Cancer Epidemiology 37 (2013) 653–659

Contents lists available at SciVerse ScienceDirect

Cancer Epidemiology

The International Journal of Cancer Epidemiology, Detection, and Prevention

journal homepage: www.cancerepidemiology.net

Pulmonary CYP2A13 levels are associated with early occurrence of lung cancer—Its implication in mutagenesis of non-small cell lung carcinoma

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ARTICLE INFO

Article history:
Received 28 January 2013
Received in revised form 1 April 2013
Accepted 26 April 2013
Available online 7 June 2013

Keywords:
CYP2A13
Early occurrence
Lung cancer
NSCLC
EGFR mutations
IHC

ABSTRACT

CYP2A13, a human pulmonary specific cytochrome P450 enzyme, plays an important role in susceptibility to tobacco-specific nitrosamines (TSNAs)-induced lung cancer in humans. The pattern of CYP2A13 distribution in respiratory tract affects the susceptibility of the lung to carcinogens. CYP2A13 is expressed in the epithelium of trachea and bronchi; however its pattern of expression in human lung cancer remains largely unknown. This study aimed to determine the CYP2A13 expression in specimens from human non-small cell lung carcinomas (NSCLCs), i.e., adenocarcinoma and squamous carcinoma, by immunohistochemical (IHC) analysis and to identify the potential linkage between tumor CYP2A13 levels and some clinicopathological characteristics of NSCLC patients in Taiwan. The tumor CYP2A13 IHC staining signal was strong in 76% of the 112 study subjects. Study subjects (especially non-smoking or lung adenocarcinoma patients) with higher tumor CYP2A13 levels were younger. Multiple logistic regression analysis revealed that in younger subjects (age $\leq$ 66) and heavy smokers (pack-years $\geq$ 40), the odds ratio (OR) for positive tumor CYP2A13 staining was significantly higher than that for negative tumor CYP2A13 staining. Moreover, the association of *EGFR* gene mutations and positive tumor CYP2A13 staining was also revealed. In conclusion, these findings suggest the potential involvement of pulmonary CYP2A13 in the early occurrence of NSCLC as well as in the development of *EGFR* gene mutations.

1. Introduction

CYP2A13, a pulmonary-specific cytochrome P450 enzyme in humans [1], is involved in the metabolism of various xenobiotics, including tobacco-specific nitrosamines (TSNAs), such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N′-nitrosonornicotine (NNN) [1–3], aflatoxin B_1 [4], and 4-aminobiphenyl [5]. Epidemiological studies have revealed that the CYP2A13 C3375T polymorphism that results in an Arg257Cys variant with reduced enzyme activity is associated with an reduced lung cancer risk, particularly in smokers [6,7]. Moreover, our previous in vitro study has shown that heterogeneous expression of human CYP2A13 in mammalian cells further enhances the TSNA-induced mutagenicity [8]. These results suggest that CYP2A13 may play an important role in cigarette smoking-induced lung cancer development.

Analysis of CYP2A13 mRNA levels indicate that CYP2A13 is expressed mainly in the human respiratory tract [1,5] with the highest to lowest levels being in the nasal mucosa > trachea > lung [1]. Immunohistochemical (IHC) studies reveal that CYP2As are expressed in several types of human cancerous tissue, including hepatocellular carcinoma [9], colon tumors [10], and non-small cell lung carcinomas (NSCLCs) [11]. In lung adenocarcinoma, the CYP2As levels are positively associated with the features of cancer progression, i.e., tumor size and lymph node metastasis [11]. In those studies, there was no antibody available to specifically recognize each CYP2A homologue, i.e., CYP2A6, CYP2A7, or CYP2A13. Thus, it was difficult to differentiate the distribution of the three CYP2A homologues in those cancers.

Because of the heterogeneity of cell types in the respiratory tract, a detailed IHC analysis is necessary to precisely evaluate if there is any cell/tissue-specific distribution of CYP2A13 in lung cancer. We have generated a CYP2A13-specific antibody with no cross-reactivity with CYP2A6 and CYP2A7 [12]. By IHC analysis

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http://dx.doi.org/10.1016/j.canep.2013.04.010

with the antibody, it is possible to unambiguously define the topological distribution of CYP2A13 in human lung cancer tissue. In the present study, we evaluated CYP2A13 expression in specimens from NSCLC patients by IHC analysis and determined the potential linkage between CYP2A13 levels and some patient clinicopathological characteristics. Pulmonary CYP2A13 was markedly expressed in the specimens of a majority of NSCLC patients. Statistical analyses indicated the possible involvement of pulmonary CYP2A13 in the early occurrence of NSCLC. Moreover, we further demonstrate the association of CYP2A13 expression with increased risk of *EGFR* gene mutations, which plays a critical role in the development of lung adenocarcinoma and has become an important issue for therapeutic decision-making in NSCLC in Taiwan.

2. Materials and methods

2.1. Tissue samples and study subjects

A lung tumor array, containing specimens from 112 patients with primary lung cancer, was kindly provided by Dr. Huei Lee (Graduate Institute of Cancer Biology and Drug Discovery, Taipei Medical University, Taiwan). The patients, 41 females and 71 males, were admitted to the Department of Thoracic Surgery of Taichung Veteran's General Hospital between 1993 and 2005 and were asked to submit a written informed consent form approved by the Institutional Review Board of Chung Shan Medical University Hospital. None of these subjects had received radiation therapy or chemotherapy before surgery. The tumor types and stages were determined according to the WHO classification system by pathologists. Demographic characters, i.e., gender, age, smoking status, and pack-years, and clinicopathological characters (i.e., type of lung cancer, clinical stage, T- and N-factor, and tumor grade) of the subjects are listed in Table 1. Gene mutations of tumor suppressor genes/oncogenes (i.e., *p53*, *K-ras*, and *EGFR*) in the study subjects was determined by direct sequencing of Exon 4–8, Exon 1–2, and Exon 18–21, respectively. Genomic DNA extraction and sequence information were previously described in detail [13,14].

2.2. IHC analysis

The IHC analysis of CYP2A13 expression was previously described in detail [12]. Briefly, formalin-fixed and paraffin-embedded tumor tissues were sectioned, and the sections were deparaffinized with xylene, rehydrated through a series of graded ethanol solutions (100%, 90%, 75%), washed with PBS, and autoclaved for 20 min in citrate buffer (pH 6.0) to retrieve antigens. Endogenous biotin was blocked with a biotin blocking reagent (Invitrogen, Carlsbad, CA, USA). IHC staining was performed using the Histostain-*Plus* (Invitrogen) according to the manufacturer's instructions. The sections were treated with 3% hydrogen peroxide for 10 min at room temperature (RT) to quench endogenous peroxidase activity, treated with 10% non-immune goat serum for 10 min at RT to block nonspecific binding of immunoglobulin, incubated with a primary antibody—rabbit polyclonal anti-CYP2A13 antibody (1:500, in blocking solution)—at RT overnight, incubated with biotinylated secondary antibody, and then with streptavidin-peroxidase conjugate. To visualize immune complex formation, the sections were stained with DAB chromogen, then counterstained by hematoxylin and mounted.

Based on the CYP2A13 levels assessed by IHC analysis, each tumor specimen was classified as "negative" (<10% of tumor cells with positive CYP2A13 staining) or "positive" (>10% of tumor cells with positive CYP2A13 staining). Positive specimens were given an *I* score (1, 2, and 3, respectively) based on the staining intensity of tumor cells (weak, moderate, and strong) and a *P* score (1, 2, and 3, respectively) based on the percentage of tumor cells with positive staining (10–50%, 50–90%, and >90%). Relative CYP2A13 IHC staining level of each positive tissue was evaluated by the product of its *I* score and *P* score ($I \times P$) and was defined as medium (+, $I \times P = 1$–4) or high (++, $I \times P = 6$–9).

Table 1
Descriptive subgroup analysis according to CYP2A13 IHC staining status of tumor specimens ($n = 112$).

	No.	CYP2A13 IHC staining		*p*-Value[d]
		Negative (%)	Positive (%)	
All cases	112	27 (24.1)	85 (75.9)	
Gender				
Male	71	20 (28.2)	51 (71.8)	0.186[a]
Female	41	7 (17.1)	34 (82.9)	
Age				
≤66	57	8 (14.0)	49 (86.0)	**0.012**[a]
>66	52	18 (34.6)	34 (65.4)	
Missing[c]	3	1	2	
Smoking status				
Non-smokers	71	16 (22.5)	55 (77.5)	0.609[a]
Smokers	41	11 (26.8)	30 (73.2)	
Pack-years				
<40	25	10 (40.0)	15 (60.0)	0.064[b]
≥40	12	1 (8.3)	11 (91.7)	
Missing[c]	4	0	4	
Cancer type				
Adenocarcinoma	56	11 (19.6)	45 (80.4)	0.269[a]
Squamous carcinoma	56	16 (28.6)	40 (71.4)	
Stage				
I	42	11 (26.2)	31 (73.8)	0.753[a]
II + III + IV	68	16 (23.5)	52 (76.5)	
Missing[c]	2	0	2	
T-factor				
1 + 2	84	21 (25.0)	63 (75.0)	0.702[a]
3 + 4	28	6 (21.4)	22 (78.6)	
N-factor				
0	56	13 (23.2)	43 (76.8)	0.825[a]
1 + 2 + 3	56	14 (25.0)	42 (75.0)	
Grade				
Low/medium	60	12 (20.0)	48 (80.0)	0.290[a]
High	30	9 (30.0)	21 (70.0)	
Missing[c]	22	6	16	

[a] The Chi-square test was used for statistical analysis.
[b] The Fisher's exact method was used for statistical analysis.
[c] Missing means data missing.
[d] *p*-Values in bold indicate statistically significant ($P < 0.05$).

2.3. Statistics

Between-group differences in age, gender, smoking status, and clinicopathological characteristics of lung cancer patients were evaluated by Chi-square tests. The nonparametric Jonckheere–Terpstra test and Student's *t*-test were used to determine the significance of differences in patient age between groups divided on the basis of CYP2A13 IHC staining level. Multiple logistic regression models were used to test different categorical variables. All statistical analyses were performed using SPSS Version 18. All *p*-values were two-sided and differences were considered significant at $p < 0.05$.

3. Results

3.1. Descriptive analysis of patient characteristics according to tumor CYP2A13 IHC staining levels

IHC analysis showed that squamous lung carcinoma (Fig. 1A) and lung adenocarcinoma (Fig. 1B) were immunopositive for