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一、中文摘要

本計劃為兩年期計劃的第二期計劃，首先探討影響週邊血液 CYP1A1 被誘導性之因素，我們以 quantitative RT-PCR 定量 32 位健康人之周邊淋巴細胞 AhR, Arnt, CYP1A1 基因表現及 CYP1A1 之被誘導性。將淋巴細胞與含 benzanthracene 之培養基培養以誘導 CYP1A1 基因表現。我們發現性別是一重要影響因素。AhR 與 CYP1A1 表現程度在女性非抽煙者顯著比男性非抽煙者高($p<0.05$)。然而 CYP1A1 被誘導性在女性非吸煙者較低，而 CYP1A1 被誘導性在男性吸煙者比男性非吸煙者高($p<0.05$)。經控制性別及抽煙習慣後，AhR 表現程度與 CYP1A1 被誘導性成正相關($p<0.05$)。這些結果顯示，淋巴細胞的 AhR 表現程度與 CYP1A1 被誘導性之個體差異有相關性，而且性別及抽煙習慣是重要影響因素。最後我們比較肺癌病人及健康人的淋巴細胞中 CYP1A1 基因表現情形，發現並無顯著差異，因此推測 CYP1A1 表現程度並非台灣肺癌發生的危險因子。

關鍵詞：cytochrome P4501A1 (CYP1A1) 、多環芳香烴類受器、肺癌

Abstract

The relationships between gene expression of aryl hydrocarbon receptor (AhR), aryl hydrocarbon receptor nuclear translocator (Arnt), cytochrome P4501A1 (CYP1A1), *CYP1A1* and the inducibility of *CYP1A1* were determined in 5 lung cancer cell lines and 32 cultivated human lymphocytes. Cytochrome P450 induction was performed by incubating lymphocytes with benzanthracne. The relative gene expression levels were determined by quantitative real-time RT-PCR assay. We found that gender is an important confounding factor for gene expression in cultivated lymphocytes. *AhR*, and *CYP1A1* levels in non-induced lymphocytes were significantly higher in female nonsmokers than in male nonsmokers ($p<0.05$). Nevertheless, *CYP1A1* inducibility was lower in female nonsmokers. *CYP1A1* inducibility was higher in male smokers than in male nonsmokers ($p<0.05$). After controlling for gender and cigarette smoking, *AhR* levels positively correlated with *CYP1A1* inducibility ($p<0.05$). These data indicate that *AhR* expression associates with individual variation of *CYP1A1* inducibility in cultivated lymphocytes. Furthermore, gender and cigarette smoking are important confounding factors for gene expression levels in cultivated lymphocytes. Finally we compared CYP1A1 expression in peripheral lymphocytes from lung cancer patients and control subjects. There was no significant difference in CYP1A1 expression between two groups. Therefore, CYP1A1 expression was not the susceptibility factor for lung cancer in Taiwan.

Keywords: cytochrome P4501A1 (CYP1A1), aryl

hydrocarbon receptor (AhR), lung cancer

二、緣由與目的

Several studies have indicated that drug metabolizing enzyme activities are highly variable in the human population (Nebert, 1991). Some drug metabolizing enzymes are responsible for metabolic activation of environmental carcinogens. Thus, individual variation in metabolic activation or detoxification of environmental carcinogens partially explains the host susceptibility to chemical-induced toxicity (Nebert, 1991; Perera, 1996).

The cytochrome P450 family1 is one of the major cytochrome P450 families involved in xenobiotic metabolism. One of the well-known examples is cytochrome P4501A1 (CYP1A1) which has been shown to participate in metabolic activation of polycyclic aromatic hydrocarbons (PAHs) (Shimada *et al.*, 1992). PAHs are one of the major carcinogens found in the environment as pollutants. Heavy exposure to PAHs contaminated air pollutants has been associated with the increased risk of lung cancer (Boffetta *et al.*, 1997). It has been demonstrated that CYP1A1 activity and expression are inducible by PAHs through activation of aryl hydrocarbon receptor (AhR) (Whitlock, 1999). CYP1A1 inducibility measured in cultivated lymphocytes has correlated well with that measured in lung tissue explants (Jacquet *et al.*, 1997). Therefore, it was suggested that CYP1A1 inducibility is a susceptibility marker for PAH-induced lung carcinogenesis (Kellermann *et al.*, 1973; Kiyohara *et al.*, 1998). Although, the association between CYP1A1 inducibility and lung cancer risk is still controversial (Kellermann *et al.*, 1973; Ward *et al.*, 1978; Prasad *et al.*, 1979; Stucker *et al.*, 2000). CYP1A1 inducibility by PAHs is variable in the human population (Kellermann *et al.*, 1973). Little information has been available on the molecular mechanism of variation in CYP1A1 inducibility. *CYP1A1* genetic polymorphisms have been demonstrated to correlate with CYP1A1 inducibility (Kiyohara et al., 1996; Kiyohara et al., 1998). However, the genotypes of "high CYP1A1 inducibility" are extremely rare in the some ethnic groups (Tefre *et al.*, 1991; Hirvonen *et al.*, 1992; Xu *et al.*, 1996) and other factors should contribute to individual variation in CYP1A1 inducibility (Smart and Daly, 2000).

Differential CYP1A1 inducibility was also reported in different strains of mice and was correlated with PAH-induced carcinogenesis in these mice (Nebert, 1989). The "high CYP1A1 inducibility" mice had a higher AhR ligand binding capacity. The PAHs are AhR ligands. The liganded AhR translocates from the cytosol to the nuclei, heterodimerizes with AhR nuclear translocator (Arnt), binds to the cognate enhancer sequence, and subsequently transactivates gene expression of *CYP1A1* (Whitlock, 1999). It has been demonstrated that AhR activation is required for PAH-induced toxicity (Nebert, 1989; Shimizu *et al.*, 2000). The study by Hayashi et al. (Hayashi *et al.*, 1994) has demonstrated individual difference in the mRNA levels of AhR and Arnt in human liver and lung tissues. The mRNA levels of *CYP1A1* from the blood of healthy subjects correlated with that of *AhR* and *Arnt* (Hayashi *et al.*, 1994). Nevertheless, it is still uncertain whether the differential expression of *AhR* and *Arnt* contributes to the interindividual variation in *CYP1A1* inducibility.

CYP1A1 inducibility by PAHs can be measured in cultivated lymphocytes. Aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin-O-deethylase (EROD) assays were utilized to determine CYP1A1 activity in cultivated lymphocytes (Kellermann *et al.*, 1973; Jacquet *et al.*, 1997). However, a few problems exist in these assays. For example, the

AHH assay is not specific to CYP1A1 activity and the EROD assay is not sensitive enough to detect basal levels of CYP1A1 activity (Jacquet *et al.*, 1997). It is worthwhile to explore more accurate and specific quantitative assays for CYP1A1 induction in lymphocytes. It is well known that the PAHs induce CYP1A1 activity at the transcription level (Whitlock, 1999). Therefore, quantification of *CYP1A1* mRNA levels is a potential tool for measuring CYP1A1 inducibility in lymphocytes. In the present study, we developed the quantitative real-time RT-PCR assay to quantify the relative mRNA levels in cultivated lymphocytes. We propose that the expression levels of *AhR* and *Arnt* contribute to the differences in *CYP1A1* inducibility in humans. Utilizing this technique, we investigated whether the expression levels of *AhR* and *Arnt* correlated with inducibility of *CYP1A1* in cultivated peripheral lymphocytes isolated from 32 healthy subjects. Other factors, such as gender and cigarette smoking, were also considered. Information generated from this study will be helpful in the elucidation on the mechanism of interindividual variation in *CYP1A1* inducibility and expression. This technique was further utilized to evaluate whether CYP1A1 is a susceptibility factor for lung cancer in Taiwan.

三、結果與討論

The linear range of the quantitative real-time RT-PCR assay was determined by amplifying serial dilutions of cDNA converted from lung cancer cell lines NCI-H1355. The standard curve was examined by amplifying 4-fold serial dilutions of the cDNA using primers for *AhR, Arnt, CYP1A1* and *β-actin*. The standard curve is plotted by the log of the template dilution fold versus the Ct. The standard curve of *AhR*, *Arnt*, and *CYP1A1* were all parallel to that of *β-actin*, which indicated that the amplification efficiency of *β-actin* and target genes was equal.

The time course of gene expression in cultivated lymphocytes was determined after exposure to 12 μM BA for 0 to 3 days. Lymphocytes were isolated from four donors: two male smokers, one male nonsmoker and one female nonsmoker. As shown in Figure 1, *CYP1A1* levels were increased at day 1 and continued to increase within 3 days. *AhR* and *Arnt* levels remained consistent after BA treatment. Lymphocytes isolated from 2 of 4 subjects died on day 4 after BA treatment (data not shown). Therefore, we decided to measure the induction of *CYP1A1* expression on day 3 in the following study. However, We also compared gene expression levels in uncultured lymphocytes and 3-day cultivated lymphocytes. We found that *CYP1A1* levels were significantly lower in uncultivated lymphocytes than in cultivated lymphocytes (data not shown). However, *AhR*, and *Arnt* levels in uncultivated lymphocytes were not significantly different from those in cultivated lymphocytes (data not shown).

Isolated peripheral lymphocytes were treated with 0.1% DMSO or 12 μM BA for 3 days and then harvested to quantify the relative gene expression levels. Cell viability in DMSO- or BA-treated cells was respectively 85% or 75% (data not shown). The mRNAs of *AhR*, *Arnt*, and *CYP1A1* were all detectable in lymphocytes (Table 1). *CYP1A1* levels in DMSO-treated cells varied ~200 and 100 fold respectively. Individual variations of *AhR* and *Arnt* levels in DMSO-treated cells were respectively 50- and 13- fold. *CYP1A1* levels were significantly increased after BA treatment, but *AhR* and *Arnt* levels were not significantly changed. The average *CYP1A1* induction fold was 45.86.

The effects of smoking and gender on gene expression levels were evaluated by analyzing log-transformed data with Student's t test. The non-transformed data

was presented in Table 2. No significant difference in gene expression levels was found between male smokers and male nonsmokers, but *CYP1A1* inducibility was significantly higher in smokers than in nonsmokers. *AhR*, and *CYP1A1* levels in DMSO-treated cells from female nonsmokers were significantly higher than those from male nonsmokers. On the other hand, *CYP1A1* inducibility was significantly higher in males than in females. However, *Arnt* levels did not differ by gender or smoking status. Nonparametric analysis (Mann-Whitney U-test) for untransformed data was consistent with the above results (data not shown).

It is well known that AhR and Arnt regulate *CYP1A1* gene expression. Therefore, we further investigated whether *AhR* and *Arnt* levels are associated with expression levels and the inducibility of *CYP1A1*. Log-transformed data were analyzed with the Pearson correlation. We found that *Arnt* levels positively correlated with *AhR* levels in DMSO-treated cells (Figure 2). However, the correlation was not observed between *CYP1A1*, *Arnt* and *AhR* (data not shown). When subjects were stratified according to smoking status, *CYP1A1* inducibility positively correlated with *AhR* levels in DMSO-treated cells from smokers (Figure 2). The correlation between *CYP1A1* inducibility and *AhR* levels was not observed in nonsmokers (data not shown).

Since gene expression levels and inducibility differed by gender and smoking status (Table 2), the correlation between gene expression levels was further assessed by multiple linear regression. The effects of gender and smoking status were controlled in this model. During data analysis, we observed that cigarette smoking had significant interaction with *AhR* levels (Table3). The interaction meaned that smoking modified the association between *AhR* and *CYP1A1* levels in DMSO-treated cells and *CYP1A1* inducibility. Other interaction terms, such as AhR*gender, Arnt*gender and Arnt*smoking, were not significant in all models (data not shown). After controlling for gender, smoking and their interaction, *AhR* levels negatively correlated with *CYP1A1* levels in the DMSO-treated cells, but positively correlated with *CYP1A1* inducibility.

We further evaluated the association between *CYP1A1* expression and lung cancer risk with the logistic regression analysis. Gene expression levels were stratified into high versus low expressers As shown in Table 4, elders and smokers were significantly at higher risk of lung cancer. After age, smoking status and gender were controlled in the analysis, *CYP1A1* expression was not associated with lung cancer risk.

Discussion

CYP1A1 inducibility has been considered as a susceptibility factor for lung cancer and is usually determined by measuring enzyme activity, such as AHH or EROD (Kellermann *et al.*, 1973; Kiyohara *et al.*, 1998). Induction of *CYP1A1* is dependent on AhR activation (Whitlock, 1999). CYP1A1 inducibility was highly variable in human population. Therefore, we hypothesized that *AhR* and *Arnt* expression might count for interindividual variation in CYP1A1 inducibility. Our study showed that *CYP1A1* inducibility was affected by gender and cigarette smoking in cultivated lymphocytes. We also noticed that cigarette smoking had interaction with *AhR* levels and modified the association between *AhR* levels and *CYP1A1* inducibility. After controlling for gender, cigarette smoking, and their interaction, *CYP1A1* inducibility correlated with *AhR* levels in non-induced lymphocytes. These data partially support our hypothesis that the differences in *AhR* expression, but not *Arnt* expression, associate with individual variation in *CYP1A1* inducibility. In

the present study, we proposed that individual variation in *CYP1A1* expression would associate with the risk of lung cancer. However, our study showed that *CYP1A1* expression was not associated with lung cancer risk. In another study, we found that *AhR* expression was not associated with lung cancer risk either. Since *CYP1A1* inducibility correlated with *AhR* expression in cultivated lymphocytes, we predict that *CYP1A1* inducibility was not the risk factor for lung cancer in Taiwan.

四、計畫成果自評

本計劃部分研究成果已被 Toxicologic Sciences 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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183-192.

Table 1. Gene expression levels and inducibility of *AhR, Arnt,and CYP1A1* in DMSO- and BA-treated lymphocytes.

	Male		Female
	Smokers	Nonsmokers	Nonsmokers
Subject numbers	12	10	10
DMSO-treated cells			
AhR	1.83 ± 1.38[a]	1.14 ± 0.72	3.16 ± 2.03[c]
Arnt	4.75 ± 2.78	5.05 ± 4.18	3.67 ± 2.37
CYP1A1	0.18 ± 0.47	0.08 ± 0.08	0.53 ± 0.67[c]
BA-treated cells			
AhR	1.21 ± 0.99	1.38 ± 1.03	2.01 ± 1.24
Arnt	4.50 ± 3.66	3.92 ± 3.39	6.19 ± 4.44
CYP1A1	3.97 ± 5.72	1.65 ± 1.01	1.26 ± 0.70
Inducibility			
CYP1A1	87.77 ± 83.11[b]	31.31 ± 21.52	10.12 ± 2.75[c]

[a] Mean ± standard deviation of relative gene expression in the original scale.

[b] Range of relative gene expression in the original scale.

[c] Compared with DMSO-treated cells, p<0.05, Student's t test.

Table 2. Gene expression levels and inducibility in lymphocytes after stratified by gender and cigarette smoking status

	Treatment		
Genes	DMSO	BA	Inducibility
AhR	2.03 ± 1.64[a]	1.51 ± 1.11	-
	(0.12 - 6.50)[b]	(0.11 - 3.63)	
Arnt	4.53 ± 3.12	04.84 ± 3.83	-
	(4.53 - 10.38)	(0.67 - 14.31)	
CYP1A1	0.26 ± 0.50	2.40 ± 3.69 [c]	45.86 ± 61.59
	(0.01 - 2.07)	(0.33 - 21.46)	(0.24 - 282.09)

[a] Mean ± standard deviation of gene expression or inducibility.

[b] Comparison between male nonsmokers and male smokers, p< 0.05, Student's t test in natural logarithm transformed scale.

[c] Comparison between male nonsmokers and female nonsmokers, p< 0.05, Student's t test in natural logarithm transformed scale.

Table 3. Associations between *AhR* and *Arnt*, respectively, and *CYP1A1* and *CYP1B1* gene expression levels and induciblity, controlling for gender and cigarette smoking status in the multiple linear regression analysis

Dependent variable	Independent variable [a]	Parameter estimate (se)[b]	p value for *AhR* or *Arnt*
In DMSO-treated cells			
CYP1A1	*AhR*[c]	-0.55 (0.13)	< 0.01
CYP1A1	*Arnt*	-0.04 (0.04)	0.42
In BA-treated cells			
CYP1A1	*AhR*	-0.06 (0.06)	0.27
CYP1A1	*Arnt*	-0.01 (0.02)	0.64
CYP1A1 inducibility	*AhR* in DMSO-treated cells[c]	0.25 (0.11)	0.02[d]
CYP1A1 inducibility	*Arnt* in DMSO-treated cells	0.02 (0.03)	0.46[d]

[a] Other independent variables included in the model were smoking and gender.

[b] Parameter estimate (standard error) in natural logarithm transformed scale.

[c] Adjusted for smoking and gender, and included the interaction term AhR*smoking in the model. P value for AhR*smoking in this model was <0.05.

[d] P value for gender in this model was <0.05.

Table 4. Logistic regression analysis of *CYP1A1* expression for lung cancer risk.

Factors	n[a] (patients / controls)	OR (95% C.I.)[b]
Age	44 / 59	1.11 (1.05-1.18)[c]
Smoking		
No	22 / 48	1.00
Yes	22 / 11	3.20 (1.04-9.84)[c]
Gender		
Male	32 / 30	1.00
Female	12 / 29	1.29 (0.44-3.75)
CYP1A1 expression levels		
Low	19 / 33	1.00
High	25 / 26	1.17 (0.62-2.23)

[a] Subject numbers

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

[c] $P < 0.05$

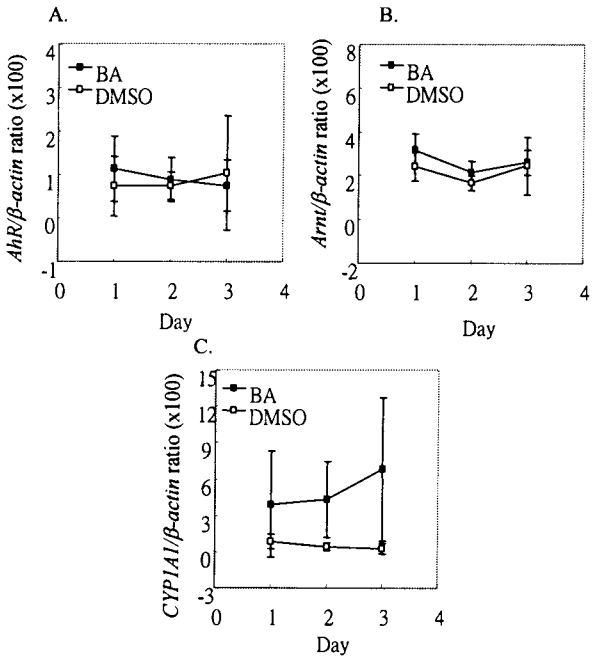

Figure 1. Time course of gene expression levels in DMSO- and BA-treated lymphocytes. Peripheral lymphocytes isolated from four donors were cultivated in the presence of 0.1% DMSO or 12 μM BA for 1, 2 and 3 days. The relative gene expression levels of (A) *AhR*, (B) *Arnt*, and (C) *CYP1A1* were determined with the quantitative real-time RT-PCR assay. The relative gene expression levels were normalized with those of *β-actin* and calculated from $2^{-\Delta Ct}$.

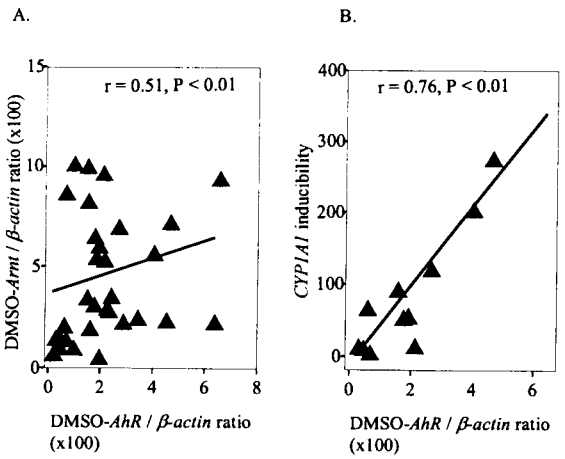

Figure 2. Correlation of gene expression levels of *AhR, Arnt, CYP1A1, CYP1B1*, inducibility of *CYP1A1* and *CYP1B1* in lymphocytes. Gene expression levels were normalized with those of *-actin* and calculated from $2^{-\Delta Ct}$ as described in Materials and Methods. Inducibility was the fold change in gene expression levels of BA- versus DMSO-treated cells. These data were fitted using linear regression analysis. In DMSO-treated cells, *AhR* levels correlated with (A) *Arnt* level and (B) *CYP1A1 inducibility*.