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一、中文摘要

已知暴露多種空氣中之污染物與肺癌的發生有關，例如多環芳香烴類，而多環芳香烴類受器(AhR)為多環芳香烴類化合物產生毒性所必須的，因此本計劃目的為探討AhR在台灣地區肺癌易感性所扮演之角色。我們以組織免疫染色檢測肺腫瘤組織中 AhR, Arnt, CYP1A1, CYP1B1 蛋白表現情形並以 ELISA 檢測 B[a]PDNA 鍵結物，以 real-time RT-PCR 方法定量肺腫瘤及非腫瘤組織中 AhRmRNA。本研究結果顯示 AhR, Arnt, CYP1A1, CYP1B1 均位於 bronchiolar epithelium 及腫瘤細胞，而且 AhR, CYP1A1 及 CYP1B1 位於細胞質而 Arnt 位於細胞核，肺腺癌細胞之 AhR, CYP1A1 and CYP1B1 免疫染色深度比肺鱗癌細胞深。CYP1B1 與 AhR 免疫染色深淺具相關性，在非吸煙者之肺腺癌中 CYP1A1, B[a]P DNA adduct 與 AhR 表現程度具有正相關，而且 AhR 在肺腺癌中表現比週邊正常上皮細胞高，以 real-time RT-PCR 亦發現腫瘤組織 AhR mRNA 較正常組織高，以研究結果推論，AhR 過度表現與非吸煙者之肺腺癌組織中 B[a]PDNA adduct 形成，CYP1A1、CYP1B1 表現有關。最後我們比較肺癌病人與正常人周邊血液中 AhR 基因表現情形，發現並無顯著差異，因此 AhR 基因表現並非台灣肺癌發生之易感性因子。而 AhR 基因在肺腺癌過度表現可能是細胞癌化所造成。

關鍵詞：多環芳香烴受器、肺癌

Abstract

The objective of this project is to evaluate the role of AhR in susceptibility to lung cancer in Taiwan. We have examined AhR, Arnt, CYP1A1 and CYP1B1 expression in human lung-tumor tissues using ∨之 immunohistochemistry method. B[a]P DNA adducts were measured with ELISA. AhR mRNA levels in tumors versus non-tumor tissues were measured with the quantitative real-time RT-PCR assay. AhR, Arnt, CYP1A1 and CYP1B1 were mainly found in the bronchiolar epithelium and neoplasm. AhR, CYP1A1 and Cyp1B1 staining were located in the cytosol, irrespective of Arnt in the nuclei of epithelial cells. AhR, CYP1A1 and CYP1B1 immmunostaining was more intense in adenocarcinoma (AD) than in squamous cell carcinoma (SQ). CYP1B1 levels correlated with AhR levels in lung tumors. CYP1A1 and B[a]P DNA adducts positively correlated with AhR levels in AD of nonsmokers. Furthermore, AhR protein expression was higher in AD than in adjacent bronchial epithelial cells. The increase in AhR expression was also detected at the mRNA levels with the quantitative real-time RT-PCR assay. Our data indicate that AhR was overexpressed in lung AD and suggest that AhR should play a role in B[a]P adduct formation, CYP1A1 and CYP1B1 expression in nonsmokers who developed lung adenocarcinoma. We also compared AhR gene expression in peripheral lymphocytes from lung cancer patients and control subjects with the real-time RT-PCR assay. No significant difference was found between two groups. Therefore, AhR expression was not the susceptibility factor for lung cancer in Taiwan.

Keywords: aryl hydrocarbon receptor (AhR), lung

cancer

二、緣由與目的

Lung cancer is currently the leading and the second-leading cause of cancer deaths among women and men, respectively, in Taiwan (1). Epidemiological studies suggested that 80 to 90% of all cancers are probably related to environmental factors, tobacco smoke or diet (2). Air-borne pollutants, such as cigarette smoke, asbestos, and polycyclic aromatic hydrocarbons (PAH), are considered to be contributory factors in the development of lung cancer (2). Benzo[*a*]pyrene (B[a]P), a major PAH in cigarette smoke (3) and a prominent air-borne particulate in Taiwan (4), has been shown to induce lung tumor development in rodents (5, 6).

AhR is the prototypical member of the basic helix-loop-helix/Per-Arnt-Sim class of transcription factor (7). Ligands for AhR include PAH, halogenated aromatic hydrocarbons and plant products such as indole carbinols. However, the endogenous ligand for the AhR has not been identified yet. The liganded AhR translocates to the nuclei, heterodimerizes with AhR nuclear translocator and subsequently transactivates downstream gene expression (8). Xenobiotic metabolizing enzymes regulated by AhR, include cytochrome P4501A1 (CYP1A1), cytochrome P4501A2 and 1B1 (CYP1B1), quinone oxidoreductase, glutathione s-transferase Ya subunit and UDP-glucuronosyltransferase (8). In animal models, aryl hydrocarbon receptor (AhR) has been shown to play a role in PAH-induced toxicity, including wasting, lymphoid involution, hepatotoxicity, endocrine dysfunction, embryotoxicity and carcinogenicity (9,10)

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a halogenated aromatic hydrocarbon, is one of the most potent AhR agonists. It has been reported that AhR-deficient mice were found to be relatively unaffected by TCDD even at doses 10 fold higher than those which would cause severe toxic and pathologic effect in mice with functional AhR (9). Other study has also demonstrated that B[a]P failed to induce carcinogenesis in AhR-deficient mice (11). A recent study by Hayashi *et al* (12) indicated that AhR mRNA was abundantly expressed in lung and AhR activation may be related to TCDD-induced bronchial metaplasia and hyperplasia in the lung (13). All these studies strongly suggested that AhR plays an important role in PAH-induced carcinogenesis. However, the identification and localization of AhR in the lung tissues as well as the AhR expression in normal vs. cancer-bearing lung tissues remain to be demonstrated and evaluated.

In this project we examined AhR expression in lung tumors and peripheral lymphocytes from lung cancer patients. AhR expression in lung tumors was compared with B[a]P DNA adducts, CYP1A1 and CYP1B1 expression. AhR expression in peripheral lymphocytes was compared between lung cancer patients and control subjects.

三、結果與討論

CYP1A1 expression in lung cancer tissues

CYP1A1 expression was detected in 55 lung tumor tissue samples. The CYP1A1 immunostaining was mainly localized in the cytoplasm of bronchial epithelial cells and neoplastic cells. Type II alveolar epithelial cells were also stained in a few cases. As with AhR immunostaining intensity grading, we classified tumor cells as high or low CYP1A1. The relationship between CYP1A1 expression levels, age, smoking status and histology was examined. As shown in Table I, high CYP1A1 expression was detected more frequently

in adenocarcinomas (65%, 22/34) than in squamous cell carcinomas (29%, 6/21, p = 0.013). Smoking status was not associated with CYP1A1 expression levels (Table I).

Correlation between AhR, Arnt, and CYP1A1 in adenocarcinomas A total of sixty patients were recruited in this study. However, not every specimen was enough to be examined for three proteins, AhR, Arnt, and CYP1A1. AhR expression levels and locations were examined immunohistochemically in the adenocarcinoma (n = 34) and squamous cell carcinoma (n = 21) specimens. AhR protein is found mainly in the bronchiolar epithelium and neoplasm. In bronchiolar epithelial cells and neoplastic cells the staining located in the cytoplasm was of grade-1 or -2 intensity. Immunopositive staining corresponding to grade-2 intensity was also seen among smooth muscle cells. Scattered weak reactivity was observed in type-II alveolar epithelial cells, most of which was of grade-1 intensity. Examination of endothelial cells and fibroblasts yielded negative results. One tissue section contained adenocarcinoma and squamous cell carcinoma morphological characters. In it, intensive staining (of grade-3 intensity) was observed in adenocarcinoma area, however, weak staining (of grade-1 intensity) was observed in the nonneoplastic bronchial epithelial cells and squamous cell carcinoma. We classified tumor cells showing grade-2 or -3 intensity as high expressers of AhR. AhR expression levels to be significantly associated with histology (p = 0.013). Diffuse and significantly higher AhR expressions were frequently detected in 65% of the adenocarcinoma samples (22/44) (Table I). Smoking behavior was not associated with AhR expression levels. Arnt expression was examined in 54 lung tumor tissues and 5 non-tumor lung tissues (including 3 tuberculosis, 1 emphysema and 1 interstitial lung disease samples). Nuclear immunostaining of Arnt proteins occurred predominantly in bronchiolar epithelial cells and neoplastic cells. Occasional immunoreactivity was observed in stromal cells and type II alveolar epithelial cells. Results from endothelial cells and smooth muscle cells were negative. Grade-1 Arnt protein levels were detected in all five non-tumor lung specimens. Among lung-tumor tissues grade-0 and grade-1 expression were categorized as low Arnt, and grade-2 as high Arnt for statistical analysis. The relationship between Arnt expression levels, age, smoking behavior and histology was examined. No association was found between Arnt and these factors (Table I). *CYP1A1* gene expression is regulated by AhR and Arnt. Furthermore, our data showed that AhR and CYP1A1 expression levels were high in adenocarcinomas (Tables I). Thus, we examined the correlations between these protein levels among adenocarcinomas. AhR, Arnt, and CYP1A1 expression levels were determined in 49 lung tumor tissues, 31 of them adenocarcinoma samples (Table II). We found that AhR expression was borderlinely correlated with CYP1A1 expression in the adenocarcinomas (p = 0.056). After stratification according to smoking behavior, AhR expression was significantly correlated with CYP1A1 expression in the non-smoker adenocarcinomas (p = 0.004). However, Arnt expression was not correlated with CYP1A1 expression in adenocarcinomas. No correlations among AhR, Arnt, and CYP1A1 expression levels were found in squamous cell carcinomas.

The correlation of B[a]P DNA adduct and AhR expression in lung tumors. B[a]P DNA adduct and AhR expression were measured in 79 lung tumors. Overall, B[a]P DNA adducts were not correlated with AhR expression levels (data not shown). When

subjects were stratified by smoking status and histological types, we found that B[a]P DNA adduct levels were positively correlated with AhR expression levels. (Table III).

Correlation of AhR and CYP1B1 expression in lung tumors. CYP1B1 protein was measured with immunohistostaining too. We classified neoplastic cells showing grade-2 or -3 anti-AhR immunnostaining intensity as high expressers of AhR and cells showing CYP1B1 immunostaining as CYP1B1 "positive". CYP1B1 and AhR protein expression was examined in 89 specimens (Table IV). We found that AhR and CYP1B1 levels were positively correlated ($p = 0.001$).

AhR expression in lung cells. AhR mRNA levels in the seven lung cancer cell lines and human bronchial cell lines BEAS-2B were quantified with the quantitative real-time RT-PCR assay. *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 in the cytosol homogenates of these cell lines was also examined using Western blot analysis. The specificity of anti-AhR was confirmed by detecting a single band of approximately 100-110 kDa protein in the cytosol homogenates of mouse lung and H1355 cells. Consistent with AhR mRNA levels, AhR protein was also found to be high in H1355, CL3 and CL5 cells.

Comparison of AhR expression in non-tumor lung and lung tumors. AhR protein expression levels and locations were examined with immunohistostaining method in 10 non-tumor lung specimens (one from fetal lung tissue and nine from normal adult lung tissue) and 95 lung tumors. The immunoreactivity of anti-AhR in these tissues is summarized in Table V. In all of the specimens examined, high immunostaining was primarily located in the cytosol of bronchiolar epithelial cells. Other cell types, such as bronchial epithelial cells, showed weak or no staining activity. In general, fetal tissue presented a weaker AhR staining than adult tissues. AhR is 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 VI, 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 (42.6%, 23/54) than in SQ (9.7%, 3/31) ($p<0.01$). Overexpression of AhR in these specimens was quantified and confirmed by the image analysis system Image Pro Plus (data not shown). Gender and smoking status have no significant effect on the increase of AhR (Table VI). 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 VII). This result further confirmed the increase of AhR expression in tumors.

Comparison of AhR gene expression in peripheral lymphocytes from lung cancer patients and control subjects. A total of 44 lung cancer patients and 59 non-cancer subjects were recruited in this study. The average age was greater in the patient group than in the control group. The proportion of males was significantly higher in the patient group than in the control group. Cigarette smoking was more

common in the patient group than in the control group. We further evaluated the association between *AhR* expression and lung cancer risk with the logistic regression analysis. Gene expression levels were stratified into high versus low expressers. As shown in Table VIII, elders and smokers were significantly at higher risk of lung cancer. After age, smoking status and gender were controlled in the analysis, *AhR* expression was not associated with lung cancer risk (Table VIII).

Conclusions

1. AhR, CYP1A1 and CYP1B1 protein levels were significantly higher in AD than in SQ.
2. B[a]P DNA adduct levels positively correlated with AhR levels in adenocarcinomas from nonsmokers.
3. AhR protein levels were positively correlated with CYP1B1 levels in lung tumors. Nevertheless, the correlation between AhR and CYP1A1 was only observed in AD of nonsmokers.
4. Arnt levels were not associated with the levels of AhR, CYP1A1, and CYP1B1 in lung tumors.
5. AhR protein and mRNA levels were higher in tumors than in non-tumor tissues.

These data suggest that AhR was up-regulated during lung carcinogenesis, especially in AD. AhR expression was responsible for CYP1A1 and CYP1B1 expression, and B[a]P DNA adducts formation in AD of nonsmokers. Therefore, AhR may play a role in the development of lung adenocarcinoma in nonsmokers.

四、計劃成果自評

本計劃部分研究成果已被 Toxicologic Pathology 期刊接受即將發表，此期刊在毒理學期刊排名 20%以內，另外目前正在撰寫其他部分 data 將投稿於國外毒理學 SCI 雜誌。

五、參考文獻

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Table I. AhR、Arnt、CYP 1A1 expression in lung tumors

	AhR			AhR			CYP1A1		
	Low	High	p-value	Low	High	p-value	Low	High	p-value
Number of cases	27	28		19	35		27	28	
Somoking behavior			0.054			0.257			0.171
Smoker	14	7		5	15		13	8	
Non-smoker	13	21		14	20		14	20	
Histology			0.013			0.139			0.013
Adenocarcinnoma	12	22		9	25		12	22	
Squamous cell carcinoma	15	6		10	10		15	6	

Table II. Correlation between AhR, Arnt and CYP1A1 expression in adenocarcinoma

	Ahr			Arnt		
	Low	High	P-value	Low	High	P-value
			0.056			1.000
Low CYP1A1	7	4		3	8	
High CYP1A1	5	15		5	15	
Non-smoker			0.004			1.0000
Low CYP1A1	5	1		2	4	
High CYP1A1	2	14		5	11	
Smoker			0.524			1.000
Low CYP1A1	2	3		1	4	
High CYP1A1	3	1		0	4	

Table III. Correlation between AhR expression and PAH-DNA adduct among nonsmoking adenocarcinomas

AhR expression	PAH-DNA adduct		P value[a]
	Low	High	
Low	11	12	
			0.037
High	3	15	

[a] Pearson chi-square analysis

Table IV. The correlation between AhR and C Y P 1 A 1 expression in lung tumors

CYP1B1	AhR		P[a]
	Low	High	
–	20	27	0.001
+	5	37	

Table V . AhR expression in human lung as well as lung tumor tissues

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

a n represented the case numbers
b SCLC represented small cell lung cancer
c NSCLC represented non-small cell cancers
d AD represented squamous cell
e SQ represented squamous cell
f AhR expression was significantly higher in AD than in SQ

Table VI .Increased AhR expression inAD 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%)
	SQ	31	3(9.7%)
Smoking atatus[d]			
	Smoker	20	4(20.0%)
	Non-smoker	61	21(34.4%)

a Represented the case numbers

b Reprsented the case numbers of AhR overexpression

c Ahr overexpression was more common in AD than SQ (Fisher's exact, $p<0.01$)

d Information of cigarette smoking status was not available for four patients

Table VII. AhR mRNA levels in paured tumor versus non-tumor tissues

Patien number	Tissue types	AhR C_T (Mean ± SD)	β-actin C^T (Mean ± SD)	AhR in tumors relative to non-tumor tissues ($2^{-\Delta\Delta C}$)
3	Tumor	21.01 ± 0.6	16.80 ± 0.10	
	Nontumor	33.90 ± 0.4	29.53 ± 0.71	1.12
4	Tumor	24.57 ± 0.6	21.23 ± 1.12	
	Nontumor	27.18 ± 0.1	22.67 ± 0.16	2.23
11	Tumor	27.68 ± 0.4	23.10 ± 0.08	
	Nontumor	23.40 ± 0.1	18.71 ± 0.12	1.07
22	Tumor	26.91 ± 0.1	24.67 ± 0.23	
	Nontumor	22.07 ± 0.0	18.57 ± 0.22	2.39

The mRNA levels were determined with the quantitative real-time RT-PCR assay

Table VIII. Logistic regression analysis of *AhR* expression for lung cancer risk.

Factors	n[a] (patients / controls)	OR (95% C.I.)[b]
Age	44 / 59	1.11 (1.05-1.19)[c]
Smoking		
No	22 / 48	1.00
Yes	22 / 11	3.29 (1.07-10.11)[c]
Gender		
Male	32 / 30	1.00
Female	12 / 29	1.32 (0.46-3.82)
AhR expression levels		
Low	21 / 31	1.00
High	23 / 28	1.02 (0.54-1.93)

[a] Subject numbers

[b] OR, odds ratio ; 95% C.I., 95% confidence interval

[c] $P < 0.05$