



Association of aryl hydrocarbon receptor and cytochrome P4501B1 expressions in human non-small cell lung cancers

Pinpin Lin^{a,*}, Han Chang^b, William L. Ho^c, Meng-Hsiang Wu^a, Jang-Ming Su^d

^a *Institute of Toxicology, Chung-Shan Medical University, No. 110, Sec 1, Chien-Kuo N. Rd., Taichung 402, Taiwan, ROC*

^b *Department of Pathology, Chung-Shan Medical University Hospital, Taichung, Taiwan*

^c *Department of Pathology, Veterans General Hospital, Taichung, Taiwan*

^d *Division of Thoracic Surgery, Department of Surgery, Chung-Shan Medical University Hospital, Taichung, Taiwan*

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Summary It has been shown that cytochrome P4501B1 (CYP1B1) is over-expressed in variety of tumors. The objective of this study was to examine the expression and localization of CYP1B1 in normal lungs and tumor tissues of non-small cell lung cancers (NSCLC). CYP1B1 was detectable in the microsomes of normal lungs and lung tumors with Western immunoblotting. Utilizing immunohistochemical staining, we found that CYP1B1 was constitutively expressed in the cytoplasm of smooth muscle cells, but not in pneumocytes. Bronchiolar epithelia from three out of 19 normal lungs expressed CYP1B1. Among 89 NSCLC tumor tissues, more than 50% expressed CYP1B1. In addition, CYP1B1 expression was more common in adenocarcinomas than in squamous cell carcinomas. Recently, we reported over-expression of aryl hydrocarbon receptors (AhR) in lung adenocarcinomas. In the present study, we observed an association between CYP1B1 and AhR expression in 89 NSCLC tumor tissues. These results suggest that CYP1B1 overexpression in NSCLC is accompanied by up-regulation of AhR expression.

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1. Introduction

Epidemiological studies have demonstrated that exposure to environmental pollutants, such as environmental tobacco smoke and other polycyclic aromatic hydrocarbons (PAH) contaminated

air, contributes to the development of lung cancers [1]. Metabolic activation is the first critical step for chemical carcinogen-associated lung carcinogenesis. The cytochrome P450 family 1 is one of the major cytochrome P450 families involved in xenobiotic metabolism. Cytochrome P4501B1 (CYP1B1) is a recently identified member of cytochrome P450 family 1 [2]. CYP1B1 is involved in metabolic activation of some exogenous and endogenous carcinogens, such as 17-β

*Corresponding author. Fax: +886-4-24751101.

E-mail address: ppl@csmu.edu.tw (P. Lin).

estradiol, 7,12-dimethylbenzo[*a*]anthracene and 1-nitropyrene [3–5]. For example, CYP1B1 hydrolyzes 17- β estradiol to 4-hydroxylated estradiol, which is an animal carcinogen [6]. In addition, CYP1B1 was mainly expressed in extrahepatic tissues [2]. Utilizing an immunohistostaining method, Murray et al. [7] demonstrated that CYP1B1 protein was expressed in human lung tumors, but not in non-tumor tissues. However, a recent study by Spivack et al. [8] showed that CYP1B1 protein was detectable in both lung tumors and non-tumor tissues with Western immunoblotting. These results suggest that CYP1B1 may be involved in the metabolic activation of carcinogens in lungs. However, the localization of CYP1B1 in normal lungs is still unclear.

Cytochrome P450 is also involved in the metabolism of anticancer drugs. The consequence of metabolism results in either inactivation or activation of anticancer drugs. CYP1B1 protein is overexpressed in various human malignant tumors, such as cancers of the breast, colon, lung, esophagus, skin, lymph node, brain and testis [7]. Later, McFadyen et al. [9] demonstrated that CYP1B1-overexpressed cells became more resistant to docetaxel. Docetaxel is used as the first and second line treatment for non-small cell lung cancers (NSCLC) [10]. Therefore, CYP1B1 was also implicated in the resistance to anticancer drugs.

Human lung cancers are highly heterogeneous and differ by histological types. Adenocarcinoma (AD) and squamous cell carcinoma (SQ) are classified as NSCLC. In Taiwan, more than 80% of lung tumors are either AD or SQ [11]. Recently, we showed that a high expression of aryl hydrocarbon receptors (AhR) was more common in lung AD than in SQ [12]. Several studies suggested that AhR regulates *CYP1B1* gene expression [13,14]. Therefore, CYP1B1 expression may be higher in lung AD than in SQ. On the other hand, cigarette smoke activated the AhR and increased the *CYP1A1* expression in animal studies [15]. Thus, it is possible that CYP1B1 expression may be higher in smokers than in non-smokers. However, SQ was more strongly associated with cigarette smoking than AD [16] and AhR expression was similar in lung tumors from smokers and non-smokers [12]. Therefore, it is unclear how histological types, cigarette smoking, and AhR expression may affect CYP1B1 expression in lung tumors.

In the present study, we examined CYP1B1 expression in a larger numbers of NSCLC tumor tissues, a total of 89 lung AD and SQ, with the immunohistostaining method. The expression and localization of CYP1B1 in normal lungs was also studied with Western immunoblotting and immunohistostaining. Finally, we evaluated the correlation between AhR

and CYP1B1 expression in lung tumors. We believe that the results of the present investigation offer information about CYP1B1 expression in lung cancers and its mechanism.

2. Materials and methods

2.1. Patient samples

A panel of 89 primary lung cancer specimens, including 59 AD and 30 SQ, were examined in the present study. Tumor types were classified according to 1999 WHO classification [17]. TNM staging was used [18]. There were, respectively, 36, 16, 31 and 6 patients in Stages I–IV. Neither chemotherapy nor radiation therapy was performed before operation for these patients. The ages of 89 patients ranged from 33 to 84-year-old, including 48 males and 41 females. Among them, 19 are current smokers, 7 former smokers and 63 non-smokers. We classified current smokers and former smokers as ever smokers. All specimens were obtained from patients undergoing surgery for lung cancer. These specimens were fixed in 10% neutral-buffered formalin and embedded in paraffin. The other six fresh lung tumors and paired non-tumor tissues were stored at -20°C immediately after operation and analyzed with Western immunoblotting later. Twenty-three normal lung tissues were obtained from patients with spontaneous pneumothorax. Western immunoblotting was performed on four and immunohistochemical staining on 19. This project was approved by the Human Subjects Committee in Chung Shan Medical University Hospital, Taichung, Taiwan.

2.2. Tissues preparation for Western immunoblotting

Lung tissues were stored at -20°C after operation. To prepare whole tissue extracts, tissues were ground in liquid nitrogen and directly extracted with sample buffer for electrophoresis. To prepare microsomes, grounded tissues were extracted with 20 mM Tris, pH 7.4, 0.5 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 50 $\mu\text{g}/\text{ml}$ leupeptin, 50 $\mu\text{g}/\text{ml}$ aprotinin, 2.5 $\mu\text{g}/\text{ml}$ pepstatin A, 0.2 mM phenylmethylsulfonyl fluoride and protease inhibitors cocktail (Roche Applied Science, Mannheim, Germany). Tissue homogenates were centrifuged at $10,000 \times g$, 4°C , for 20 min and then the supernatant was further centrifuged at $100,000 \times g$ for 1 h. The microsome pellets were dissolved in sample buffer for electrophoresis. Whole tissue extracts and microsomes in sample buffer were boiled, resolved by denaturing electrophoresis on discontinuous polyacrylamide gel, electrotrans-

ferred to PVDF membrane and immunostained with CYP1B1 antibody (Gentest Corp., Woburn, MA; catalog number A211). Bands were visualized with the enhanced chemoluminescence kit according to the manufacturer's instructions (Amersham, Buckinghamshire, UK).

2.3. Immunohistochemistry

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded lung tissue using the LSAB kit (DAKO, Glostrup, Denmark) and the DAB-plus substrate kit (ZYMED, San Francisco, CA). Tissue sections (4–5 μ m) were immersed in 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity, then microwaved in 0.01 M citrate buffer (pH 6.0) for 3×5 min. After washing, the sections were incubated at 4°C overnight with anti-AhR (90-fold dilution; from Biomol, Plymouth Meeting, PA) or anti-CYP1B1 (1500-fold dilution; from Gentest Corp., Woburn, MA; catalog number A211) antibody and followed by a standard staining procedure using the LSAB kit.

2.4. Evaluation of immunostaining

The immunointensity of vascular smooth muscle cells, which showed consistently positive anti-CYP1B1 immunostaining in each section examined, were defined as an internal positive control. Non-specific rabbit serum, instead of primary antibody anti-CYP1B1, was used as the negative control staining. The pattern in positively stained cells revealed cytoplasmic staining. Neither nuclear nor membranous staining was identified. CYP1B1 immunostaining in the tumor cells was scored as negative (–) or positive (+). The positivity of anti-CYP1B1 immunostaining was determined by three blinded examiners (performed by Chang, Wu, and Lin). At least 100 cells were counted and evaluated per specimen. If the majority of cells showed similar or stronger intensity than smooth muscle cells they were recorded as positive. Tissue samples showing undetectable or weaker immunostaining intensity than those of smooth muscle cells were scored as negative.

The AhR expression in lung tissues was measured with an AhR immunostaining technique as previously described [12]. IHC scoring for AhR was graded on a scale of 0–3. AhR staining intensity in tumor cells was evaluated using reactions in smooth muscle cells as internal, built-in controls and basal cells of the prostate gland as an external control. Tissue samples showing undetectable or negligible expression were scored as Grade-0 intensity. Cells showing similar intensity with smooth muscle cells

were recorded as Grade-1 intensity. When positive reactions in cells were similar to those in prostate basal cells, they were scored as Grade-2 intensity. Cells showing significantly more intense staining than prostate basal cells were recorded as Grade-3 intensity. Grades-0 and -1 were combined as low expressers, and Grades-2 and -3 as higher expressers. In addition to visual evaluations by three investigators, staining gradings for CYP1B1 and AhR were also verified by the image analysis system Image Pro Plus (Media Cybernetics Inc., Silver Spring, MD). Statistical analysis: Pearson's χ^2 -test was used to examine the difference in CYP1B1 expression between AD and SQ. The correlation between AhR, and CYP1B1 expression levels were evaluated using Pearson's χ^2 -test too.

3. Results

The specificity of CYP1B1 antibodies used in our study was confirmed with Western immunoblotting (Fig. 1). The anti-CYP1B1 antibody detected one single band of 52 kDa protein in human CYP1B1 expressed in insect cells (Gentest, Woburn, MA), in microsomes of benzo[*a*]pyrene-treated human lung adenocarcinoma cell line NCI-H1355, in homogenates of human lung tumors, and in microsomes of human lung tumors (Fig. 1). These protein bands were consistent with the known human CYP1B1 molecular weight.

CYP1B1 protein was detectable in microsomes prepared from four normal human lungs with Western immunoblotting (Fig. 2(A)). We further examined CYP1B1 protein in microsomes of six paired lung tumors and non-tumor tissues those were adjacent to tumors. As shown in Fig. 2(B), CYP1B1 protein was abundant in all tumors and non-tumor tissues.

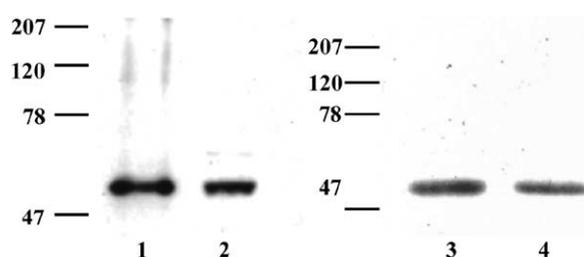


Fig. 1 The specificity of CYP1B1 antibodies. (Lane 1) Microsomes of human CYP1B1 expressed in insect cells (Gentest, Woburn, MA); (lane 2) microsomes of 10 μ M benzo[*a*]pyrene-treated H1355 cells; (lane 3) whole tissue homogenates of lung tumors; and (lane 4) microsomes of lung tumors were electrophoresed, and Western immunoblotted with anti-CYP1B1 as described in Section 2.

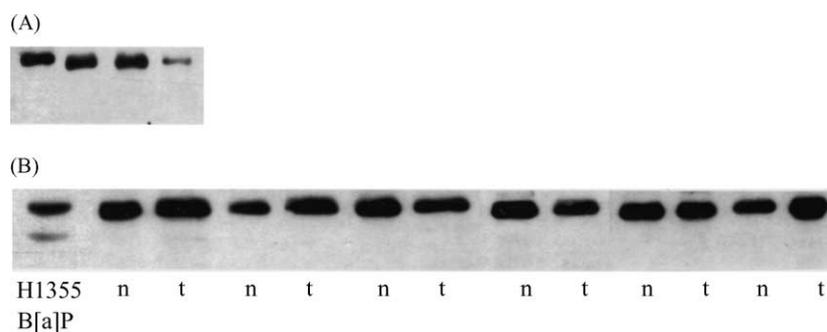


Fig. 2 Western immunoblotting of CYP1B1 in non-tumor and tumor lung tissues. Microsomes of: (A) normal lungs; (B) lung tumors (*t*) and paired non-tumor tissues (*n*) were prepared and examined for CYP1B1 expression with Western immunoblotting as previously described in Section 2.

CYP1B1 protein expression levels and locations were examined with the immunohistostaining method in 19 normal lung tissues and 89 tumor tissues of NSCLC. In normal lung tissues, CYP1B1 protein was consistently expressed in the cytoplasm of smooth muscle cells under the bronchiolar walls and blood vessels. Compared to smooth muscle

cells under the bronchiolar walls, the bronchiolar epithelia of most specimens revealed negative or weak immunostaining intensity (Fig. 3(A)). Type I or Type II pneumocytes showed no immunoreactivity (Fig. 3B). Anti-CYP1B1 showed specific staining in the cytoplasm of AD cells (Fig. 3(C)). Non-specific reactions were not observed in the negative control

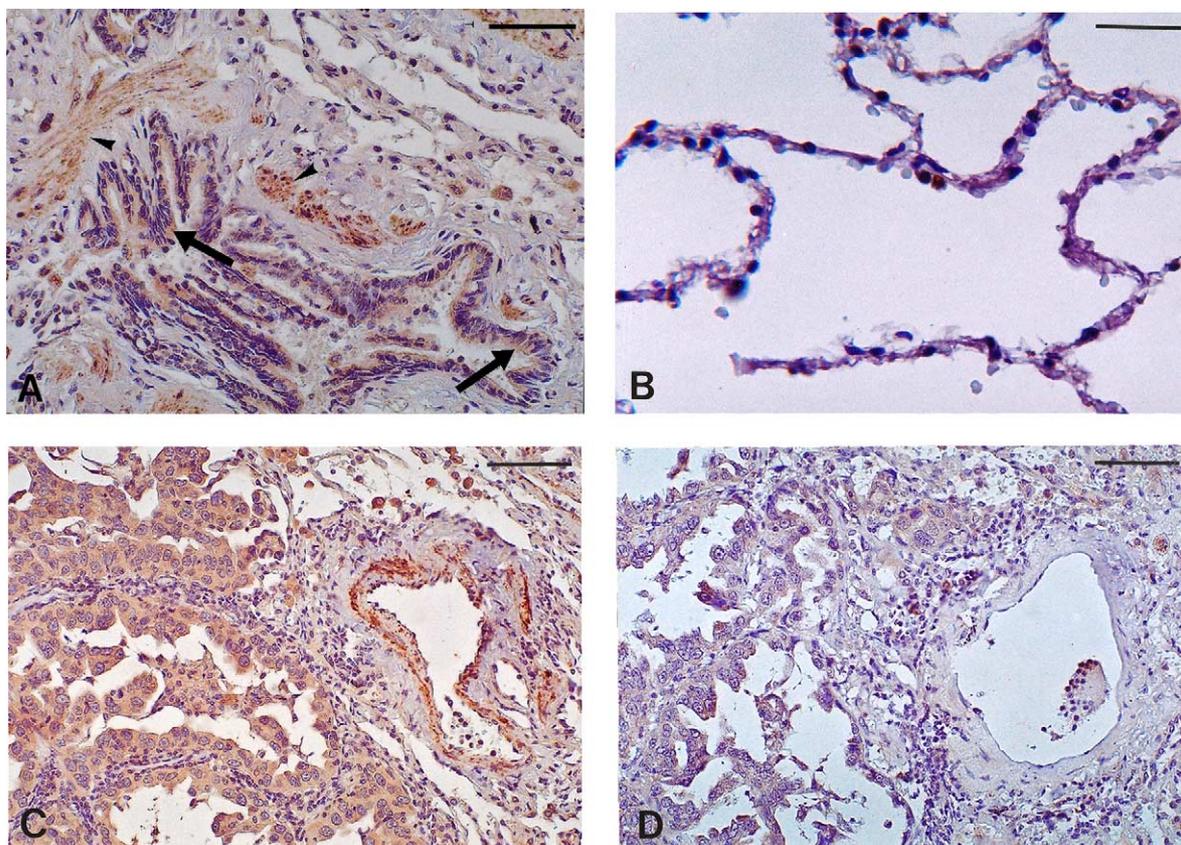


Fig. 3 CYP1B1 immunostaining microphotographs of normal lung tissues and lung tumors. Tissue sections were stained with anti-AhR and the reactivity was visualized as brown color by DAB as described in Section 2. (A) The (arrow) bronchiolar epithelial cells showed (arrow head) weaker cytoplasmic immunostaining than smooth muscle cells (scale: bar = 25 μ m). (B) Negative CYP1B1 immunostaining occurred in pneumocytes (scale: bar = 12.5 μ m). (C) Lung adenocarcinoma was stained with anti-CYP1B1 (scale: bar = 25 μ m). (D) Lung adenocarcinoma was stained with non-specific rabbit serum as a negative control (scale: bar = 25 μ m).

Table 1 CYP1B1 expression in human normal lung as well as non-small cell lung cancers^a

	CYP1B1 expression		
	<i>n</i> ^b	–	+
Normal lung tissues			
Bronchiolar epithelium	19	16	3
Type I or Type II pneumocyte	19	19	0
Smooth muscle cells of bronchiolar walls and vessels	19	0	19
Non-small cell lung cancers	89	47	42 ^c

^a CYP1B1 expression was determined with immunohistochemical staining.

^b *n*, The case numbers.

^c CYP1B1 expression was more common in non-small cell lung cancers than in bronchiolar epitheliums (χ^2 -test, $P < 0.05$).

using a serial section (Fig. 3(D)). The immunoreactivity of anti-CYP1B1 in tissues was summarized in Table 1. The prevalence of anti-CYP1B1 immunoreaction was low (15.8%) in bronchiolar epithelia. In addition, CYP1B1 was not detected in pneumocytes. However, 42 of 89 (47.2%) NSCLC showed positive CYP1B1 immunoreactivity. These included 34 AD and 8 SQ. Thus, CYP1B1 expression was more common in NSCLC than in bronchiolar epithelia ($P < 0.05$). CYP1B1 expression in NSCLC

Table 2 CYP1B1 expression in relation to gender, histology and smoking behavior in non-small cell lung cancers^a

	<i>n</i> ^b	CYP1B1		<i>P</i> ^e
		–	+	
Number of cases	89	47	42	
Gender				
Male	48	27	21	0.482
Female	41	20	21	
Histology				
AD ^c	59	25	34	0.006
SQ ^d	30	22	8	
Smoking behavior				
Ever smoker	26	18	8	0.046
Non-smoker	63	29	34	

^a CYP1B1 expression was determined with immunohistochemical staining.

^b *n*, Specimen numbers.

^c AD represented adenocarcinoma.

^d SQ represented squamous cell carcinoma.

^e *P*-value after Pearson χ^2 analysis.

Table 3 Correlation between CYP1B1 and AhR expression in lung cancers^a

CYP1B1	AhR		<i>r</i> ^b
	Low	High	
–	20	27	0.59 ^c
+	5	37	0.59 ^c

^a CYP1B1 and AhR expression was determined with immunohistochemical staining.

^b Spearman rank correlation coefficients.

^c $P < 0.001$.

was not differed by gender (Table 2). After stratification by histology, more than 50% (34/59) of AD expressed CYP1B1 protein (Table 2). Furthermore, CYP1B1 expression was more common in AD than in SQ ($P < 0.05$). Similarly, CYP1B1 expression was more common in non-smokers than in ever smokers ($P < 0.05$). Among ever smokers, the prevalence of CYP1B1 expression was similar between current and former smokers (data not shown).

The AhR immunostaining assay in this study was an extension from our previous study. Some of these specimens were immunostained with anti-AhR in our previous study. Among 89 tumor tissues of NSCLC, 64 expressed high levels of AhR protein (Table 3). More than 50% (37/64) of high AhR expressers were CYP1B1 positive. Similarly, the majority of AhR low expressers (80%, 20/25) were CYP1B1 negative. Data from statistical analysis showed that CYP1B1 expression positively correlated with AhR expression in NSCLC ($P < 0.001$).

4. Discussion

The objective of this study was to examine the expression and localization of CYP1B1 in normal lungs and tumor tissues of NSCLC. In normal lungs, CYP1B1 was expressed in the cytoplasm of smooth muscle cells, but weakly expressed in bronchiolar epithelial cells. CYP1B1 also expressed in the cytoplasm of tumor cells. Therefore, our data are consistent with Murray et al.'s results [7] that CYP1B1 over-expressed in lung tumor cells. The more important finding in our study was that CYP1B1 expression was more common in AD than in SQ. It is plausible to develop anticancer drugs those are metabolically activated by CYP1B1. CYP1B1-activated drugs will be specific to tumor cells, especially AD.

Shehin et al. [14] have demonstrated that the dioxin response elements in the enhancer region of *CYP1B1* were responsible for the constitutive and induced expression of *CYP1B1*. Previously, we reported that AhR was over-expressed in lung tumors and its expression was more prevalent in lung AD than SQ [12]. We also demonstrated that benzo[*a*]pyrene, a carcinogenic components found in cigarette smoke, activated AhR and increased *CYP1B1* expression in lung cancer cell lines [19]. On the other hand, a study by Spivack et al. [8] reported that *CYP1B1* was elevated in human lung cancer patients who were active smokers. Spivack et al.'s [8] finding suggested that cigarette smoke might induce *CYP1B1* expression in lung tumors. In the present study, we showed that *CYP1B1* expression correlated well with AhR expression in lung tumors. However, *CYP1B1* expression was more common in non-smokers than in ever smokers. This discrepancy may rest on the duration between active smoking and sampling of the tissues. Our samples were obtained from smokers who probably had ceased smoking for a considerable period of time before lung tissues were obtained during surgery (in Taiwan, all patients with lung cancers are advised to cease cigarette smoking from time of diagnosis). The cessation of active smoking may lessen the influence of cigarette smoke on *CYP1B1* expression as claimed by Spivack et al. [8]. These data strongly suggest that *CYP1B1* expression in lung tumor cells is accompanied by up-regulation of AhR expression, but not by exposure to cigarette smoke in these specimens.

Our analyses on the interrelationship between the AhR and *CYP1B1* expressions revealed a very interesting and important phenomenon. Our data showed that 88.1% (37/42) of the positive *CYP1B1* expression was associated with an elevated AhR expression and 80.0% (20/25) of the specimens with low AhR expression were also *CYP1B1* negative. However, our study also showed that a reverse relationship did not occur. That is, tumors with an up-regulation of AhR were not necessarily accompanied by an elevation of *CYP1B1* (only 37/64). These findings indicate that the causal relationship between AhR and *CYP1B1* proteins is a complex one. While *CYP1B1* elevation may depend on the status of AhR in the cells, other "factors" may also be involved in interlinking or activating this process. That is, it is likely that other factors may influence AhR activation and the subsequent up-regulation of *CYP1B1* in lung tumors. AhR is a ligand-activated transcription factor. However, endogenous ligands for AhR have not yet been identified. Recently, Song et al. [20] isolated an AhR ligands from porcine lungs. It is

possible that AhR activation by endogenous ligands is required for the constitutive expression of *CYP1B1* in lungs. Thus, the mere presence or elevation of AhR alone will not necessarily result in an elevation or induction of *CYP1B1* in the cells.

It is well-recognized that AhR regulates *CYP1A1* gene expression. Our previous study have reported that mRNA levels of *CYP1A1* and *CYP1B1* were simultaneously increased by B[*a*]P in human lung cancer cells [19]. This observation was later confirmed by Mollerup et al. [21] in TCDD-treated bronchial epithelial cells [22]. *CYP1A1* expression in lung tumors has been reported by Anttila et al. [22] and Saarikoski et al. [23]. Both of these investigators reported that both *CYP1A1* mRNA and protein expression were more intense in peripheral AD than in SQ. Compared to our data, it is likely that overexpression of AhR simultaneously up-regulates *CYP1A1* and *CYP1B1* in lung AD.

Previous studies reported contradictory results for *CYP1B1* expression in normal lungs. Our data indicated that this inconsistency might result from the difference in techniques. Consistent with the study by Murray et al. [7], utilizing immunohistostaining method we confirmed that *CYP1B1* was barely detectable in the bronchiolar cells of normal lungs. However, *CYP1B1* protein was abundant in microsomes prepared from non-tumor lungs detected with Western immunoblotting. Lung tissues contain a variety of cell types, such as epithelial cells, smooth muscle cells, pneumocytes and stromal cells. It is likely that *CYP1B1* protein detected in tissue microsomes mainly comes from smooth muscle cells.

Some studies have shown that *CYP1B1* expression and polymorphisms are associated with breast cancer risk [12,24,25]. The association was explained by the ability of *CYP1B1* to convert 17 β -estradiol into carcinogenic metabolites [26]. However, our study and the study by Spivack et al. [8] did not detect any gender difference in *CYP1B1* expression. *CYP1B1* also metabolically activates polycyclic aromatic hydrocarbons [3]. In our present study, however, we failed to observe an association between *CYP1B1* expression in tumors and history of cigarette smoking in patients. Thus, further investigations are needed to define the contribution of *CYP1B1* to lung carcinogenesis.

In the present study, we demonstrated that *CYP1B1* is prevalently expressed in lung AD. This finding inspires us to explore the possibility of *CYP1B1* as a diagnosis biomarker for lung AD in the future. In addition, targeting *CYP1B1* will be a potential method for the design of anticancer drugs for lung AD in the future.

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