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Increased cytochrome P450 and aryl hydrocarbon receptor in bronchial epithelium of heavy smokers with non-small cell lung carcinoma carries a poor prognosis

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1. ABSTRACT

Smoking induces mutations via the formation of DNA-adducts in the bronchial and alveolar epithelium and contributes to the development of lung cancer. Benz(a)pyrene and nitrosamine, typical carcinogens in cigarette smoke, undergo metabolic activation by the phase I enzymes, such as cytochrome P450 (CYP) 1A1, CYP2A6 and CYP2E1. The transcriptional regulation of these phase I enzymes is regulated by arylhydrocarbon receptor (AH-R) which binds many well-known carcinogens. To identify a cause and effect relationship, the expression of cytochrome CYP and AH-R in the bronchial epithelium was correlated with the history of cigarette smoking in patients with non-small cell lung carcinoma (NSCLC). Although $CYP3A^+$ cells were absent in the bronchial epithelium of all patients, there were many $CYP2E1^+$ cells in heavy ($\geq$1000 cigarette/day × year) smokers (38.5%). In contra-distinction, there was significantly less number of $CYP2E1^+$ cells in light (<1000 cigarette/day × year) smokers (15.6%) or non-smokers (10.0%). Similarly, there were more $CYP1A1^+$ (19.2%) and $CYP2A6^+$ cells in heavy (65.4%) smokers as compared to non-smokers. The number of $AH\text{-}R^+$ cells was also significantly higher in cases with *p53* mutation (62.5%) than those without (12.2%) mutation. Since in patients with early NSCLC, CYP positivity showed a close correlation with a poor survival ($p < 0.01$), expression of CYP in bronchial epithelium has a prognostic potential.

2. INTRODUCTION

Despite a poor survival rate, lung cancer is a preventable disease since many forms of this cancer are developed due to a longstanding exposure to inhaled carcinogens (1-2). In the past several years, the pathway for the metabolic activation of these carcinogens and the underlying basis for their carcinogenicity have been characterized in detail (3-7). Benz(a)pyrene and nitrosamine, two well known carcinogens present in cigarette smoke, enter the cytoplasm, bind arylhydrocarbon receptor (AH-R) and form a complex with HSP90. After translocation to the nucleus, AH-R dissociates from HSP90 and forms a heterodimer with the arylhydrocarbon receptor nuclear translocator (Arnt). The newly formed AH-R/Arnt complexes bind cis-acting DNA elements, known as xenobiotic responsive element (XRE) or dioxin response element (DRE), and enhance the transcription of a number of drug-metabolizing enzyme genes, also known as phase I genes. These enzymes include cytochrome P450 (CYP) 1A1, CYP2A6 and CYP2E1 (3-6, 8-9). Thus, the increased levels of phase I enzymes leads to an accelerated activation of carcinogens.

For the most part, carcinogens activated on the endoplasmic reticulum by phase I enzymes are converted to inactive metabolites by the phase II enzyme, glutathione S-transferase (GST), particularly the Mu class (GSTM1) (10). However, when the activated carcinogens incidentally

Table 1. List of antibodies

Antigen	Source	Type	Dilution
AH-R	Santa Cruz biotechnology, Santa Cruz, CA	Polyclonal goat anti-human	400
CYP1A1	Affiniti research products Ltd., Devon, England	Polyclonal rabbit anti-human	200
CYP2A6	Gentest Corporation., Franklin Lakes , NJ	Monoclonal mouce anti-human	200
CYP2E1	Gentest Corporation., Franklin Lakes , NJ	Monoclonal mouse anti-human	100
CYP3A (CYP3A4, CYP3A5, CYP3A7)	Gentest Corporation., Franklin Lakes , NJ	Monoclonal mouse anti-human	1000

enter into the nucleus, they covalently bind to DNA and form DNA adducts. The carcinogen-DNA adducts introduce mutations in genes such as *p53* which leads to errors in DNA repair at an accelerated pace, and causes carcinogenesis (11, 12). The susceptibility to this chemical induced carcinogenesis, however, varies in different individuals since not every smoker develops lung cancer (13). Identification of markers that underlie this differential susceptibility, therefore, is important for the prevention and management of lung cancer (14-19). Here, we show that the expression of CYP and AH-R in bronchial epithelium of smokers, bears prognostic potential in patients with non-small cell lung carcinoma.

3. MATERIALS AND METHODS

3.1. Tissue samples

Formalin fixed paraffin embedded tissues of seventy eight patients with non-small cell lung carcinoma prior to receiving chemotherapy or radiotherapy were included in this study. The TNM staging revealed twenty-six patients with stage I, 10 with stage II, 39 with stage III, and 3 with stage IV disease (20). Histological typing of the tumors was performed according to the WHO classification (21). For detection of *p53* mutation, genomic DNA was extracted from fresh tumor samples.

3.2. Immunoshitochemical staining

Immunohistochemical staining was performed by the avidin-biotin complex (ABC) method with a labeled streptavidin-biotinylated antibody (LSAB) kit (Dako Corp., Carpinteria, USA) (12, 22)(Table 1). Briefly, 3 μm sections were incubated first with a 1:100 - 1000 dilution of primary antibodies for 40 minutes at room temperature, followed by 10 min incubation with secondary antibodies and peroxidase-conjugated streptavidin (23). Staining was completed after a 15 minutes incubation with a freshly prepared substrate-chromogen solution. Cases with <10% positive BE were defined as negative and those with >10% positive BE were defined as positive.

3.3. Detection of *p53* mutation

The *p53* point mutations occurring in exons from 5 through 8 were identified in tumors by automated sequencing using an ABI 373A DNA Sequencer (Applied Biosystems, Foster City, CA) (24).

3.4. Statistical analysis

Statistical differences were determined by analysis of variance (ANOVA). The survival rate was calculated using the Kaplan-Meier method and the significance of survival rate was evaluated by the log rank test.

4. RESULTS AND DISCUSSION

CYP1A1, CYP2A6, CYP3A, CYP2E1 and AH-R were immunolocalized in tumor sections. CYP3A was absent in sections of all 78 patients with NSCLC while it was strongly present in hepatocytes in control human liver sections. Among the 78 cases, 17 specimens had CYP2E1+ BE cells (Figure 1A), while the others were all CYP2E1- (Figure1B). The rate of positivity for AH-R (Figure1D), CYP1A1 (Figure 1E), and CYP2A6 were 20.5%, 15.4%, and 52.6%, respectively. Since CYPs are induced by smoking in pulmonary tissues (26-28, 31, 34), it was hypothesized that the activation of carcinogens from cigarette smoke might be enhanced by the induction of CYP enzymes. Although, most CYPs are primarily expressed in the liver, presence of CYP1A1 and CYP1B1, CYP2A6, CYP3A4, CYP3A5, CYP2B6, CYP2B7, CYP2E1, CYP2F1 and CYP2J2 has been reported in the bronchial epithelium (25-31). Although, CYP3A was absent in BE in patients with small cell lung carcinoma, we previously reported that CYP3A was found in tumor tissue in 40% of patients with adenocarcinoma of the lung (23). In the liver, the CYP3A gene is transcriptionally regulated by pregnane X receptor (PXR) that interacts with a broad range of chemicals and forms a heterodimer with retinoid X receptor (RXR). The PXR/RXR heterodimer enhances the transcription of the CYP3A4 gene by interacting with the PXR response DNA elements (32). The regulation of PXR and CYP3A in BE cells, therefore, is important in understanding the difference in the CYP3A expression in NSCLC cells and BE cells.

The immunopositivity was then correlated with history of cigarette smoking, clinicopathological parameters and *p53* mutation (Table 2). Parameters such as sex, age, T or N stage of the disease, had no impact on the rate of AH-R, CYP1A1, CYP2A6, and CYP2E1 positivity. However, CYP2E1 positivity was correlated with history of smoking. $CYP2E1^+$ BE cells were present in 38.5% in heavy smokers (≥1000 cigarette/day × year) as compared to that in light (<1000 cigarette/day × year) smokers (15.6%) or non-smokers (10.0%). The CYP1A1 and CYP2A6 positive rates were higher (19.2% and 65.4%, respectively) in heavy smokers as compared with those in non-smokers. We also found a significantly positive ($p = 0.04$) relationship between numbers of $CYP1A1^+$, $CYP2A6^+$ and $CYP2E1^+$ BE and smoking (Figure 2).

The rate of AH-R positivity was significantly ($p < 0.01$) higher (62.5%) in samples with than those without *p53* mutation (12.2%). A significant relationship was not found for the other CYPs (Table 2).

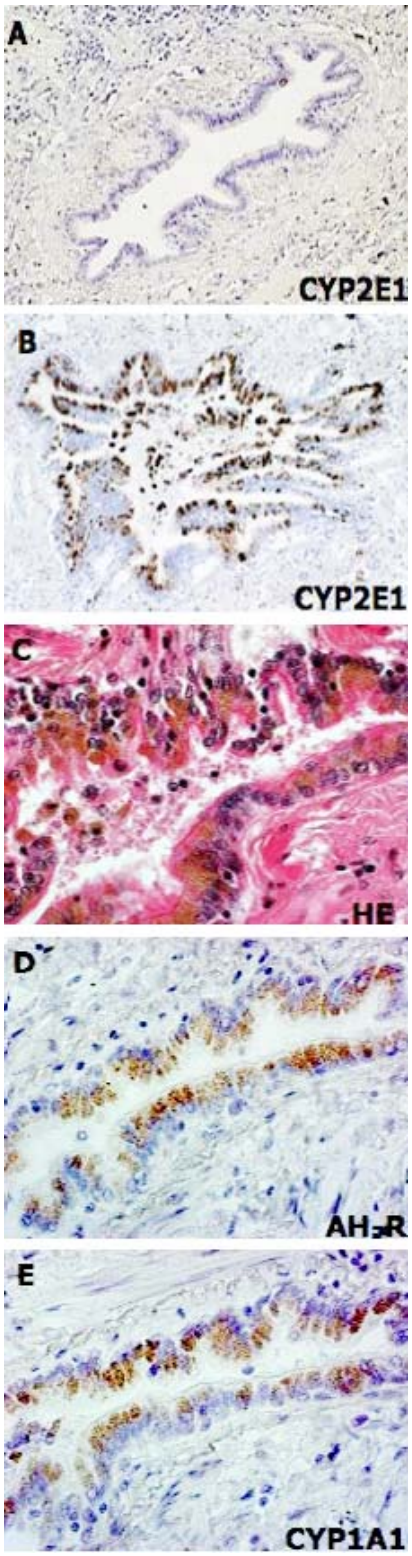

Figure 1. The expression of AH-R and CYP enzymes in bronchial epithelium. Representative immunohistochemical images of bronchial epithelia from patients with non-small cell lung cancer were shown; (A) no CYP2E1 positive cells in BE, (B) CYP2E1 strongly expressed in BE, (C) HE staining reveals BE, (D) AH-R strongly expressed, and (E) CYP1A1 strongly expressed in the same BE.

Table 2. Correlation of Immunopositivity with cigarette smoking, clinicopathological parameters and *p53* mutations in patients with non-small cell lung cancer

Parameter		AH-R (%)			CYP-1A1 (%)			CYP2A6 (%)			CYP2E1 (%)		
	n[1]	+	-	P[2]	+	-	p	+	-	p	+	-	p
Sex													
Female	22	4 (18.2)	18		2 (9.1)	20		8 (36.4)	14		2 (9.1)	20	
Male	56	12 (21.4)	44	0.75	10 (17.9)	46	0.33	33 (58.9)	23	0.07	15 (26.8)	41	0.09
Age													
≤ 65	37	8 (21.6)	29		4 (10.8)	33		20 (54.1)	17		8 (21.6)	29	
> 65	41	8 (19.5)	33	0.82	8 (17.9)	33	0.29	21 (51.2)	20	0.80	9 (22.0)	32	0.97
Smoking [3]													
0	20	5 (25.0)	15		1 (5.0)	19		7 (35.0)	13		2 (10.0)	18	
0 - 1000	32	5 (15.6)	27		6 (18.8)	26		17 (53.1)	15		5 (15.6)	27	
≥ 1000	26	6 (23.1)	20	0.66	5 (19.2)	21	0.33	17 (65.4)	9	0.12	10 (38.5)	16	0.04
T [4]													
1	21	4 (19.0)	17		4 (19.0)	17		8 (38.1)	13		5 (23.8)	16	
2	26	7 (26.9)	19		5 (19.2)	21		16 (61.5)	10		6 (23.1)	20	
3, 4	31	5 (16.1)	26	0.59	3 (9.7)	28	0.53	17 (54.8)	14	0.26	6 (19.4)	25	0.91
N [5]													
0	36	7 (19.4)	29		7 (19.4)	29		17 (57.7)	19		10 (27.8)	26	
1, 2, 3	42	9 (21.4)	33	0.83	5 (11.9)	37	0.36	24 (18.2)	18	0.38	7 (16.7)	35	0.24
Stage													
I	26	6 (23.1)	20		5 (19.2)	21		13 (50.0)	13		8 (30.8)	18	
II - IV	52	10 (19.2)	42	0.69	7 (13.5)	45	0.51	28 (53.8)	24	0.75	9 (17.3)	43	0.17
Adenocarcinoma	48	9 (18.8)	39		5 (10.4)	43		23 (47.9)	25		11 (22.9)	37	
Squamous cel carcinoma	30	7 (23.3)	23	0.63	7 (23.3)	23	0.12	18 (60.0)	12	0.30	6 (20.0)	24	0.76
***p53* mutation**													
Positive	29	10 (62.5)	19		3 (33.3)	26		17 (41.5)	12		4 (13.8)	25	
Negative	49	6 (12.2)	43	0.02	9 (18.4)	40	0.33	24 (49.0)	25	0.41	13 (26.5)	36	0.19
Total	78	16 (20.5)	62		12 (15.4)	66		41 (52.6)	37		17 (21.8)	61	

[1] n: number of patients, [2] p: *p* Value, [3] Smoking; Number of cigarette/day × year, 0; non-smoker, 0 - 1000; light smoker, >1000; heavy smoker, [4] T ; Tumor stage, [5] N; Nodal stage

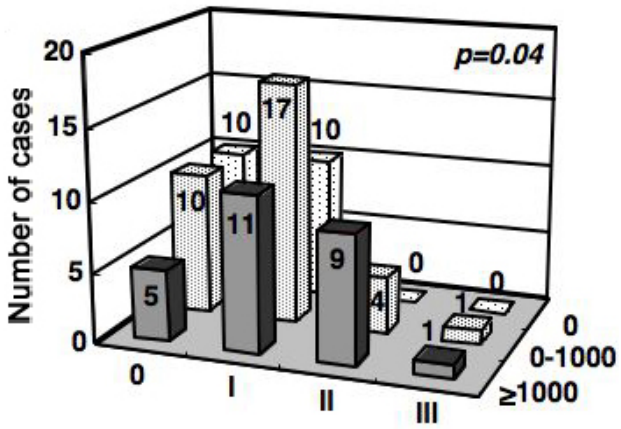

Figure 2. Relationship between co-expression of all three CYP (CYP1A1, CYP2A6 and CYP2E1) and smoking. X- axis: sample groups: 0: none of three CYP were expressed, I: samples with one of three CYP expressed, II: samples with two of three CYP expressed, III: all three CYP expressed. Z-axis: Non-smokers:0, Light smokers: 0 – 1000 cigarette/day × year, Heavy smokers: ≥ 1000 cigarette/day × year.

As shown in Table 2, the induction of the CYPs (CYP1A1, CYP2A6, and CYP2E1) in BE showed a correlation with history and extent of smoking. Moreover, there was a significant ($p = 0.04$) relationship between co-expression of all three CYP and smoking. As expected from the known regulatory mechanism of CYP1A1, there was a significantly positive ($p < 0.01$), relationship between AH-R and CYP1A1 expression in BE of NSCLC suggesting that AH-R may be the primary regulator of CYP1A1 in BE cells as it is in other cell types (Table 3). However, no significant relationship in any combination of the CYPs was observed, suggesting that each CYP enzyme may be independently regulated in the bronchial epithelial cells.

Interestingly, we found a significant ($p < 0.01$) relationship between rate of co-expression of CYP1A1, CYP2A6, and CYP2E1 in BE cells in early NSCLC and poor survival (Figure 3). Five of 19 patients with positive CYP cases died from metastasis of NSCLC. Since the

Table 3. *p* values for AH-R, CYP1A1, CYP2A6 and CYP2E1 co-expressions

	N[1]	*p* value			
		AH-R	CYP1A1	CYP2A6	CYP2E1
AH-R	16		<0.01	0.82	0.34
CYP1A1	12			0.85	0.29
CYP2A6	41				0.97
CYP2E1	17				

N: Number of positive cases

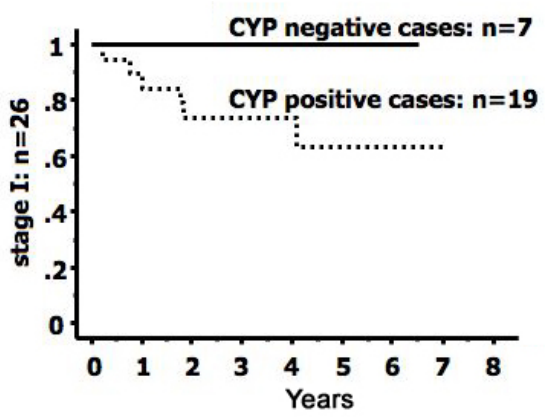

Figure 3. Survival curves of NSCLC patients with stage I disease. CYP negative cases: cases without CYP^+ cells in bronchial epithelium. CYP positive cases: cases with one or more CYP^+ cells in bronchial epithelium.

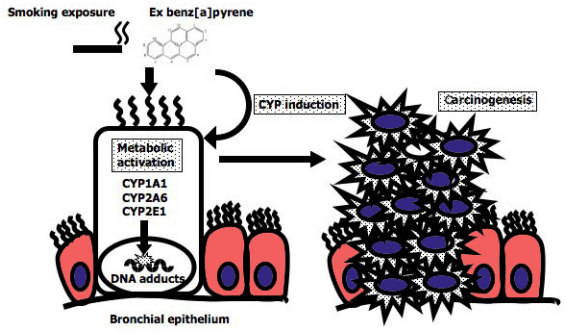

Figure 4. Schematic model for CYP metabolic activation and carcinogenic effect in bronchial epithelium (see text for details).

$CYPs^+$ BE appear in chronic obstructive lung disease as emphysema (26, 33), it is unclear why CYP positive cases are associated with metastasis in patients with NSCLC.

The possible relationship between the expression of CYP enzymes in BE cells and development of the lung cancer is shown in Figure 4. Further studies are needed to confirm the relationship between the CYP expression profile in the bronchial epithelium and the risk of development of lung cancer. Based on profiling of the enzyme expression, it should be possible to accurately predict the risk of cancer development and formulate individually tailored plans for the prevention and management of lung cancer.

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Abbreviations: CYP: Cytochrome P450, AH-R: Aryl hydrocarbon receptor, Arnt: Aryl hydrocarbon nuclear translocator, BE: Bronchial epithelium cell, NSCLC: non-small cell lung cancer

Key Words: Cytochrome P450, Aryl Hydrocarbon Receptor, Bronchial Epithelium, Respiratory tract, Carcinogen

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