: Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J Pharmacol Exp Ther*, 270, 414-23(1994)

16. Oyama, T., N. Kagawa, N. Kunugita, K. Kitagawa, M. Ogawa, T. Yamaguchi, R. Suzuki, T. Kinaga, Y. Yashima, S. Ozaki, T. Isse, Y. D. Kim, H. Kim & T. Kawamoto: Expression of cytochrome P450 in tumor tissues and its association with cancer development. *Front Biosci*, 9, 1967-76 (2004)

17. Guengerich, F. P.: Cytochrome P-450 3A4: regulation and role in drug metabolism. *Annu Rev Pharmacol Toxicol*, 39, 1-17 (1999)

18. Kivisto, K. T., E. U. Griese, P. Fritz, A. Linder, J. Hakkola, H. Raunio, P. Beaune & H. K. Kroemer: Expression of cytochrome P 450 3A enzymes in human lung: a combined RT-PCR and immunohistochemical analysis of normal tissue and lung tumours. *Naunyn Schmiedebergs Arch Pharmacol*, 353, 207-12 (1996)

19. Hukkanen, J., O. Pelkonen, J. Hakkola & H. Raunio: Expression and regulation of xenobiotic-metabolizing cytochrome P450 (CYP) enzymes in human lung. *Crit Rev Toxicol*, 32, 391-411 (2002)

20. Mountain, C. F. & C. M. Dresler: Regional lymph node classification for lung cancer staging. *Chest*, 111, 1718-23 (1997)

21. Brambilla, E., W. D. Travis, T. V. Colby, B. Corrin & Y. Shimosato: The new World Health Organization classification of lung tumours. *Eur Respir J*, 18, 1059-68 (2001)

22. Oyama, T., T. Osaki, N. Nose, Y. Ichiki, M. Inoue, H. Imoto, T. Yoshimatsu, M. Kodate, H. Uramoto, T. Mizoue, K. Yano & K. Yasumoto: Evaluations of p53 immunoreactivity, nucleolar organizer regions, and proliferating cell nuclear antigen in non-small cell lung carcinoma. *Anticancer Res*, 20, 505-10 (2000)

23. Dikov, M. M., T. Oyama, P. Cheng, T. Takahashi, K. Takahashi, T. Sepetavec, B. Edwards, Y. Adachi, S. Nadaf, T. Daniel, D. I. Gabrilovich & D. P. Carbone: Vascular endothelial growth factor effects on nuclear factor-kappaB activation in hematopoietic progenitor cells. *Cancer Res*, 61, 2015-21 (2001)

24. Nishimura, M., H. Yoshitsugu, S. Naito & I. Hiraoka: Evaluation of gene induction of drug-metabolizing enzymes and transporters in primary culture of human hepatocytes using high-sensitivity real-time reverse transcription PCR. *Yakugaku Zasshi*, 122, 339-61 (2002)

25. Oyama, T., T. Kawamoto, T. Mizoue, K. Nishida, T. Osaki, K. Sugio, K. Yasumoto & T. Mitsudomi: p53 mutations of lung cancer are not significantly affected by CYP1A1 or GSTM1 polymorphisms. *Int J Oncol*, 11, 305-309 (1997)

26. Oyama, T., T. Kawamoto, T. Mizoue, K. Sugio, Y. Kodama, T. Mitsudomi & K. Yasumoto: Cytochrome P450 2E1 polymorphism as a risk factor for lung cancer: in relation to p53 gene mutation. *Anticancer Res*, 17, 583-7 (1997)

27. Mitsudomi, T., T. Oyama, T. Kusano, T. Osaki, R. Nakanishi & T. Shirakusa: Mutations of the p53 gene as a predictor of poor prognosis in patients with non-small-cell lung cancer. *J Natl Cancer Inst*, 85, 2018-23 (1993)

28. Oyama, T., T. Osaki, T. Mitsudomi, R. Ogawa, R. Nakanishi, K. Sugio & K. Yasumoto: p53 alteration, proliferating cell nuclear antigen, and nucleolar organizer regions in thymic epithelial tumors. *Int J Mol Med*, 1, 823-6 (1998)

29. Kato, S., P. G. Shields, N. E. Caporaso, R. N. Hoover, B. F. Trump, H. Sugimura, A. Weston & C. C. Harris: Cytochrome P450IIE1 genetic polymorphisms, racial variation, and lung cancer risk. *Cancer Res*, 52, 6712-5 (1992)

30. Wolbold, R., K. Klein, O. Burk, A. K. Nussler, P. Neuhaus, M. Eichelbaum, M. Schwab & U. M. Zanger: Sex is a major determinant of CYP3A4 expression in human liver. *Hepatology*, 38, 978-88 (2003)

31. Paine, M. F., S. S. Ludington, M. L. Chen, P. W. Stewart, S. M. Huang & P. B. Watkins: Do men and women differ in proximal small intestinal CYP3A or P-glycoprotein expression? *Drug Metab Dispos*, 33, 426-33 (2005)

32. Shields, P. G.: Epidemiology of tobacco carcinogenesis. *Curr Oncol Rep*, 2, 257-62 (2000)

33. Siegfried, J. M.: Women and lung cancer: does oestrogen play a role? Lancet Oncol, 2, 506-13 (2001)

34. Thummel, K. E. & G. R. Wilkinson: In vitro and in vivo drug interactions involving human CYP3A. *Annu Rev Pharmacol Toxicol*, 38, 389-430 (1998)

35. McFadyen, M. C., P. H. Rooney, W. T. Melvin & G. I. Murray: Quantitative analysis of the Ah receptor/cytochrome P450 CYP1B1/CYP1A1 signalling pathway. *Biochem Pharmacol*, 65, 1663-74 (2003)

36. Peng, H. M. & M. J. Coon: Promoter function and the role of cytokines in the transcriptional regulation of rabbit CYP2E1 and CYP2E2. *Arch Biochem Biophys*, 382, 129-37 (2000)

37. Gerbal-Chaloin, S., L. Pichard-Garcia, J. M. Fabre, A. Sa-Cunha, L. Poellinger, P. Maurel & M. Daujat-Chavanieu: Role of CYP3A4 in the regulation of the aryl hydrocarbon receptor by omeprazole sulphide. *Cell Signal*, 18, 740-50 (2006)

38. Saito, S., A. Iida, A. Sekine, S. Kawauchi, S. Higuchi, C. Ogawa & Y. Nakamura: Catalog of 680 variations among eight cytochrome p450 (CYP) genes, nine esterase genes, and two other genes in the Japanese population. *J Hum Genet*, 48, 249-70 (2003)

39. Hirvonen, A., K. Husgafvel-Pursiainen, S. Anttila, A. Karjalainen & H. Vainio: The human CYP2E1 gene and lung cancer: DraI and RsaI restriction fragment length polymorphisms in a Finnish study population. *Carcinogenesis*, 14, 85-8 (1993)

40. Uematsu, F., S. Ikawa, H. Kikuchi, I. Sagami, R. Kanamaru, T. Abe, K. Satoh, M. Motomiya & M. Watanabe: Restriction fragment length polymorphism of the human CYP2E1 (cytochrome P450IIE1) gene and susceptibility to lung cancer: possible relevance to low smoking exposure. *Pharmacogenetics*, 4, 58-63 (1994)

41. Hayashi, S., J. Watanabe & K. Kawajiri: Genetic polymorphisms in the 5'-flanking region change transcriptional regulation of the human cytochrome P450IIE1 gene. *J Biochem (Tokyo)*, 110, 559-65 (1991)

42. Hege, K. M. & D. P. Carbone: Lung cancer vaccines and gene therapy. *Lung Cancer*, 41 Suppl 1, S103-13(2003)

43. Oyama, T., T. Osaki, T. Baba, Y. Nagata, M. Mizukami, T. So, S. Nakata, Y. Ichiki, H. Uramoto, M. Sugaya, T. Yoshimatsu, M. Morita, T. Hanagiri, K. Sugio, T. Kawamoto & K. Yasumoto: Molecular genetic tumor markers in non-small cell lung cancer. *Anticancer Res*, 25, 1193-6 (2005)

44. Oyama, T., A. Matsumoto, T. Isse, Y. D. Kim, S. Ozaki, T. Osaki, K. Sugio, K. Yasumoto & T. Kawamoto: Evidence-based prevention (EBP): approach to lung cancer prevention based on cytochrome 1A1 and cytochrome 2E1 polymorphism. *Anticancer Res*, 23, 1731-7 (2003)

Abbreviations: AH-R: aryl hydrocarbon receptor, CYP: cytochrome P450, NSCLC: non-small cell lung cancer

Key Words: Cytochrome P450, Non-small cell lung cancer, NSCLC, Survival

Send correspondence to: Tsunehiro Oyama, M.D., Ph.D., Department of Environmental Health, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu, 807-8555, Japan, Tel: 93-691-7429, Fax : 93-692-9341, E-mail: oyama@med.uoeh-u.ac.jp