Differential Distribution of CYP2A6 and CYP2A13 in the Human Respiratory Tract

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Key Words

CYP2A6 · CYP2A13 · Metabolic activation · Respiratory tract · Immunohistochemistry

Abstract

Background: Human CYP2A6 and CYP2A13 play important roles in metabolic activation of many pulmonary carcinogens and thus their expression and distribution may determine the pulmonary susceptibility to metabolically activated carcinogens and the following lung cancer development. Because of the 93.5% of amino acid identity between CYP2A6 and CYP2A13, generation of antibodies specific to CYP2A6 or CYP2A13 has limited immunohistochemical (IHC) analysis of CYP2A6 and CYP2A13 levels in the respiratory tract. **Objectives:** This study aimed to determine the differential distribution of CYP2A6 and CYP2A13 in human respiratory tissue with IHC analysis. **Methods:** With computer-aided protein sequence analyses, candidate epitopes of 15 amino acids in the C-terminal domains of CYP2A6 and CYP2A13 were selected for antibody generation. Specificity of these two antibodies was confirmed with immunoblot and immunofluorescence analyses. With these two selective antibodies, the differential distribution of CYP2A6 and CYP2A13 in human respiratory tissues, including tracheae, bronchi, bronchioles and alveoli, was determined. **Results:** IHC re-

sults showed that both CYP2A6 and CYP2A13 were markedly expressed in epithelial cells of tracheae and bronchi and that only CYP2A6 was detected in bronchiolar epithelial cells of peripheral lungs. A limitation of the present study is the cross-reactivity of our CYP2A6 antibody to the functional inactive CYP2A7. **Conclusions:** The differential distribution patterns of CYP2A6 and CYP2A13 in the respiratory tract are of importance in considering the pulmonary susceptibility to carcinogens and the following lung cancer development.

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Introduction

Cytochrome P450 enzymes play important roles in the pathogenesis of various pulmonary diseases, including chronic obstructive pulmonary disease and lung cancer [1, 2]. Epidemiological studies have shown the linkage between the loss of CYP2As enzyme activity and the reduced risk of lung cancer occurrence, particularly for cigarette smoking-induced lung cancer [3–6]. Human CYP2As play the major role in metabolic activation of several pulmonary carcinogens including tobacco-spe-

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cific nitrosamines (NNK and NNN) [7–9], aflatoxin B1 [10, 11] and 1,3-butadiene [12]. Expression of human CYP2As in bacterial or mammalian cells potentiates the mutagenicity induced by tobacco-specific nitrosamines [13–15] or aflatoxin B1 [10]. These studies suggested the critical role of CYP2As in xenobiotic-induced pulmonary toxicity and carcinogenicity.

The human CYP2A subfamily includes two functional enzymes – CYP2A6 and CYP2A13 [16]. Because cigarette smoke is inhaled through the respiratory tract, it is extremely important to determine the differential expression patterns of these two CYP2A enzymes in the tract. Analysis of mRNA levels showed that both CYP2A6 and CYP2A13 were detected in the lungs [17] as well as in the respiratory tract, i.e. nasal mucosa and trachea [7]. In the respiratory tract, the levels of CYP2A6 mRNA detected were very low [7, 17, 18] and relative levels of CYP2A13 mRNA are nasal mucosa > trachea > lungs [7]. Because of the heterogeneity in cell types of the respiratory tract, detailed IHC analyses were necessary to precisely evaluate any cell/tissue-specific distribution of these two enzymes. However, the 93.5% of amino acid identity between CYP2A6 and CYP2A13 would be the major limitation to generate a CYP2A6 or CYP2A13 specific antibody. IHC results revealed that CYP2A13 was markedly detected in epithelial cells of tracheae and bronchi [19, 20] and was barely expressed in peripheral lung tissue – bronchioles and alveoli [19]. Although two CYP2A13-specific antibodies were generated [19, 20], a CYP2A6 specific antibody was lacking.

Susceptibility to pulmonary carcinogens and the following lung cancer development may mainly depend on exposure routes of the carcinogens and tissue distribution patterns of those CYPs involved in metabolic activation of the carcinogens, such as CYP2A6 and CYP2A13. The present study aimed to determine the differential tissue distribution of CYP2A6 and CYP2A13 in human respiratory tissues by generation of specific antibodies for CYP2A6 and CYP2A13. Our IHC results revealed that both CYP2A6 and CYP2A13 were markedly expressed in epithelial cells of tracheae and bronchi and that only CYP2A6 was detected in bronchiolar epithelial cells of peripheral lungs.

Materials and Methods

Cells, Antibodies, Plasmids and Reagents

Human embryonic kidney 293 cells (HEK-293) and human lung adenocarcinoma A549 cells were routinely maintained

in DMEM (HyClone, Logan, UT) and RPMI-1640 medium (HyClone), respectively, at 37°C with a humidified mixture of 5% CO₂ and 95% air. Both media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin.

Mouse monoclonal anti-CYP2A6 antibody (BD Gentest, Woburn, Mass., USA) was shown to cross-react with human CYP2A13 [19] and thus was named as mouse anti-CYP2As antibody in this study. Mouse monoclonal anti-calnexin antibody and a normal rabbit IgG were from GeneTex (Irvine, Calif., USA). Mouse monoclonal anti-hemagglutinin (HA) antibody, HRP-labeled goat anti-mouse and anti-rabbit IgG antibodies, FITC-labeled goat anti-mouse IgG antibody and TRITC-labeled goat anti-rabbit IgG antibody were from Santa Cruz Biotechnology (Santa Cruz, Calif., USA).

pcDNA5 plasmid was from Invitrogen (Carlsbad, Calif., USA). CYP2A6 and CYP2A13 cDNA clones were from OriGene (Rockville, Md., USA). CYP1A1, CYP2A7, CYP2D6 and CYP3A4 cDNA clones were gifts from ICON Biotechnology (Hsinchu, Taiwan). Western Lightning Chemiluminescence Reagent Plus was from PerkinElmer Life Sciences (Boston, Mass., USA). Lipofectamine 2000, Platinum *pfx* DNA polymerase, biotin blocking reagent, Histostain-Plus, mounting solution containing 4',6-diamidino-2-phenylindole (DAPI) and hematoxylin were all from Invitrogen.

Computer-Aided Protein Sequence Analysis

CYP2A6 (NP_000753) and CYP2A13 (NP_000757) protein sequences were collected from NCBI GeneBank. Sequence similarity (by setting the window size of 5 amino acids) and hydrophobicity were analyzed with the program AlignX and the Kyte and Doolittle method [21], respectively, with the Vector NTI Suite version 10 (Invitrogen). Antigenicity was analyzed with the semi-empirical method [22] from the IEDB Analysis Resource (http://tools.immuneepitope.org/tools/bcell/iedb_input). Hydrophobicity and antigenicity scores were calculated by setting the window size of 7 amino acids. The scores of protein similarity, hydrophobicity and antigenicity were plotted against the amino acid position with the SigmaPlot Windows version 9 (Systat Software Inc., San Jose, Calif., USA).

Peptide Synthesis and Antibody Generation

On the basis of the computer-aided protein sequence analysis, two peptides – ‘FGDVIPMSLARRVKK’ and ‘FGDMLPMGLAHRVKNK’ – corresponding to amino acid position 362–376 of CYP2A6 and CYP2A13, respectively, were synthesized as immunogens for generating rabbit polyclonal antibodies specific for CYP2A6 or CYP2A13. Peptide synthesis, rabbit immunization and polyclonal antibody purification were performed by ICON Biotechnology (Hsinchu, Taiwan).

Constructs of CYP-Expressing Plasmids

The entire open reading frames of CYP1A1, CYP2A6, CYP2A7, CYP2A13, CYP2D6 and CYP3A4 without the stop codon were amplified by PCR from the corresponding cDNA clones with the Platinum *pfx* DNA polymerase. The RE sites designed in the primer pairs were used to sub-clone the PCR products into the expression vector pcDNA5, by which the expressed protein molecules contain a 2×HA tag in their C-terminals. All the CYP-expressing constructs were *in silico* checked by Vector NTI and were confirmed by direct sequencing.

Immunoblot Analysis

HEK-293 cells were seeded on 10-cm dishes at 80% confluence and then were transfected with one of the CYP-expressing plasmids with Lipofectamine 2000 as described [23]. Microsome purification was performed as previously described [8]. Briefly, the transfected HEK-293 cells were washed twice with PBS and then were resuspended in PBS containing protease/phosphatase inhibitors (2 mM PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 2 µg/ml pepstatin, 2 mM NaF, and 2 mM Na₃VO₄). Following sonication, the cell lysates were centrifuged at 15,490 *g* for 10 min at 4°C to remove cell debris. Following ultra-centrifugation of supernatants at 196,000 *g* for 1 h at 4°C, the sedimentary microsome fractions were collected and redissolved in RIPA buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM EGTA, 1% Triton X-100 and 0.25% sodium deoxycholate) with the protease/phosphatase inhibitors. The microsome samples were subjected to SDS-PAGE and immunoblot analysis as described [24]. The blots were probed with a primary antibody against HA, CYP2A6, CYP2A13 or CYP2As. HRP-conjugated secondary antibodies were used to reveal the specific protein bands with Western Lightning Chemiluminescence Reagent Plus.

Immunofluorescence Analysis

Construction, preparation and infection of the adenoviruses were described previously [15]. A549 cells were seeded at 50% confluence on cover-slides in 12-well plates and then were infected with one of the adenoviruses (Ad-Mock, Ad-CYP2A6 and Ad-CYP2A13). Following infection for 8 h, the infected cells were washed twice with PBS and then fixed with 1% paraformaldehyde at RT for 30 min. The cells were washed twice with 100 mM glycine/PBS, permeabilized in 1% Triton X-100/PBS at RT for 10 min, blocked with PBS with 2% horse serum (hs-PBS) at RT for 30 min, and then incubated with a primary antibody – mouse monoclonal anti-calnexin antibody (1:100, hs-PBS), rabbit polyclonal anti-CYP2A13 antibody (1:100, hs-PBS) or rabbit polyclonal anti-CYP2A6 antibody (1:20, hs-PBS) – at RT overnight. Following incubation with a corresponding fluorescence-labeled secondary antibody (1:100, PBS) at RT for 1 h in the dark, the cells were mounted with a mounting solution and DAPI.

IHC Analysis

Human tissue sections of liver and respiratory tissues, including trachea, bronchus, bronchiole and alveolus, from eleven different donors (table 1) were purchased from US Biomax (Rockville, Md., USA). All human tissues were collected under IRB and HIPPA-approved protocols. The Human Ethical Committee of the National Health Research Institutes of Taiwan approved the study. The formalin-fixed and paraffin-embedded human tissue sections were deparaffinized with xylene, rehydrated through a series of graded ethanol (100, 90 and 75%), and washed with PBS. The sections were autoclaved for 20 min in citrate buffer (pH 6.0) to retrieve antigens. Endogenous biotin in the liver sections was blocked with a biotin blocking reagent (Invitrogen). IHC staining was performed with the Histostain-Plus (Invitrogen). Briefly, endogenous peroxidase activity in the sections was quenched with 3% hydrogen peroxide for 10 min at RT. Nonspecific binding was blocked with 10% nonimmune goat serum for 10 min at RT. The sections were then incubated with a primary antibody – rabbit polyclonal anti-CYP2A6 antibody (1:100, blocking solution) or rabbit polyclonal anti-CYP2A13 antibody (1:500, blocking solu-

Table 1. Summary of the IHC analysis of CYP2A6 and CYP2A13 levels in tissue sections of the human respiratory tract and liver from 11 different donors

Tissue	Donors (age, gender)	CYP2A6	CYP2A13
Trachea	I (24, male)	++	+++
	II (19, male)	+++	+++
	III (19, female)	+++	+++
Bronchus	IV (48, male)	+++	+++
	V (37, male)	+++	+++
	VI (16, female)	+++	+++
Peripheral lung			
	Bronchiole	+++	–
	Alveolus	++	–
	Bronchiole	+	–
	Alveolus	+	–
	Bronchiole	++	–
Liver	IX (48, female)	++	–
	X (40, female)	+++	–
	XI (40, male)	+++	–

IHC results of CYP2A6 and CYP2A13 expression in the human respiratory tract and liver are summarized. Intensity of IHC staining was defined as negative (–), weak (+), moderate (++) and strong (+++). The detailed IHC results are shown in the online supplementary figure.

tion) – at RT overnight. Negative controls were obtained by replacing the primary antibody with a normal rabbit IgG (GeneTex). The sections were incubated with biotinylated secondary antibody and then with streptavidin-peroxidase conjugate. Staining reaction was performed by adding the 3,3'-diaminobenzidine (DAB) chromogen. Following hematoxylin counterstaining, the sections were mounted.

Results

Design of Epitopes for Generating Antibodies Specific for CYP2A6 or CYP2A13

Because protein sequences between CYP2A6 and CYP2A13 share 93.5% identity, the design of epitopes for generating antibodies specific for CYP2A6 or CYP2A13 was a great challenge. In this study, we used the computer-aided prediction methods to select candidate epitopes for antibody generation. On the basis of similarity (fig. 1a), hydropathicity and antigenicity analyses, the region ranging from amino acid position 362–376 in CYP2A6 and CYP2A13 was identified with the most variable/antigenic property (fig. 1b). Hydropathicity analysis re-

Fig. 1. Epitope design for generating antibodies specific for CYP2A6 or CYP2A13. Following the computer-aided protein sequence analysis, CYP2A6 and CYP2A13 protein sequences were used to calculate similarity, hydropathicity and antigenicity. **a** The resulted similarity scores were plotted against the amino acid (aa) position. **b** The most variable/antigenic region was predicted between amino acid 362 and amino acid 376. The peptides were synthesized and used as immunogens for antibody generation. The different amino acids between CYP2A6 and CYP2A13 are indicated in boxes.

vealed that average hydropathicity scores of these 15 amino acids are -0.190 for CYP2A6 and -0.257 for CYP2A13. Antigenicity analysis demonstrated that average antigenicity scores of these 15 amino acids are 1.03 for CYP2A6 and 1.00 for CYP2A13. The two corresponding amino acid sequences in CYP2A6 and CYP2A13 are 'FGDVIPMSLARRVKK' and 'FGDMLPMGLAHRVNK', respectively (fig. 1b), in which 5 out of 15 amino acids are different between CYP2A6 and CYP2A13, and thus share 66.7% identity in amino acids. On the basis of these results from three different approach strategies, the 15 amino acid regions in CYP2A6 and CYP2A13 were synthesized and used as immunogens for generating antibodies specific for CYP2A6 or CYP2A13, as described in Materials and Methods.

Determination of Antibody Specificity

The human CYP2A subfamily contains three members, CYP2A6, CYP2A7 and CYP2A13. CYP1A1, CYP2D6 and CYP3A4 are detected in human respiratory tissues [25] and exhibit similar substrate specificity as CYP2As [26]. To determine the specificity of our CYP2A6 and CYP2A13 antibodies, all six HA-tagged CYP expression plasmids were constructed as described in Materials and Methods in detail. Following transfection of each plasmid into HEK-293 cells, immunoblot analysis with microsomal fraction samples was performed to evaluate the specificity of these two CYP2A antibodies.

As shown in figure 2a, by using an anti-HA antibody, all six CYPs were detected with a similar expression level, indicating that all six HA-tagged CYP expression plasmids functioned well in HEK-293 cells. Our CYP2A6 and CYP2A13 antibodies neither cross-react with each other nor react with CYP1A1, CYP2D6 and CYP3A4. It was also noted that the CYP2A6 antibody did cross-react with CYP2A7. In addition, a commercially available mouse monoclonal anti-CYP2As antibody reacted with all three members of the human CYP2A subfamily – CYP2A6, CYP2A7 and CYP2A13.

Specificity of the two CYP2A antibodies was further evaluated with immunofluorescent staining. A549 cells were infected with one of the adenoviruses (Ad-Mock, Ad-CYP2A6 and Ad-CYP2A13), incubated for another 24 h for protein expression, and then fixed for immunofluorescent staining. As shown in figure 2b, our CYP2A6 and CYP2A13 antibodies specifically recognize CYP2A6 and CYP2A13, respectively, without cross-reacting with each other. Colocalization of CYP2A6/CYP2A13 with calnexin, an endoplasmic reticulum marker, indicated the functional expression of the adenovirus-mediated CYP proteins in endoplasmic reticulum compartments. In figure 2b we also noted the inconsistent immunofluorescent stain of the CYP2As between individual cells, which could be due to the loss of antigenicity by paraformaldehyde over-fixation and/or the uneven infection of adenovirus in cells of different cell-cycle stages.

CYP2A6 and CYP2A13 Expression in the Human Respiratory Tract

Tissue sections of the human respiratory tract – trachea ($n = 3$), bronchus ($n = 3$) and peripheral lungs ($n = 3$), including bronchiole and alveolus – and liver ($n = 2$) were from eleven different donors. IHC analysis of CYP2A6 and CYP2A13 levels in these tissue sections is summarized in table 1 and a set of representative IHC staining is shown in figure 3. Because of the abundant expression of CYP2A6

Fig. 2. Determination of antibody specificity. **a** Antibody specificity was determined with immunoblot analysis. HEK-293 cells were left untreated (–) or transiently transfected with pcDNA5 (m), CYP1A1 (1A1), CYP2A6 (2A6), CYP2A7 (2A7), CYP2A13 (2A13), CYP2D6 (2D6) or CYP3A4 (3A4) expressing plasmid. Immunoblots of these microsomal extracts were probed with antibodies: mouse anti-HA antibody (α HA), rabbit anti-CYP2A6 antibody (α CYP2A6), rabbit anti-CYP2A13 antibody (α CYP2A13) and mouse anti-CYP2As antibody (α CYP2As). **b** Antibody specificity was also determined with immunofluorescence analysis. A549 cells were left untreated (–) or infected with one of the ad-

enoviruses, i.e. Ad-Mock (Mock), Ad-CYP2A6 (CYP2A6) and Ad-CYP2A13 (CYP2A13). Expression of CYP2A6 and CYP2A13 was probed with rabbit anti-CYP2A6 antibody (α CYP2A6) and rabbit anti-CYP2A13 antibody (α CYP2A13), respectively; CYP2A6 and CYP2A13 were labeled with TRITC (red; colors refer to the online version only). The endoplasmic reticulum marker calnexin was probed with mouse anti-calnexin antibody and then labeled with FITC (green). Cell nuclei were labeled with DAPI (blue). A representative immunofluorescence analysis from three independent experiments is shown.

in liver [18, 27, 28], the liver tissue section was used as a positive control for CYP2A6. Results in table 1 and figure 3 showed that CYP2A6 was mainly detected in the epithelial cells of trachea (fig. 3a), bronchus (fig. 3b) and bronchiole (fig. 3c), as well as in some pneumocytes of alveolar epithelium (fig. 3d). In addition, an inter-individual variation in bronchiolar CYP2A6 expression was observed (table 1 and online suppl. figure 1; for all online suppl. material, see www.karger.com/doi/10.1159/000339591) and

similar results have previously been reported [29, 30]. Meanwhile, ubiquitous CYP2A6 expression was detected in human liver sections (fig. 3e). We found that CYP2A13 was also markedly expressed in the epithelial cells of trachea (fig. 3f) and bronchus (fig. 3g), and was barely detected in bronchiole (fig. 3h), alveolus (fig. 3i) and liver (fig. 3j). By replacing the primary antibodies with a normal rabbit IgG, the negative IHC staining showed no marked immunostaining signal in all five tissue sections (fig. 3k–o) in

Fig. 3. IHC analysis of CYP2A6 and CYP2A13 levels in the human respiratory tract and liver. Human tissue sections included trachea (**a**, **f** and **k**), bronchus (**b**, **g** and **l**), bronchiole of peripheral lungs (**c**, **h** and **m**), alveolus of peripheral lungs (**d**, **i** and **n**) and liver (**e**, **j** and **o**). The sections were probed with rabbit anti-CYP2A6 antibody (**a–e**), rabbit anti-CYP2A13 antibody (**f–j**), or a normal rabbit IgG (**k–o**).

which some macrophages were sporadically detected with intense immunostaining, possibly due to their endogenous peroxidase activity. The detailed IHC results of these donors are also shown in the online supplementary figure.

Discussion

Because of the 93.5% homology in amino acids between CYP2A6 and CYP2A13, it was a great challenge to generate specific antibodies against CYP2A6 or CYP2A13 for the IHC analysis. Recently, two CYP2A13-specific antibodies were generated [19, 20]; however, a CYP2A6-specific antibody remained lacking. The computer-aided protein sequence analysis identified two short peptides of 15 amino acids (fig. 1b) for generating the specific antibodies against CYP2A6 or CYP2A13. The analysis involved several criteria, i.e. amino acid sequence similarity between CYP2A6 and CYP2A13, and hydrophobicity/antigenicity of the selected peptides. The antigenicity of the selected peptides was analyzed with the semi-empirical method [22], by which the potential antigenic efficacy is predicted on the basis of the physicochemical property of amino acids and their occurrence

frequency in the known epitopes. The antigen/immunogen specificity mainly depends on their peptide composition including amino acid structure [e.g. His (H) vs. Arg (R)], ionic charge [e.g. Asn (N) vs. Lys (K)], and those hydrophobic residues frequently found on the surface of protein molecules also exhibiting potent antigenicity, including Cys (C), Leu (L) and Val (V).

To the best of our knowledge, our CYP2A6 antibody is the first selective antibody that does not cross react with CYP2A13 (fig. 2a). Immunofluorescence staining of human lung cells (fig. 2b) indicated that our antibodies can be applied in IHC analysis of CYP2A6 and CYP2A13 protein distribution in human tissues. Both CYP2A6 and CYP2A13 are involved in the metabolic activation of tobacco-specific nitrosamines (TSNAs), e.g. NNK and NNN [9, 26]. Our previous study demonstrated that both CYP2A6 and CYP2A13 significantly enhanced the NNK-induced gene mutation in human lung cells [15]. Because the respiratory tract is the primary target tissue by cigarette smoking, it is suggested that the tissue-specific expression of CYP2A6 and/or CYP2A13 may significantly contribute to the tissue susceptibility to TSNAs.

CYP2A6 is mainly expressed in human liver [18, 28] and is also detected in the extrahepatic tissues at rela-

tively low levels [7, 17], i.e. breast >> lungs > ovary and adrenal gland [17]. In the human respiratory tract, CYP2A6 mRNA levels are mainly expressed in the upper respiratory tissues, i.e. nasal mucosa and trachea, and are barely detected in the peripheral lung tissues, i.e. bronchiole and alveolus [7]. However, marked CYP2A6 protein levels in the peripheral lung tissues have been reported with a high-resolution immunoblotting analysis of microsomal samples [30]. Moreover, the critical role of CYP2A6 in metabolic activation of NNK has been demonstrated in human peripheral lung microsomes [31] as well as in the type II pneumocytes and alveolar macrophages from human peripheral lung tissues [32]. The present IHC analysis using our selective CYP2A6 antibody clearly shows that CYP2A6 is markedly expressed in epithelial cells of trachea and bronchus and is also detected in bronchiolar epithelial cells of peripheral lungs. Moreover, the interindividual variation in bronchiolar CYP2A6 expression shown in table 1 is consistent in some previous studies in human peripheral lung tissue with qPCR [29] and immunoblot assay [30]. The interindividual variation in CYP2A6 may contribute to the large variation in NNK metabolism by the peripheral lung microsomes from individuals with different levels of CYP2A6 [33] and thus may significantly influence the susceptibility of individuals to TSNAs. Because of the heterogeneity of cell types in the respiratory tract, by using protein or mRNA samples from whole tissue it is extremely difficult to precisely define those protein molecules with specific expression in certain types of cells, such as epithelial or alveolar cells. On the basis of our and others' results, it is suggested that the IHC analysis is more sensitive in determining the CYP2A6 distribution in the human respiratory tract.

As shown in figure 2a, our two selective CYP2A antibodies neither cross-react with each other nor recognize the other CYPs (i.e. CYP1A1, CYP2D6 and CYP3A4); however, the CYP2A6 antibody did cross-react with CYP2A7. The CYP2A7 is a catalytic inactive enzyme [34], mainly expressed in human liver, and has been shown with no or rare expression in human lungs [17, 18]. Recently, expression of both CYP2A7 and CYP2A6 in the pulmonary parenchyma and bronchial mucosa of human respiratory tissue has also been noted [29]. Because pulmonary expression of CYP2A6 has been detected with PCR [7], immunoblot [30] and enzymatic activity assay [31, 32], our present IHC results support the expression of CYP2A6 and do not rule out the possible expression of CYP2A7 in the human respiratory tract. Because CYP2A7 is a catalytic inactive enzyme, determination of

enzyme activity could be a potential way to further differentiate CYP2A6 and CYP2A7.

Two peptides, i.e. 'F₃₆₂GDMLPMGLAHRVNK₃₇₆' (in this study) and 'G₃₆₉LAHRVNK₃₇₆' [19] from CYP2A13, and a full-length CYP2A13 protein [20] have been used as immunogens for generating CYP2A13 antibodies. Based on the IHC analyses with these three antibodies, it is generally agreed that CYP2A13 is markedly expressed in epithelial cells of trachea and bronchus and is not detected in the peripheral lungs. However, cigarette smoking-induced lung adenocarcinoma occurs mostly in peripheral lungs [35] and the role of CYP2A13 needs further elucidation. It should be also noted that, besides cigarette smoking (or TSNAs), several other pulmonary toxicants, such as aflatoxin B1 [10, 11], 1,3-butadiene [12], 4-aminobiphenyl [17], methyl *tert*-butyl ether [7, 36], naphthalene, styrene and toluene [37], are also activated through the CYP2A6- and/or CYP2A13-mediated metabolism.

In conclusion, two highly selective CYP2A6 and CYP2A13 antibodies have been generated. IHC analysis in human respiratory tissue with the antibodies shows that both CYP2A6 and CYP2A13 are expressed in epithelium of tracheae and bronchi, and only CYP2A6 is detected in bronchiolar epithelium of peripheral lungs. However, it is also noted that the limitation of the present study is the cross-reactivity of our CYP2A6 antibody to the functional inactive CYP2A7. Therefore, the present CYP2A6 staining in the human respiratory tract may be partially attributable to CYP2A7. Further studies in enzyme activity with the purified microsome samples from peripheral lung tissue would be necessary to determine the relative expression of these two proteins in the peripheral lung tissue.

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