

CYP2A13 in Human Respiratory Tissues and Lung Cancers: An Immunohistochemical Study with A New Peptide-specific Antibody

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Running title: Immunohistochemical Study of CYP2A13 in Human Tissues

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The number of text page is 21.

The number of table is 0.

The number of figure is 2.

The number of references is 33.

The number of words in the *Abstract* is 245.

The number of words in the *Introduction* is 377.

The number of words in the *Discussion* is 1209.

The abbreviations used are: CYP, cytochrome P450; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; AFB1, aflatoxin B1.

Abstract

Human Cytochrome P450 2A13 (CYP2A13) is highly efficient in the metabolic activation of a tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and another potent carcinogen aflatoxin B1 (AFB1). Although previous studies demonstrated that CYP2A13 mRNA is predominantly expressed in human respiratory tissues, expression of CYP2A13 protein in these tissues and the involved cell types have not been determined due to the lack of CYP2A13-specific antibodies. To explore the toxicological and physiological function of CYP2A13, it is important to understand the tissue/cellular distribution of CYP2A13 protein. In this study, we generated a peptide-specific antibody against human CYP2A13 and demonstrated by immunoblot analysis that this antibody does not cross-react with heterologously expressed human CYP2A6 and mouse CYP2A5 proteins, both sharing a high degree of amino acid sequence similarity with CYP2A13. Nor does the antibody cross-react with heterologously expressed human CYP3A4, CYP2S1 or any of the CYP enzymes present in the human liver microsomes. Using this highly specific antibody for immunohistochemical staining, we detected a high level of CYP2A13 protein expression in the epithelial cells of human bronchus and trachea, but a rare distribution in the alveolar cells. There was little expression of CYP2A13 protein in different types of lung cancers. In consideration of the high efficiency of CYP2A13 in NNK metabolic activation, our result is consistent with the reported observations that most smoking-related human lung cancers are bronchogenic and supports that CYP2A13-catalyzed *in situ* activation may play a critical role in human lung carcinogenesis related to NNK and AFB1 exposure.

Introduction

Encoded by a super-gene family, cytochrome P450 (CYP) enzymes play a critical role in the metabolism of many endogenous compounds and therapeutic drugs. They also catalyze the biotransformation of most environmental protoxicants and procarcinogens (Nebert and Russell, 2002; Pelkonen and Raunio, 1997). Human CYP2A gene subfamily has three members: CYP2A6, CYP2A7 and CYP2A13 (Su et al, 2000). While CYP2A6 is a major human enzyme for the metabolism of coumarin and nicotine, CYP2A7 has no catalytic activity (Ding et al, 1995). Our previous work demonstrated that CYP2A13 has the highest catalytic efficiency, among all the known human CYP enzymes, in the metabolic activation of a major tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Su et al, 2000; He et al, 2004). Recently, we demonstrated that CYP2A13 is also highly efficient in metabolizing aflatoxin B₁ (AFB₁) to its carcinogenic/toxic metabolites AFB₁-8,9-epoxide and AFM₁-8,9-epoxide (He et al, 2006). Both NNK and AFB₁ induced lung tumors in animals (Pepin et al, 1992; Donnelly et al, 1996; Tam et al, 1999) and are suspected lung carcinogens in humans (Van et al, 2001; Kamataki et al, 2005). Thus, CYP2A13-catalyzed metabolic activation may play a critical role in human lung carcinogenesis related to NNK and AFB₁ exposure.

Although previous studies demonstrated that CYP2A13 mRNA is predominantly expressed in human respiratory tract (Su et al, 2000), the expression of CYP2A13 protein in human tissues could not be determined due to the lack of CYP2A13-specific antibodies. It is well known that the mRNA expression level of a particular gene does not necessarily reflect the expression level

of the corresponding protein. To support the putative role of CYP2A13 in lung carcinogenesis, it is critical to demonstrate that the expression of CYP2A13 protein correlates with the mRNA expression in human respiratory tissues. Because the amino acid sequence of CYP2A13 shares a high degree of similarity with that of CYP2A6 (Fernandez-Salguero et al, 1995), it would not be feasible to use the entire CYP2A13 protein as an antigen for the preparation of CYP2A13-specific antibodies. We, therefore, generated a rabbit polyclonal antibody that specifically recognizes the C-terminal amino acid sequence of CYP2A13. After demonstration of its specificity by immunoblotting, we used this antibody for immunohistochemical analysis to investigate the protein expression and cellular localization of CYP2A13 in human respiratory tissues and lung tumors.

Materials and Methods

Chemicals and reagents

Normal goat serum, biotinylated goat anti-rabbit IgG, antigen unmasking solution, Vectastain ABC reagent and peroxidase substrate kit (DAB SK-4100) were purchased from Vector Laboratories Inc. (Burlingame, CA). Mayer's hematoxylin solution was purchased from Sigma-Aldrich (Saint Louis, WA). Anti-human CYP2A6 monoclonal antibody and human liver microsomes (pooled from 27 normal individual livers) were purchased from BD Gentest Corporation (Woburn, MA). Goat anti-rabbit IgG conjugated with horseradish peroxidase was obtained from BIO-RAD Laboratories (Hercules, CA). Goat anti-mouse IgG conjugated with horseradish peroxidase and the ECLTM western blotting reagents were from Amersham Biosciences Inc. (Piscataway, NJ). Human CYP3A4, CYP2S1, CYP2A6 and CYP2A13 as well as mouse CYP2A5 proteins were expressed by a baculovirus/Sf9 insect cell system as described

previously (He et al, 2004; Wang et al, 2005; He et al, 2006). Microsomes containing individually expressed CYP enzymes were prepared from the infected Sf9 insect cells by sonication and differential centrifugation as described previously (He et al, 2004).

Antibody preparation

A polyclonal antibody was raised against a C-terminal CYP2A13-specific peptide sequence covering the amino acid residues 369 to 377. The selection of this antigenic peptide was based on the hydrophilicity and the side chain properties of the amino acid residues that differ most from either CYP2A6 or CYP2A7. The peptide was covalently conjugated with keyhole limpet haemocyanin (KLH) and was then used to immunize rabbits. The peptide synthesis, KLH conjugation, and immunization of the rabbits were performed by Lampire Biological Laboratories, Inc. (Pipersville, PA).

Immunoblot analysis

Immunoblotting was conducted as described previously (He et al, 2004). Microsomal proteins, prepared either from the insect cells expressing individual CYP enzymes or from human livers, were separated by SDS-polyacrylamide gel (10%) electrophoresis. The proteins were then transferred to a nitrocellulose membrane and probed with either the mouse monoclonal antibody against human CYP2A6 (dilution 1:2400) or the rabbit polyclonal antibody against human CYP2A13 (dilution 1:2000). Goat anti-mouse IgG and anti-rabbit IgG (both conjugated with horseradish peroxidase) were used as the secondary antibodies for the detection of CYP2A6 and CYP2A13, respectively. The immunoblot was visualized by enhanced chemiluminescence (ECL) according to the manufacturer's protocol (Amersham Biosciences Inc., Piscataway, NJ). In some

experiments, the probed membranes were incubated with a stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 50°C for 30 min to remove the bound antibodies and were re-used for immunoblotting with a different primary antibody.

Tissue samples

Paraffin tissue sections (5 µM thickness) prepared from normal human trachea (T2234160), bronchus (HuFPT111) and lung (containing bronchiole areas, HuPFT131 and T2234152) were obtained from Biochain Institute, Inc. (Hayward, CA) and US Biomax, Inc. (Rockville, MD). Tissue microarrays containing normal lung alveolar tissues (NC04-01-001, n=22), lung carcinomas with self-matched margin and normal tissues (CC04-02-001, n=18), and multiple organs (NC00-01-003, including 5 samples each, for lung, heart, liver, testis and ovary) were obtained from Cybrdi Inc. (Frederick, MD). The data available for the samples included the age, gender, and pathological diagnosis of the patients.

Immunohistochemistry

Tissue sections were first de-paraffinized with xylene (8 min x 2) and hydrated gradually through a series of graded ethanol (100%, 95%, 70%, 50%). After washes with phosphate-buffered saline (PBS), the sections were pretreated for antigen retrieval in a microwave oven for approximately 10 min using the antigen unmasking solution. To quench the endogenous peroxidase activity, the sections were incubated with 3% H₂O₂ for 15 min. Nonspecific binding was blocked with 3% normal goat serum for 1 hr at room temperature. The sections were then incubated with the rabbit anti-CYP2A13 antiserum (1:1200 dilution for trachea sections, and 1:800 dilution for the sections of bronchus, lung and other tissues) in a humidified chamber

overnight at 4°C. On the next day, the sections were rinsed three times in PBS for 5 min each, incubated with biotinylated goat anti-rabbit IgG as the secondary antibody (1:200 dilution) for 30 min at room temperature. After rinsing with PBS, the sections were incubated for 30 min with Vectastain ABC reagent. CYP2A13 proteins were localized by the development of substrate-chromogen mixture (3,3'-diaminobenzidine, DAB). The sections were counterstained by Mayer's hematoxylin solution and were dehydrated and mounted immediately with gum. Negative controls were performed by replacing the primary antibody with PBS buffer. Staining specificity was appraised by substitution of the primary antibody with the pre-immune serum from the same rabbits. The immunohistochemical staining was performed at least twice with several sections per tissue and the results were confirmed independently by three authors (K.R. Reuhl, G-Y Yang, and L-D Wang), who are pathologists experienced in immunohistochemical analysis.

Results

To determine the specificity of the anti-CYP2A13 peptide antibody we generated, immunoblot analysis was conducted on heterologously expressed human CYP2A13, CYP2A6, CYP3A4, CYP2S1 and mouse CYP2A5 as well as human liver microsomes (pooled from 27 normal individual livers). The result clearly demonstrated that the anti-CYP2A13 antibody is highly specific and only reacts with the expressed CYP2A13 protein (Fig.1). It did not cross-react with human CYP2A6 and mouse CYP2A5, both are composed of the same number of amino acid residues (494 a.a.) as CYP2A13 and have a high degree of identity in amino acid sequence. Nor did the antibody cross-react with human CYP3A4 and CYP2S1 or any of the CYP forms in human liver microsomes (Fig.1). Since both CYP3A4 and CYP2A6 are major CYP enzymes in

human livers, the immunoblot result with human liver microsomes is consistent with the lack of cross-reactivity of anti-CYP2A13 antibody to heterologously expressed CYP2A6 and CYP3A4 proteins. The anti-human CYP2A6 antibody was used as a positive control in immunoblot analysis. CYP2A6 proteins, either expressed heterologously or in the human liver microsomes, were clearly detected by the anti-CYP2A6 antibody. Consistent with our previous reports (He, et al, 2004), it also cross-reacted with CYP2A13 and mouse CYP2A5 but not with human CYP3A4 and CYP2S1. While this anti-CYP2A6 antibody appears to have an equal efficiency in binding to CYP2A6 and CYP2A13, it has a much weaker cross-reaction with mouse CYP2A5.

After confirmation of its specificity, we used the anti-CYP2A13 antibody in immunohistochemical study to examine the expression of CYP2A13 protein in human respiratory tissues. Strong immunostaining was mainly observed in the epithelia cells of the bronchus and trachea, but was weak in the smooth muscle layers of the trachea and bronchus. The staining was rare in the centrilobular area of the peripheral lung tissues (n=27 in total). There was no CYP2A13-specific staining in the type I pneumocytes and the staining was either weak or undetectable in most type II pneumocytes in all the lung parenchymal tissues examined. While positive staining of macrophages was observed in the alveolar spaces of the lung, it was most likely non-specific as macrophages are known to have a high level of endogenous peroxidase activity. No CYP2A13-specific staining was observed in the negative controls of all the respiratory tissues (including the bronchus and trachea), in which the anti-CYP2A13 antibody was replaced by either PBS buffer or the pre-immune rabbit serum. This result demonstrates the specificity of our anti-CYP2A13 antibody in immunohistochemical analysis. The antibody specificity was further evidenced by the lack of immunostaining in human liver,

heart, testis and ovary tissues, which is consistent with our previous report that there is little or no expression of CYP2A13 mRNA in the human liver and heart (Su et al, 2000) (Fig. 2).

Because some CYP enzymes were reported to be overexpressed in tumors (Oyama et al, 2004; Wastl et al, 1998; Downie et al, 2005; Wenzlaff et al, 2005), we also examined the expression of CYP2A13 protein in different types of human lung carcinomas. The tissue array used for immunohistochemical analysis included carcinoma tissue, margin of carcinoma tissue and normal tissue from each patient (n=18). The tumor types included squamous carcinoma (n=3), adenocarcinoma (n=6), large cell carcinoma (n=3), adenosquamous carcinoma (n=2), alveolar carcinoma (n=2), basaloid carcinoma (n=1) and papillary bronchiolar carcinoma (n=1). Expression of CYP2A13 protein was not detected in any of these samples (Fig. 2).

Discussion

The cDNA and protein sequences of CYP2A13 are highly similar to CYP2A6 with only 4.7% and 6.5% difference in the nucleotide coding sequence and amino acid sequence, respectively. In addition, the distribution of most different amino acid residues or nucleotides in the proteins or cDNAs of CYP2A13 and CYP2A6 are scattered. Therefore, it is a great challenge to develop specific antibodies or nucleotide probes that can distinguish between CYP2A13 and CYP2A6 for immunological or hybridization analysis. Prior to the present study, specific antibodies against CYP2A13 were not available. Several anti-CYP2A antibodies, including anti-CYP2A10/11 (Ding and Coon, 1990), anti-CYP2A5 (Gu et al, 1998) and anti-CYP2A6 antibody (Gentest), all cross-react with both CYP2A6 and CYP2A13 proteins in immunoblotting. While these antibodies have been used to detect CYP2A13 protein in cDNA-mediated heterologous

expression systems, none of them can be used for specific immunological detection of CYP2A13 protein in human tissues.

In the present study, we decided to develop a polyclonal antibody that targets a CYP2A13-specific peptide sequence. The selection of the antigenic peptide for the production of CYP2A13-specific antibodies was based on the following logic. It is well known that antibodies that distinguish among closely related family members (such as CYP2A13, CYP2A6 and CYP2A5) rarely recognize only a single amino acid difference in an epitope; but generally recognize areas that include two or more differences. Also, a continuous epitope (sequential amino acids in a single peptide) most commonly does not exceed 6-7 amino acids. Lastly, differences in amino acid sequence most likely responsible for antibody specificity include residues which are structurally quite different and/or where there is a change in ionic charge (e.g. Asn *versus* Lys = neutral *versus* positive charge at pH 7). Of course, best of all would be a switch in ionic charge from positive to negative or vice versa (e.g. Lys *versus* Glu). Taking into account the above requirements there are only three areas in CYP2A13 protein that qualify and they are the residues of 25-30, 372-375 and 403-409. The first site, residues 25-30, is part of the transition from the very hydrophobic amino terminal end that is in the endoplasmic reticulum to the remaining globular heme-containing region. Experience showed that few antibodies recognize this region. The next region, residues of 372-375, appears to be the most promising since comparing CYP2A13 with CYP2A6 (His *versus* Arg at residue 372 and Asn *versus* Lys at residue 375) there are changes in charge and significant differences in structure. Inclusion of the residue #369 adds another difference between CYP2A13 and CYP2A6, (Gly *versus* Ser, which is a very conservative change but could help. Therefore, the peptide GLAHRVNKD (covering

residues 369-377 of CYP2A13) was selected to generate the CYP2A13 specific antibody. The amino acid residues at the corresponding positions are SLARRVKKD for CYP2A6 and GLARRVTKD for mouse CYP2A5. Since we were successful in generating the CYP2A13-specific antibody with this peptide sequence, we did not pursue the residues 403-409 site for the antibody production. Immunoblot analysis clearly demonstrated that our CYP2A13 peptide antibody does not cross-react with CYP2A6 protein (either expressed heterologously or in human liver microsomes), or any of the CYP proteins that are mainly expressed in human liver that include CYP1A1, CYP1A2, CYP2C9, CYP2C19, CYP2E1 and CYP3A4 (Parkinson, 2001). Moreover, the anti-CYP2A13 antibody does not cross-react with CYP2S1, which is a non-hepatic CYP with high expression in human respiratory tract (Rylander et al, 2001; Rivera et al, 2002; Saarikoski et al, 2005), or mouse CYP2A5, which also shares a high degree of amino acid sequence similarity with both human CYP2A13 and CYP2A6. To our knowledge, this is the first specific antibody that can distinguish CYP2A13 from CYP2A6. It should be a powerful tool in studying the distribution of CYP2A13 and CYP2A6 proteins in human tissues, which is important in assessing their relative contributions to xenobiotic metabolism *in situ*.

Previous RT-PCR analysis with total tissue RNA revealed that CYP2A13 mRNA is predominantly expressed in human respiratory tissues (nasal mucosa > trachea > lung, rank order of expression level) but has no or little expression in the heart or liver (Su et al, 2000). While human nasal mucosa samples were not available for the present study, our immunohistochemical study demonstrated that the expression of CYP2A13 protein in these human tissues is consistent with its mRNA expression. Such a consistency also provides additional support to the specificity of our anti-CYP2A13 antibody.

A unique advantage of the immunohistochemical approach is its capability of identifying the cellular localization of an interested protein. Our results demonstrated that in human respiratory tract, the expression of CYP2A13 protein is almost exclusively localized in the epithelial cells in the bronchus and trachea with little expression in the bronchiolar and alveolar cells. The presence of several CYP enzymes, mostly assayed at the mRNA level, in human respiratory tract has been reported (Su et al, 2000; Saarikoski et al, 2005; Castell et al, 2005). Among them, immunohistochemical studies revealed that CYP2S1 protein is highly expressed in both bronchi and bronchioli but is very low in the alveolar lining cells (Saarikoski et al, 2005). In contrast, the expression of CYP1A1 protein was intense in bronchiolar epithelium of peripheral lung and type II alveolar epithelial cells (Saarikoski et al, 1998). The cellular localization pattern of CYP2A13 protein in human respiratory tract is much more similar to that of CYP2S1. It is also very similar to the expression of CYP2A5 protein in mouse respiratory tract (Piras et al, 2003), although mouse CYP2A5 but not CYP2A13 is mainly expressed in the liver (Ulvila et al, 2004). The tissue- and cell-specific expression of human CYP enzymes is believed to play a critical role in the metabolism *in situ* of various CYP substrates including the metabolic activation of chemical carcinogens in the target tissues/cells. For CYP2A13, its high level expression in human bronchial epithelium and high efficiency in the metabolic activation of tobacco-specific carcinogen NNK are consistent with the observations that most smoking-related lung cancers are bronchogenic (Williams and Sandler, 2001).

It has been reported that some CYP enzymes are overexpressed in tumors. Examples include CYP2S1 in human lung squamous cell carcinoma (Saarikoski et al, 2005;) and CYP2A5 in

mouse liver tumors induced by chemical carcinogen (Wastl et al, 1998; Ulvila et al, 2004). A significant alteration in CYP expression profiling in tumors might be used as a prognostic biomarker (Downie et al, 2005; Kumarakulasingham et al, 2005) and could affect the efficacy of chemotherapeutic drugs if they are metabolized by the involved CYP enzymes (Roy and Waxman, 2006; Oberndorfer et al, 2005; Lopes et al, 2004). Nevertheless, the present study showed that the expression of CYP2A13 protein is not increased in various human lung carcinomas, suggesting the regulation mechanism involved in CYP2A13 gene expression is not altered in lung cancer cells.

In summary, we have developed a highly specific anti-CYP2A13 antibody that can distinguish CYP2A13 from its closely related human CYP2A6. With an increased research interest in CYP2A13, this antibody should be very useful in studying the function of CYP2A13 in human tissues. We have also demonstrated a high expression of CYP2A13 protein in human bronchial epithelial cells, which provides a strong support to the hypothesis that CYP2A13-catalyzed metabolic activation *in situ* plays an important role in human lung carcinogenesis related to NNK and AFB1 exposure.

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Footnotes:

This study was partially supported by NIH grant ES-10048 (J-Y. Hong).

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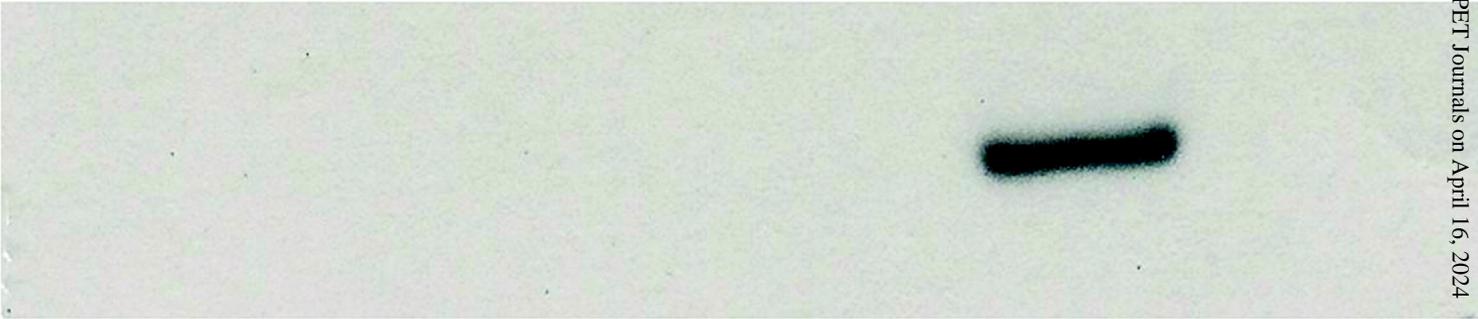
Fig. 1 Immunoblot analysis of expressed human CYP proteins and human liver microsomes. For heterologously expressed CYP3A4, CYP2A5, CYP2A6, CYP2A13 and CYP2S1, 10 µg of microsomal proteins were used for each samples. For human liver microsomes (pooled from 27 normal liver samples), 100 µg of proteins were used. From left to right, 1. human liver microsomes, 2. CYP3A4, 3. CYP2A5, 4. CYP2A6, 5. CYP2A13, and 6. CYP2S1. (A) Probed with anti-CYP2A13 antibody. The anti-CYP2A13 antibody only reacts with the CYP2A13 protein. (B) Probed with anti-CYP2A6 antibody. The anti-CYP2A6 antibody detects CYP2A6 protein in human liver microsomes and CYP2A6 protein heterologously expressed. It cross-reacts with CYP2A13 and also has a weak cross-reaction with mouse CYP2A5.

Fig. 2 Immunohistochemical analysis of CYP2A13 protein in human tissues (magnification x 200). A and B, trachea; C and D, bronchus; E and F, peripheral lung tissues; G and H, liver; I, lung squamous carcinoma; J, lung adenocarcinoma. A, C, E, G, I and J used the anti-CYP2A13 antibody. Strong immunostaining was mainly observed in the epithelial cells of the bronchus and trachea, but was rare in peripheral lung tissue. Positive staining of macrophages was observed in the alveolar space of the lung. No staining was observed in the liver. Pre-immuno rabbit serum, as a negative control, was used for B, D, F and H.

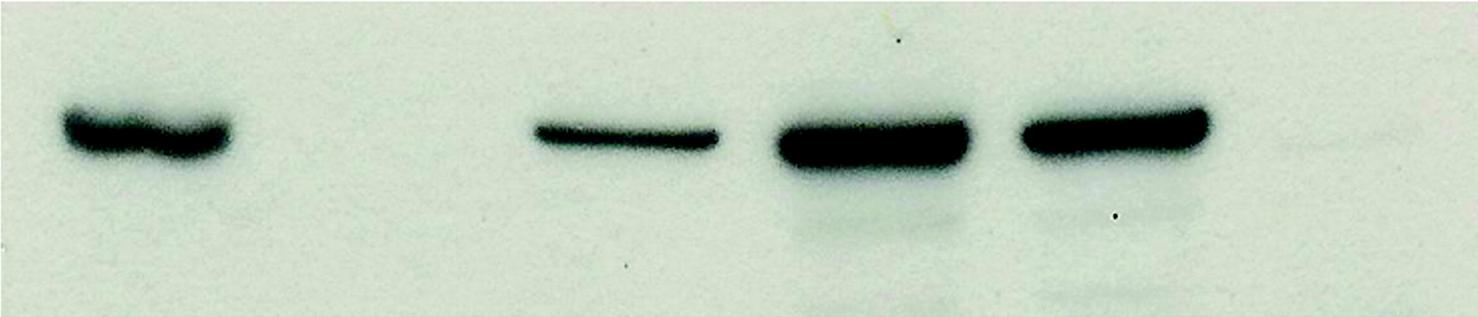
Fig.1

1 2 3 4 5 6

A



B



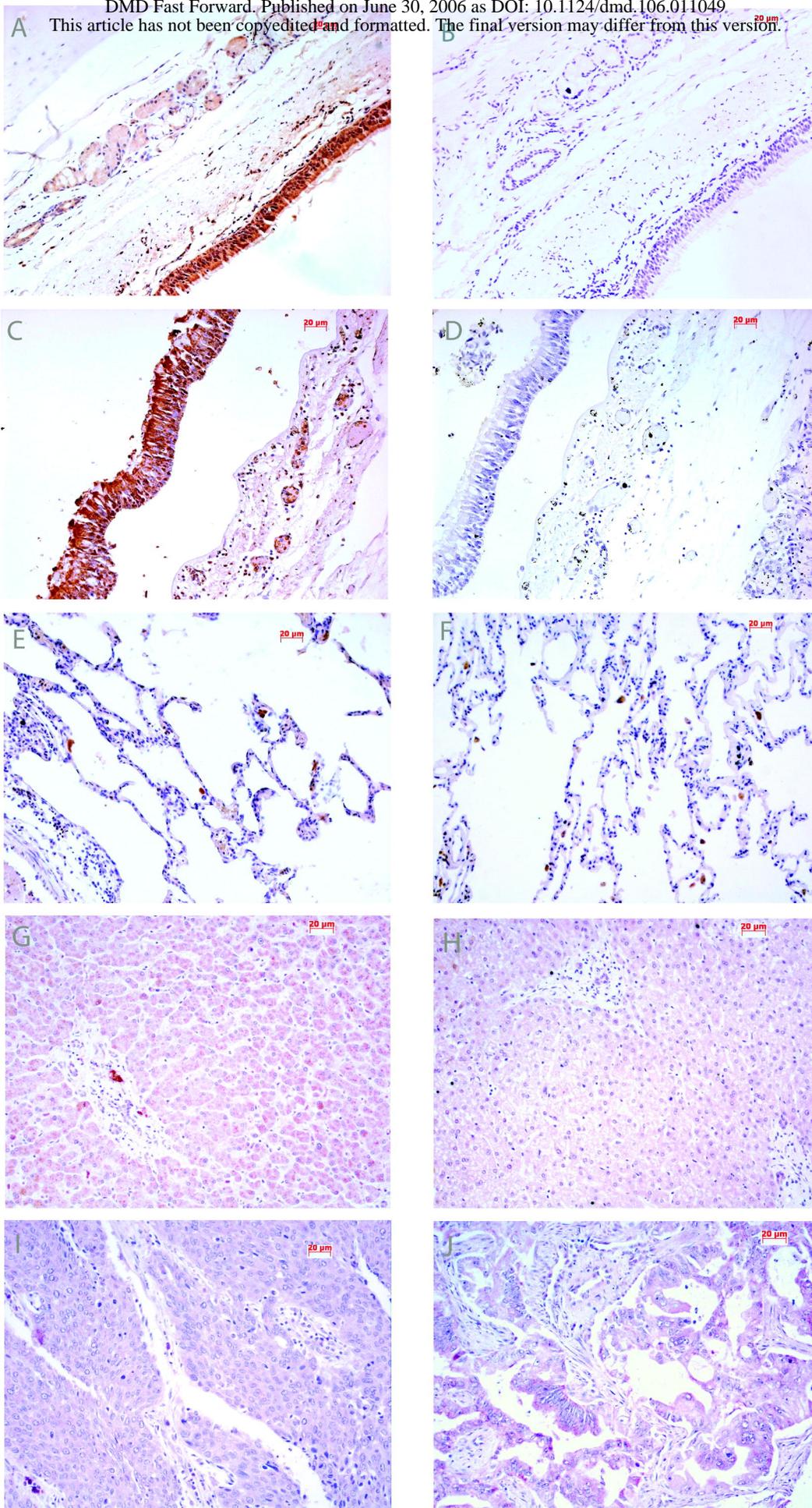


Fig.2