OVEREXPRESSION OF ARYL HYDROCARBON RECEPTOR IN HUMAN LUNG ADENOCARCINOMAS

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Introduction

Lung cancer is the leading cause of cancer death worldwide. Epidemiological studies suggest that exposure to environmental pollutants, such as environmental tobacco smoke, polycyclic aromatic hydrocarbons (PAH) contaminated air pollutants and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), contributes to the development of lung cancers (1). Benzo[a]pyrene (B[a]P) is the major PAH found in environmental tobacco smoke and air pollutants. Both B[a]P and TCDD increase the incidence of lung tumors in rodents. Although the mechanisms of B[a]P and TCDD-induced lung carcinogenesis may be different, they share the same receptor – aromatic hydrocarbon receptor (AhR)(2).. AhR activation is dependent on ligand binding. PAH and halogenated aromatic hydrocarbons are AhR ligands, within which TCDD is the most potent. AhR regulates genes code for xenobiotic metabolizing enzymes, such as cytochrome P4501A1 (CYP1A1) and growth-regulatory proteins (8). Several studies have demonstrated that AhR activation is required for B[a]P and TCDD-induced toxicity (3,4). AhR mRNA was abundantly expressed in lung (11). Furthermore, Tritscher *et al*. (5) demonstrated that AhR protein expressed in lung epithelial cells and TCDD exposure induced alveolar-bronchiolar metaplasia in the lung. Thus, AhR may also play an important role in the molecular mechanism of B[a]P and TCDD induced lung carcinogenesis. Nevertheless, AhR protein expression has never been examined in human lung tissues and lung tumors. As previously described, CYP1A1 gene expression is regulated by AhR signaling pathway. It is possible that cell type-specific CYP1A1 expression in lung tissues is mediated by AhR expression. Thus, identification and localization of AhR in the lung tissues as well as the AhR expression in normal vs. cancer-bearing lung tissues deserve further investigation. Our present study provides the identification and localization of AhR in normal lung and in lung cancers. AhR expression in these tissues will be evaluated and compared. We believe that results from our present investigation would serve to further elucidate the relationship between AhR and lung cancer development in human.

Methods and Materials

Cell culture. Seven human lung cancer cell lines, four adenocarcinoma (AD) cells NCI-H1355, NCI-H23, CL3, CL5, and three squamous cell carcinoma (SQ) cells NCI-H226, CH27 and Calu-1, were studied. BEAS-2B cells were human bronchial epithelial cell lines immortalized with SV40.

Western immunoblot analysis. The cytosolic homogenates of cells were prepared and analysed with Western blot as previously described (6). Bands were visualized using an enhanced chemiluminescence kit.

Quantitative real-time reverse transcription- polymerase chain reaction (RT-PCR) assay. Quantitative PCR was performed using the QuantiTect SYBR Green PCR kit Qiagen, Hilden, Germany and analyzed on a ABI PRISM 7700 Sequence Detector System Perkin-Elmer Applied Biosystem,

Foster City, CA. Quantitative values were obtained from the threshold PCR cycle number C_T at which the increase in signal associated with an exponential growth for PCR product starts to be detected. Final results expressed as fold changes in tumor *AhR* gene expression in tumors relative to that in non-tumor tissues were determined as follows:

$$\text{Fold change} = 2^{-\triangle\triangle C_T}$$

Where $\triangle\triangle C_T = (C_{T\text{target}} - C_{\text{Tb-actin}})_{\text{tumor}} - (C_{T\text{target}} - C_{\text{Tb-actin}})_{\text{non-tumor}}$

Patient samples. A panel of 85 primary lung cancer specimens including 4 small cell carcinomas, 54 AD, 31 SQ, 4 large cell carcinomas and 2 adenosquamous cell carcinoma were examined in the present study. Specimens of small cell lung carcinoma were obtained by bronchoscopic or needle biopsies. Normal lung tissues were obtained from 7 autopsies, 1 taken from patient with metastatic prostate cancer and 1 taken from patient with metastatic breast cancer. One fetal lung was obtained from a fetus with a gestational age of 24 weeks who was artificially aborted because of congenital anomaly in routine sonographic examinations.

Immunohistochemistry. Immunohistochemical staining was performed on paraffin-embedded tissue using the LSAB Kit (Dako, Glostrup, Denmark) and the DAB-plus substrate kit (Zymed, San Francisco, CA). Tissue sample sections included tumor and non-tumor areas.

Evaluation of immunostaining. Since AhR was detectable in prostate tissues (7), human hyperplastic prostate sections were used as the positive control slides in all tests. The positivity of samples stained with anti-AhR was graded by three blinded observers (performed by H. Chang, M.-H. Wu, and P. Lin). AhR staining intensity in normal and tumor cells were evaluated using reactions in smooth muscle cells as internal, built-in controls and basal cells of the prostate gland as an external control. When positive reactions in cells were similar to or more intense than those in prostate basal cells, they were classified as high expressers. Cells showing similar intensity with smooth muscle cells or undetectable expression were recorded as low expressers.

Statistical analysis. Pearson's chi-square test was used to examine the difference in AhR expression between AD and SQ. Fisher's exact test was used to examine the difference in the prevalence of increased AhR expression between AD and SQ.

Results and Discussion

As shown in Figure 1A, AhR mRNA levels were higher in AD cells (H1355, CL3 and CL5 cells) than in BEAS-2B and SQ cells (Calu-1, H226 and CH27 cells). AhR protein expression was also examined in the cytosol homogenates of these cell lines with Western blot analysis. The specificity of anti-AhR was confirmed by detecting one single band of 110 kDa protein in the cytosol homogenates of rat lung and H1355 cells (Figure 1B). Consistent with AhR mRNA levels, AhR protein was also found to be high in H1355 and CL5 cells (Figure 1C and 1D). AhR protein expression levels and locations were examined with immunohistostaining method in 10 non-tumor lung specimens and 95 lung tumors. The immunoreactivity of anti-AhR in these tissues is summarized in Table 1. In all of the specimens examined, the high immunostaining was primarily located in the cytosol of bronchiolar epithelial cells. The localization of AhR is consistent with that of CYP1A1 reported by Anttila et al (8) and Saarikoski et al (9). In general, the fetal tissue presented a weaker AhR staining than the adult tissues. AhR highly expressed in small cell lung cancers and AD. Furthermore, high expression of AhR was more common in AD (42 of 54) than in SQ (16 of 31) ($p<0.05$). To understand whether this discrepancy occurred between tumor and non-tumor areas of the same specimens, AhR immunostaining intensity was examined in AD and SQ. As shown in Table 2, AhR expression in neoplastic cells was

increased among 26 out of 85 (30.6%) specimens. Of these, increased expression of AhR was more common in AD than in SQ ($p<0.01$). The relative levels of AhR mRNA were quantified in 4 specimens and their paired control tissues devoid of cancer cells with the quantitative real-time RT-PCR assay. The data were calculated and presented according to the method described in Materials and Methods. AhR mRNA levels in 2 of 4 lung tumors were increased to approximately two fold of the levels in the paired non-tumor tissues (Table 3). It has been demonstrated that AhR mRNA and protein levels were elevated in,12-dimethylbenzo[a]anthracene-induced rat mammary tumors (10). In the present study, we also observed an elevation of AhR protein in lung AD. Consistent with the up-regulation of AhR protein in lung tumors, AhR mRNA levels were elevated in 2 of 4 paired specimens. In addition, AhR protein expressions in 2 of 4 AD cell lines were higher than those in bronchial epithelial cells BEAS-2B and SQ cell lines. AhR protein levels in cell lines corresponded well with the relative mRNA levels. These results suggested that AhR expression may be up regulated in accompanied with PAH–induced carcinogenesis and PAH exposure may contribute to the development of lung AD. Furthermore, the mechanism was at least partially at the transcription level.

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Table 1. AhR expression in human normal lung as well as lung tumor tissues

		Immunostaining intensity	
	n[a]	Low	High
Normal lung tissues			
Bronchial epithelium	4	3	1
Bronchiolar epithelium	8	2	6
Type I or Type II pheumocyte	9	9	0
Smooth muscle cells of vessels	9	9	0
Fetal bronchial epithelium	1	1	0
Lung cancer			
SCLC[b]	4	1	3
NSCLC[c]			
AD[d]	54	12	42[f]
SQ[e]	31	15	16
Large cell carcinoma	4	4	0
Adenosquamous carcinoma	2	1	1

[a] n represented the case numbers.
[b] SCLC represented small cell lung cancer.
[c] NSCLC represented non-small cell lung cancers.
[d] AD represented adenocarcinoma.
[e] SQ represented squamous cell carcinoma.
[f] AhR expression was significantly higher in AD than in SQ (Chi-square test, p<0.05).

Table 2.Increased AhR expression in AD and SQ

			AHR
		n[a]	n[b] (% of overexpression)
Total		85	26 (30.6%)
Sex			
	Male	54	14 (25.9%)
	Female	31	12 (38.7%)
Histology			
	AD	54	23 (42.6%)[c]
	SQ	31	3 (9.7%)
Smoking status[d]			
	Smoker	20	4 (20.0%)
	Non-smoker	61	21 (34.4%)

[a] represented the case numbers.
[b] represented the case numbers of AhR overexpression.
[c] AhR overexpression was more common in AD than in SQ (Fisher's exact test, p<0.01).
[d] Information of cigarette smoking status was not available for four patients.

Table 3. *AhR* mRNA levels in paired tumor versus non-tumor tissues.

Patient number		AhR C_T (Mean ± S.D.)	β-actin C_T (Mean ± S.D.)	$\Delta C_{T(AhR - \beta\text{-actin})}$	$\Delta\Delta C_{T(tumor - non\text{-}tumor)}$	AhR in tumors relative to non-tumor tissues ($2^{-\Delta\Delta C_T}$)
3	Tumor	21.01 ± 0.10	16.80 ± 0.10	4.21		
	Non-tumor	33.90 ± 0.45	29.53 ± 0.71	4.37	-0.16	1.12
4	Tumor	24.57 ± 0.66	21.23 ± 1.12	3.35		
	Non-tumor	27.18 ± 0.14	22.67 ± 0.16	4.51	-1.16	2.23
11	Tumor	27.68 ± 0.45	23.10 ± 0.08	4.59		
	Non-tumor	23.40 ± 0.17	18.71 ± 0.12	4.69	-0.10	1.07
22	Tumor	26.91 ± 0.19	24.67 ± 0.23	2.24		
	Non-tumor	22.07 ± 0.08	18.57 ± 0.22	3.50	-1.26	2.39

The mRNA levels were determined with the quantitative real-time RT-PCR assay. Each measurement was repeated for four times.

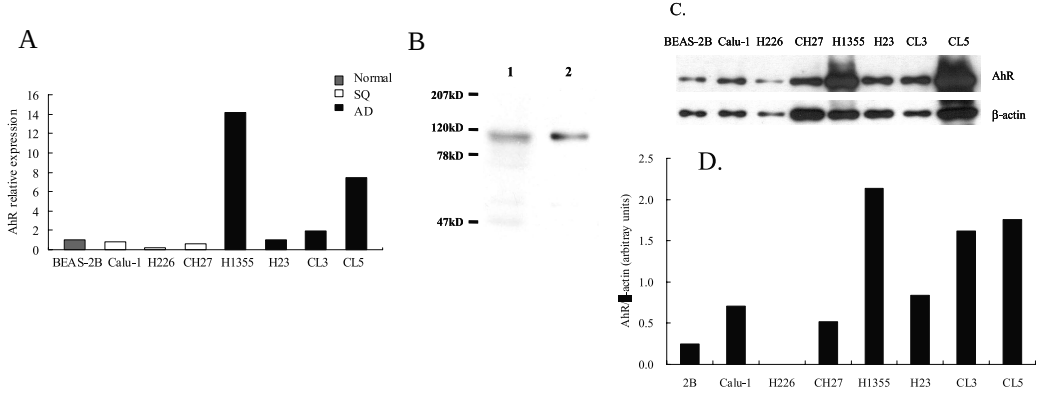

Figure 1. AhR expression levels in lung cancer cell lines and bronchial epithelial cell lines BEAS-2B. (A) Total RNA was isolated from four AD (H1355, CL3, CL5, and H23), three SQ (CH27, H226, and Calu-1) cell lines and BEAS-2B cells. (B) AhR protein was detected in the cytosol homogenates of rat lung (lane 1) and H1355 cells (lane 2) with Western immunoblot. (C) AhR protein was detected in the cytosol homogenates of lung cancer cell lines and BEAS-2B cells with Western immunoblot. (D) Band intensities in C were quantified using the Imaging Analysis System 120 (Kodak Digital Science, New Heaven, CT)