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Immunohistochemical analysis of CYP2A13 in various types of human lung cancers

Tatsuki Fukami,[1] Miki Nakajima,[1,4] Isao Matsumoto,[2] Yoh Zen,[3] Makoto Oda[2] and Tsuyoshi Yokoi[1]

[1]Drug Metabolism and Toxicology, Faculty of Pharmaceutical Sciences, Kanazawa University, Kakuma-machi, Kanazawa; [2]Department of General and Cardiothoracic Surgery, Kanazawa University School of Medicine, Takara-machi, Kanazawa; [3]Division of Pathology, Kanazawa University Hospital, Takara-machi, Kanazawa, Japan

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Human CYP2A13, which is expressed in the respiratory tract, is the most efficient enzyme for the metabolic activation of tobacco-specific nitrosamines such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). The relevance of CYP2A13 in carcinogenicity and toxicity in the respiratory tract has been suggested, but the expression of CYP2A13 protein in lung cancer tissues remains to be determined. We first prepared a mouse monoclonal antibody against human CYP2A13. The antibody showed no cross reactivity with the other CYP isoforms including CYP2A6. Using the specific antibody, we performed immunohistochemical analysis for human lung carcinomas. In adenocarcinomas (n = 15), all specimens were positive for the staining and five samples showed strong staining. In squamous cell carcinomas (n = 15) and large cell carcinomas (n = 15), each 14 samples were positive for the staining and two and three samples showed strong staining, respectively. In small cell carcinoma samples (n = 15), eight samples were negative for the staining and five samples showed weak or moderate staining. In conclusion, we first found that the expression of CYP2A13 was markedly increased in non-small cell lung carcinomas. The high expression might be associated with the tumor development and progression in non-small cell lung carcinomas. (*Cancer Sci* 2010; 101: 1024–1028)

The human cytochrome P450 2A (CYP2A) subfamily comprises three members: CYP2A6, CYP2A7, and CYP2A13.[1] Among them, CYP2A6 and CYP2A13 are functional enzymes.[2,3] They are composed of 494 amino acids with a high degree of identity (93.5%). It has been reported that CYP2A6 is expressed in liver, whereas CYP2A13 is predominantly expressed in the respiratory tract, with the highest level in the nasal mucosa, followed by the lung and trachea.[3–5] The expression has been analyzed at the mRNA level. The tissue distribution of CYP2A13 at the protein level has not been determined because a specific antibody against CYP2A13 is not commercially available.

CYP2A13 is the most efficient enzyme in the metabolism of nicotine and cotinine as well as the metabolic activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) with similar substrate specificity as CYP2A6.[3,6] Recently, we found that CYP2A13 efficiently metabolizes various environmental chemicals in air pollutants or tobacco smoke such as 4-aminobiphenyl, naphthalene, styrene, and toluene.[7,8] Since these chemicals are easily absorbed by inhalation, CYP2A13 in the lung plays an important role in the local metabolism of the chemicals. It is considered that CYP2A13 would be relevant to carcinogenicity and toxicity in the lung.

The human P450 isoforms of which the expression in lung cancer has been most studied are CYP1A1 and CYP1B1, because cigarette smoking containing polycyclic aromatic hydrocarbons induces their expression.[9] Especially, CYP1B1 is highly expressed in lung non-small cell carcinomas compared with normal tissues.[10,11] CYP1A1 and CYP1B1 catalyze the metabolic activation of polycyclic aromatic hydrocarbons, which would be one of the causal factors of lung cancer. Since CYP2A13 is involved in the metabolic activation of environmental chemicals, it is important to know the expression level of CYP2A13 protein in lung cancer. Previously, Zhu *et al.* reported that CYP2A13 protein could not be detected in lung cancers by immunohistochemical analysis using a polyclonal antibody they made.[12] In the present study, we originally prepared an antibody against CYP2A13. Using the antibody, we evaluated the expression of CYP2A13 protein in various types of human lung cancers.

Materials and Methods

Chemicals and reagents. Normal mouse IgG and biotinylated goat anti-mouse IgG were obtained from Santa Cruz biotechnology (Santa Cruz, CA, USA) and Zymed (South San Francisco, CA, USA), respectively. Mayer's hematoxylin solution was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Recombinant human P450 enzymes. Recombinant human CYP1A1, CYP1B1, CYP2B6, CYP2D6, CYP2E1, and CYP3A4 expressed in baculovirus-infected insect cells (Supersomes) were purchased from BD Gentest (Woburn, MA, USA). *E. coli* membranes expressing human CYP2A6 and CYP2A13 were previously prepared.[13,14] The expression system of human CYP2S1 in *E. coli* was constructed according to the method by Wu *et al.*[15] and *E. coli* membranes expressing CYP2S1 were also prepared. The P450 content and protein concentration were determined according to the method described previously.[13]

Preparation of antibody against CYP2A13. Recombinant human CYP2A13 expressed in *E. coli* was purified according to the method described previously.[16] Mouse monoclonal antibody against human CYP2A13 was prepared by Kohjin Bio (Saitama, Japan). The hybridomas producing the antibodies were screened by ELISA with the purified recombinant CYP2A13 and CYP2A6. The clones reacted with CYP2A13, but not with CYP2A6 were selected. Among them, a clone showing highest reactivity was expanded by intraperitoneal injection into mineral oil-primed mice. Monoclonal antibodies from mouse ascitic fluids were partially purified by precipitation with 33% ammonium sulfate. Finally, the specificity of the antibody was confirmed by immunoblot analysis as described below.

Immunoblot analysis. SDS-polyacrylamide gel electrophoresis and immunoblot analysis were performed according to Laemmli.[17] The recombinant CYP1A1, CYP1B1, CYP2A6, CYP2A13, CYP2B6, CYP2D6, CYP2E1, CYP2S1, and CYP3A4 (each 1 pmol) were separated on 10% polyacrylamide gel and transferred electrophoretically to a polyvinylidene difluoride membrane, Immobilon-P (Millipore, Billerica, MA, USA).

[4]To whom correspondence should be addressed.
E-mail: nmiki@kenroku.kanazawa-u.ac.jp

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The membrane was blocked in 3% non-fat dry milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 for 12 h at room temperature. The membranes were incubated with the prepared mouse monoclonal anti-CYP2A13 antibody (1:2000, PBS) for 1 h at room temperature. Biotinylated anti-mouse IgG and the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, USA) were used for diaminobenzidine staining.

Tissue samples. Fifteen specimens each of adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and small cell carcinoma obtained from surgically removed lung tissues were used. These specimens were collected between 1997 and 2005 at the Pathology Departments of Kanazawa University Hospital, Japan. The age and sex of the patients were as follows: adenocarcinomas, 44–75 years, 10 men and five women; squamous cell carcinomas, 36–83 years, 12 men and three women; large cell carcinomas, 36–80 years, 14 men and one woman; small cell carcinomas, 56–83 years, 14 men and one woman. These specimens were fixed in neutral formalin and then embedded in paraffin for immunohistochemistry. This study was approved by the Ethics Committee of Kanazawa University (Kanazawa, Japan).

Immunohistochemistry. The sections described above were first deparaffinized with xylene three times for 5 min each and hydrated gradually through a series of graded ethanol (100%, 99.5%, 90%, 70%). After the washes with distilled water for 5 min, the sections were treated with a liberated antibody binding solution (Polysciences, Warrington, PA, USA) for 8 min to liberate the antigen-epitope site. Endogenous peroxidase activity in the sections was blocked with 3% hydrogen peroxide in PBS for 30 min. Nonspecific binding was blocked with 1.5% normal rabbit serum for 30 min at room temperature. The sections were then incubated with the mouse monoclonal anti-CYP2A13 antibody at 4°C for 16 h. The sections were then rinsed in PBS for 5 min and incubated with biotinylated goat anti-mouse IgG as the second antibody for 30 min at room temperature. After rinsing with PBS, staining reactions were performed using the ABC-elite kit. After counterstaining with Mayer's hematoxylin solution, the sections were mounted. As a negative control, normal mouse IgG was used instead of the anti-CYP2A13 antibody. The staining intensity of tumor cells was estimated as follows: 0, no staining; 1, weakly staining; 2, moderately staining; 3, strongly staining. The estimated percentages of positive tumor cells were classified as follows: 0, none; 1, <10%; 2, 10–50%; 3, >50%. The products of the scores 0–1, 2–4, 6–9 were defined as −, +, ++, and +++, respectively.

Statistical analyses. The statistical significance of differences in the extent of staining between different types of lung carcinoma was tested by Fisher's exact method. A value of $P < 0.05$ was considered statistically significant.

Results

Specificity of the anti-human CYP2A13 antibody. The specificity of the raised monoclonal antibody against human CYP2A13 was evaluated with immunoblot analysis. A panel of recombinant CYP isoforms, CYP1A1, CYP1B1, CYP2B6, CYP2D6, CYP2E1, and CYP3A4 expressed in baculovirus-infected insect cells as well as CYP2A6, CYP2A13, and CYP2S1 expressed in *E. coli* membrane, were separated on SDS-PAGE (Fig. 1). The antibody prepared in this study specifically reacted with CYP2A13. It did not react with the other P450 isoforms including CYP2A6.

Immunohistochemical analysis of CYP2A13 for human lung cancer. The antibody was used for the immunohistochemical analysis of a total of 60 lung cancer tissues. In adjacent noncancerous tissues, strong staining was observed in the epithelial cells of the bronchus (Fig. 2a), but no staining was observed in peripheral lung tissues (Fig. 2b). We confirmed the absence of staining in human liver (data not shown), supporting the specificity of this antibody. Next, we evaluated the staining of different types of lung carcinoma (Fig. 2c–f). All of 15 adenocarcinomas showed positive immunostaining, and five and three samples were judged as +++ and ++, respectively (Table 1). Each 14 samples of 15 squamous cell carcinomas and 15 large cell carcinomas were positive for the staining, and eight squamous cell carcinomas and six large cell carcinomas were judged as +++ or ++. In contrast, in 15 small cell carcinomas, eight samples were negative for the staining and five samples were judged as + or ++. These results suggest that CYP2A13 is highly expressed in non-small cell carcinomas.

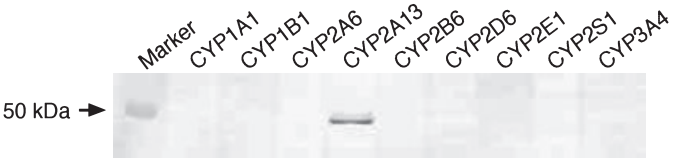

Fig. 1. Immunoblot analysis using the mouse monoclonal antibody against human CYP2A13 prepared in this study. One pmol each of recombinant P450 isoforms, CYP1A1, CYP1B1, CYP2B6, CYP2D6, CYP2E1, and CYP3A4 expressed in baculovirus-infected insect cells and CYP2A6, CYP2A13, and CYP2S1 expressed in *E. coli*, were separated on 10% SDS-PAGE.

Relationship between the CYP2A13 levels and smoking status or clinical characteristics of lung cancer. The relationship between the CYP2A13 staining levels and smoking status was evaluated (Table 2). In 15 adenocarcinomas and 15 squamous cell carcinomas, 10 and 12 samples were smokers, respectively. All of 15 large cell carcinomas and small cell carcinomas were smokers. No relationship was observed between the CYP2A13 immunostaining levels and the pack-years. We also investigated the relationship between the CYP2A13 staining levels and the extents of the primary tumor (T-factor), regional lymph node metastasis (N-factor), and distant metastasis (M-factor), and the clinical stage was also evaluated (Table 2). In adenocarcinomas, squamous cell carcinomas, and large cell carcinomas, no relationship was observed. In small cell carcinomas, significant differences were observed in the extent of CYP2A13 staining with the values of N-factor and the clinical stage. However, we could not draw definitive conclusions because of the limited number of samples.

Discussion

In previous studies, the tissue distribution of CYP2A13 was analyzed at the mRNA level, demonstrating the expression in the respiratory tract including the nasal mucosa, trachea, and lung.[3–5] Recently, Wong *et al.* successfully detected CYP2A13 protein in fetal nasal microsomes by immunoblotting using a polyclonal anti-mouse Cyp2a5 antibody.[18] Although this antibody reacted with CYP2A6, they could separate CYP2A13 from CYP2A6 by high resolution SDS-PAGE using a DNA sequencing apparatus. Zhang *et al.* detected CYP2A13 protein in lung microsomes by immunoblotting using the anti-Cyp2a5 antibody, in which immunoprecipitants from lung microsomes using the anti-Cyp2a5 antibody were separated.[19] Thus, since a specific antibody to CYP2A13 has not been available, great effort has been required to detect CYP2A13 protein. This background prompted us to prepare a specific antibody against human CYP2A13.

The amino acid identities of CYP2A13 with CYP2A6, CYP2B6, and CYP2S1 are 93.5%, 53.4%, and 47.5%, respectively. It has been demonstrated that a monoclonal antibody against CYP2A6 from BD Gentest reacts with not only CYP2A6 but also CYP2A13 and CYP2E1.[12] It seems that anti-rat CYP2A antibody reacted with CYP2S1 protein in human hepatic stellate cells.[20] We investigated whether our antibody

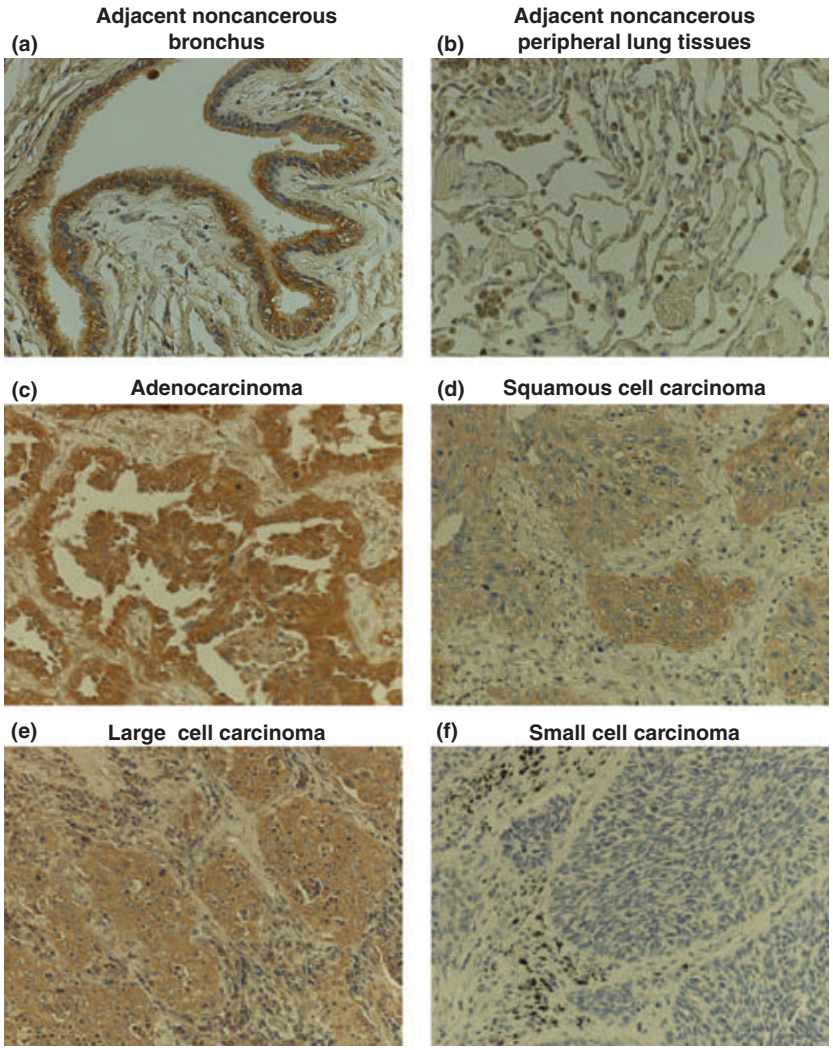

Fig. 2. Immunohistochemical analysis of CYP2A13 in human lung cancer tissues. Adjacent noncancerous (a) bronchus and (b) peripheral lung tissues; (c) adenocarcinoma; (d) squamous cell carcinoma; (e) large cell carcinoma; (f) small cell carcinoma. Strong immunostaining was observed in the epithelial cells in bronchus, but not in peripheral lung tissues. In lung carcinomas, immunostaining was positive in most of non-small cell carcinomas. In contrast, the staining was mostly negative in small cell carcinoma. Original magnification, ×200.

Table 1. CYP2A13-specific staining in lung adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and small cell carcinoma

Lung carcinoma type	Immunoreactive score			
	–	+	++	+++
Adenocarcinoma (n = 15)	0	7	3	5
Squamous cell carcinoma (n = 15)	1	6	6	2
Large cell carcinoma (n = 15)	1	8	3	3
Small cell carcinoma (n = 15)	8	4	1	2

The statistical significance of differences in the extent of staining in the different lung carcinoma types was tested by Fisher's exact method ($P < 0.05$).

against CYP2A13 may react with other P450 isoforms including CYP2A6, CYP2B6, and CYP2S1. In the results, it was demonstrated that the antibody specifically reacted with CYP2A13 (Fig. 1). Thus, we succeeded in preparing an antibody specific to human CYP2A13.

Using the specific antibody against CYP2A13, we performed immunoblot analysis using human normal lung microsomes, but we observed no band (data not shown). Then, we performed immunohistochemical analysis using lung cancer tissues. In adjacent noncancerous tissues, strong staining was observed in the epithelial cells of the bronchus, but no staining was observed in peripheral lung tissues (Fig. 2a,b). Thus, the expression of CYP2A13 is not uniform in the lung, supporting the negative results of the immunoblot analysis using microsomes prepared from whole lung tissues. The distribution of CYP2A13 in the epithelial cells of the bronchus would be physiologically reasonable, because CYP2A13 plays a role in the metabolism of environmental chemicals. When four kinds of lung carcinomas were evaluated for the CYP2A13 expression, we found differences in the extent of the immunostaining. The strong staining was observed in most samples of non-small cell carcinomas. In general, it is well known that squamous cell carcinoma and small cell carcinoma are associated with tobacco smoking.[21,22] Adenocarcinoma is the most common type of lung cancer in female nonsmokers, and it is increasingly associated with smoking as well. In contrast, no association has been reported for large cell carcinoma with smoking.[23] Recently, we found that CYP2A13 metabolically activates 4-aminobiphenyl, naphthalene, and styrene which are carcinogenic components in tobacco smoke.[7,8] CYP2A13 metabolically activates NNK, a causal factor of lung adenocarcinoma.[24] In support, there are studies showing a relationship between the genetic polymorphism of the *CYP2A13* gene and the risk of lung adenocarcinoma.[25,26] Thus, higher expression of CYP2A13 in adenocarcinoma and squamous cell carcinoma may be associated with the tumor development and progression. However, we have no conclusive explanation for low expression of CYP2A13 in small cell carcinoma. The regulation mechanism of CYP2A13 may be different in different

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Table 2. Characteristics of patients with lung adenocarcinoma, squamous cell carcinoma, large cell carcinoma, or small cell carcinoma in relation to expression level of CYP2A13

	Adenocarcinoma					Squamous cell carcinoma					Large cell carcinoma					Small cell carcinoma				
	−	+	++	+++	*P*	−	+	++	+++	*P*	−	+	++	+++	*P*	−	+	++	+++	*P*
Smoking status																				
Never smokers	0	2	1	2	1.000	1	1	1	0	0.446	0	0	0	0	1.000	0	0	0	0	1.000
Smokers	0	5	2	3		0	5	5	2		1	8	3	3		8	4	1	2	
Pack-years																				
<35		4	2	3	1.000		1	2	0	1.000	0	2	0	2	0.351	3	0	0	0	0.434
35–64		0	0	0			2	1	1		1	5	1	1		3	3	0	1	
65–94		1	0	0			1	0	0		0	1	2	0		1	1	1	0	
95<		0	0	0			1	2	1		0	0	0	0		1	0	0	1	
T-factor																				
1	0	2	1	3	0.670	1	2	1	1	0.914	0	1	0	1	0.990	4	4	0	0	0.119
2	0	4	1	1		0	1	2	1		0	3	1	0		3	0	1	2	
3	0	0	1	1		0	2	3	0		1	2	1	1		0	0	0	0	
4	0	1	0	0		0	1	0	0		0	2	1	1		1	0	0	0	
N-factor																				
0	0	3	2	5	0.400	0	4	5	1	0.188	1	4	1	1	1.000	6	0	0	0	**0.004**
1	0	2	1	0		1	0	0	1		0	1	0	1		0	1	0	2	
2	0	2	0	0		0	2	1	0		0	3	2	1		2	3	1	0	
3	0	0	0	0		0	0	0	0		0	0	0	0		0	0	0	0	
M-factor																				
0	0	6	2	5	0.667	1	5	6	2	1.000	1	8	3	3	1.000	8	4	1	2	1.000
1	0	1	1	0		0	1	0	0		0	0	0	0		0	0	0	0	
Clinical Stage																				
IA	0	1	1	3	0.908	0	1	1	1	0.752	0	1	0	1	1.000	3	0	0	0	0.052
IB	0	2	0	1		0	0	1	0		0	1	0	0		2	0	0	0	
IIA	0	1	0	0		1	0	0	0		0	0	0	0		0	1	0	0	
IIB	0	1	1	1		0	1	3	1		1	3	1	1		0	0	0	2	
IIIA	0	0	0	0		0	2	1	0		0	1	1	0		2	3	1	0	
IIIB	0	1	0	0		0	1	0	0		0	2	1	0		1	0	0	0	
IV	0	1	1	0		0	1	0	0		0	0	0	0		0	0	0	0	
I	0	3	1	4	0.907	0	1	2	1	0.717	0	2	0	1	0.930	5	0	0	0	**0.012**
II	0	2	1	1		1	1	3	1		1	3	1	1		0	1	0	2	
III	0	1	0	0		0	3	1	0		0	3	2	0		3	3	1	0	
IV	0	1	1	0		0	1	0	0		0	0	0	0		0	0	0	0	

cancer types. Furthermore, we cannot speculate the reason at the present why the cancer cells still need the function protein of CYP2A13 after carcinogenic events. Clarifying this issue would contribute to our understanding for physiological and biological significance of CYP2A13.

While performing this study, another research group also generated a specific antibody against CYP2A13.[12] In contrast to our study, they used a synthetic peptide covering C-terminal amino acid residues 369 to 377 as an antigen. They confirmed that the antibody reacted with CYP2A13 but not with CYP2A6, CYP2S1, CYP3A4, and mouse CYP2A5 by immunoblot analysis using recombinant enzymes. Using this antibody, they have reported that immunostaining was observed in normal trachea but not in normal peripheral lung tissues, supporting our study. However, in contrast to our results, they reported that the expression of CYP2A13 protein was not detected in any adenocarcinomas ($n = 6$), squamous carcinomas ($n = 3$), large cell carcinomas ($n = 3$), alveolar carcinomas ($n = 3$), basaloid carcinoma ($n = 1$), or papillary bronchiolar carcinoma ($n = 1$). The reason for this discrepancy is not clear, but it may partly depend on differences in the numbers of samples or individual differences. Another possibility is that differences in the reactivity of the antibodies used may lead to conflicting results.

In summary, we prepared a specific monoclonal antibody against CYP2A13. The immunohistochemical analysis using this antibody demonstrated that, in lung tissues, the expression of CYP2A13 is localized in epithelial cells in the bronchus. We first found that the expression of CYP2A13 was markedly increased in lung non-small cell lung carcinomas. The high expression might be associated with the tumor development and progression in non-small cell lung carcinomas.

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Disclosure Statement

None of the authors have any conflicts of interest to declare.

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