

行政院國家科學委員會專題研究計畫 成果報告

多環芳香烴受器與細胞色素 P450 1B1 用於大腸直腸癌及肺 癌早期診斷之研究 研究成果報告(精簡版)

計畫類別：個別型
計畫編號：NSC 95-2320-B-040-013-
執行期間：95年08月01日至96年07月31日
執行單位：中山醫學大學醫學系病理學科

計畫主持人：張菡

計畫參與人員：碩士級臨時工：蔡宛霖、江惠卿

處理方式：本計畫可公開查詢

中華民國 96 年 10 月 12 日

行政院國家科學委員會補助專題研究計畫 成果報告
 期中進度報告

多環芳香烴受器與細胞色素 P4501B1 用於大腸直腸癌與肺癌早期診斷之研究

計畫類別： 個別型計畫 整合型計畫

計畫編號：NSC 95-2320-B-040-013

執行期間：2006 年 08 月 01 日至 2007 年 07 月 31 日

計畫主持人：張菡

共同主持人：林嬪嬪

計畫參與人員：蔡宛霖、江惠卿

成果報告類型(依經費核定清單規定繳交)： 精簡報告 完整報告

執行單位：中山醫學大學

中 華 民 國 九 十 六 年 十 月 參 日

Introduction

Lung cancer has been the major cause of Taiwanese cancer death since 1982 [1, 2]. There are about 5,000 due to lung cancer as well as 6,000 new cases of lung cancer per year [1, 2]. By contrast, the 5 year survival rates for lung cancers are significant higher in early stages (Stage I) than advanced stage (Stage IV) in Taiwan [3]. Thus, early diagnosis is very important for improving the prognosis of lung cancers. In other words, a good detection test for early lung cancers is needed.

AhR signaling pathway is a major pathway for xenobiotic and drug metabolism in human. AhR is a basic helix-loop-helix (bHLH) protein belonging to the Per-Arnt-Sim (PAS) family of transcription factors. While ligand binding, AhR sheds the chaperon proteins and translocates to the nucleus, where it forms a heterodimer with the AhR nuclear translocator (ARNT). This heterodimer binds to the xenobiotic response element (XRE) of human CYP1A1, CYP1A2, and CYP1B1 genes activating transcription [4]. AhR ligands include polycyclic aromatic hydrocarbons (PAHs, BaP as representative), dioxin (2,3,7,8-Tetrachlorodibenzo-p-dioxin, TCDD, as representative) and cigarette smoke, which are carcinogenic for lung cancers [5-7]. In human, epidemiological evidence showed that the carcinogenic risk associated with TCDD exposure was increased for all cancers combined including non-Hodgkin's lymphoma, soft tissue sarcoma, rectal cancer and lung cancer [8]. Accordingly, AhR signaling pathway might involve the human carcinogenesis.

Lung cancers are clinically categorized into small cell cancers and non-small cell lung cancers. Squamous cell carcinoma and adenocarcinoma are two major types of histology. Concerning the tumorigenesis of human lung cancers, squamous cell carcinoma and adenocarcinoma are different. The progression of squamous cell carcinoma is recognized through squamous metaplasia, dysplasia and carcinoma in situ in the bronchi. Adenocarcinomas locate mostly in lung periphery. Peripheral lung adenocarcinomas are considered to be derived from type II pneumocytes or Clara cells, based on the phenotype of neoplastic cells [9, 10]. Kitamura and co-workers [10] reported that atypical adenomatous hyperplasia (AAH), as a precancerous lesion, was thought to be the adenoma in a putative "adenoma-carcinoma" sequence, and lead to the development of bronchioloalveolar carcinoma (BAC) and invasive adenocarcinoma [11]. Thus, AAH-BAC-adenocarcinoma may be a progression model of peripheral adenocarcinomas. This progression model may be used to understand the genetic alterations in multistage development of peripheral lung adenocarcinoma.

Sputum cytology and chest x-ray/spiral computed tomography (CT) are usually used to screen lung cancers, subsequently diagnosed by bronchoscopic or imaging-guided biopsy. Traditional sputum cytology is used to examine the malignant cells based on the cell morphology. Routine chest x-ray screening is often unable to detect lung cancers until they are of advanced stages. By spiral CT, studies found that non-calcified pulmonary nodules were 3-fold easily detected, compared with by chest radiography according to the tumor size and vasculization [12]. However, these detection techniques for lung cancers showed the high specificity and low sensitivity [13]. Detection of lung cancer-related antigens in the blood, such as carcino-embryonic antigen, failed to be of use in early detection [14]. Furthermore, detections of blood DNA levels, gene silencing and overexpression were investigated for early diagnosis of lung cancers in recent studies [14, 15]. Of these, aberrant gene methylation for gene

silencing is a highlighted method. In general, the expression of a gene is controlled through methylation of the cytosine phosphate guanosine rich regions (CpG islands) of the chromosome [16]. CpG islands are usually hypomethylated and often linked to promoter regions of genes [17]. However, an aberrant methylation in one of CpG islands induces a silencing of the gene and is associated with some cancers [18-22]. In the initiation and progression of lung cancer, DNA hypermethylation contributed to inactivate some tumor suppressor genes, including p16, p15, tissue inhibitor of metalloprotease (TIMP)-3 and glutathione S-transferase P1 (GSTP1) [14]. By contrast, CYP1B1-promoter hypomethylation lead to up-regulation of CYP1B1 in prostatic adenocarcinomas [23]. The methylation-specific PCR assay enabled the rapid identification of genes that were methylated in cancers. This assay also revealed the higher sensitivity and specificity than traditional screen tests for lung cancer detection [24, 25]. Our previously studies showed overexpression of CYP1B1 in bronchioloalveolar carcinoma and adenocarcinomas of lungs using immunohistochemistry [26, 27]. By contrast, AhR and CYP1B1 expressions were limited in normal epithelia of lung. Therefore, these differential expressions of AhR and CYP1B1 between neoplasms and normal epithelia could be used as screening biomarkers for lung cancers. Based on AhR-dependent xenobiotic metabolism, AhR gene mutations might affect the AhR activation and expressions [28]. As well, aberrant methylation in CYP1B1 promoter might affect CYP1B1 expression in cancer cells. We hypothesize the kinetic differences between normal and premalignant/cancer cells and these differences affect gene expressions of AhR and CYP1B1. Thus, this genetic change is thought to be feasible as screening biomarkers for lung cancers.

Material and Methods

Cell and Cell culture

NCI-H1355 (American Cell Type Cell Collection, Manassas, VA) was used in this study. H1355 cells were a gift from Dr. C-M. Tsai (Veterans General Hospital-Taipei, Taiwan, ROC) maintained in RPMI1640 medium containing 10% FBS. The cells were incubated in a 37°C incubator with a humidified mixture of 5% CO₂ and 95% air.

Human tissues and DNA extraction

28 frozen unfixed lung cancer tissues were used to determine the mutation status of AhR ligand binding domain. Of them, 18 lung tissues were also assayed by AhR immunohistochemistry for investigating the correlation between AhR expression and mutation status of AhR ligand binding domain. 18 formalin-fixed, paraffin-embeded lung cancer tissues were used to microdissect the tumor and non-tumor tissues in the same section and DNA was isolated for CYP1B1 methylation analysis. Genomic DNA was extracted using QIAGEN™ DNeasy tissue kit. In order to avoid the cross-contamination of the samples for CYP1B1 methylation analysis, we changed the microtome blade and cleaned the related experiments for each sample. 2 slices of 10µm thick tissues was deparaffinized and rehydrated. The DNA-containing fractions were digested with proteinase K (500µg/ml) for overnight in a buffer containing 100 mM Tris-HCl and 10 mM EDTA (pH 8.0), and followed by phenol/chloroform extraction and ethanol precipitation. The concentration of total DNA will be quantified by spectrophotometry (OD 260/280). All

genomic DNA were stored at -20°C until used.

Mutation of AhR ligand bind domain

Ligand-binding domain of AhR contains two portions, PAS1 71 amino acids (111-181 aa) and PAS2 68 amino acids (275-342 aa). Compared to AhR DNA, 4 fragments of AhR DNA were analyzed. We designed 4 sets of primers (Table 1) for these DNA fragments which then sequencing. Finally, we examined these DNA sequences of lung tumor to compare with constitutive AhR (NC000007).

AhR immunohistochemistry

AhR immunohistochemistry was described as our previous reports. 5-µm thick paraffin sections (cases as the same as study for mutation status of AhR ligand binding domain) were deparaffinized and rehydrated. The primary antibody was anti-AhR (1:90 dilution; Biomol, Plymouth Meeting, PA). After incubation with the primary antibodies overnight at 4°C, a streptavidin-biotin peroxidase method was carried out according to the manufacturer's instructions (Universal LSAB2 kit, DakoCytomation, Glostrup, Denmark). Finally, these sections were counterstained with hematoxylin. The hyperplastic prostate sections were used as positive controls. When AhR staining intensity of lung tumor was stronger than that of prostate, such lung tumor was rated as a high expresser; inversely lung tumor showing less intensity than prostate was scored as a low expresser.

Methylation PCR (MSP)

DNA from lung cancer sections were modified with sodium bisulfite to convert unmethylated Cs to Us using a DNA modification kit (Intergen, Oxford, United Kingdom), and subjected to PCR amplification. According to the functional promoter/enhancer sequence of the CYP1B1, CYP1B1 enhancer sequence (-1185— -709) contains 57 CpG sites including 5 DREs and 2 Sp1 sites. The promoter region contains 24 CpG sites including 2 Sp1 sites, a TATA-like box, and the transcription ignition site. Primers (Table 1) were designed using methprimer (<http://www.urogene.org/methprimer>) to detect the methylation status of CpG sites. PCR were performed by 2 sets of primers used to amplify methylated (M) and unmethylated (U) allele respectively. PCR products are separated by 2% agarose gel electrophoresis and stained with ethidium bromide. The density of each band was calculated by Image J software (<http://rsb.info.nih.gov/>). The methylation level in each sample will be determined using the following formula: $MSP\ ratio\ (\%) = \frac{MSP}{MSP + USP} \times 100$. A MSP ratio of 5% was used as cutoff value to assess whether samples is MSP positive or negative.

.Polymerase Chain Reaction (PCR) and DNA Sequencing

Each PCR reaction was performed in a total volume of 50µl with 1.25 unit of EX Taq DNA polymerase (TaKaRa). The initial step of PCR was 95°C 4 min and cycles of denaturation at 95°C 45 sec, annealing at 50°C to 61°C 1 min and elongation at 72°C 1 min. The final extension at 72°C 4 min was made for termination. The templates contain 0.5µg genomic DNA for 32 to 45 cycles of the PCR. Contaminated controls consist of samples amplified in absence of template DNA. All PCR products for AhR ligand

binding domain and CYP1B1 promoter methylation were sequenced.

Results and Discussion

Aberrant methylation of CYP1B1 promoter was observed in human lung tumor and non-tumor tissues (Figure 1).

The methylation status of the *CYP1B1* promoter gene was studied in 10 lung cancer samples and 8 non-cancerous samples. We found the hypomethylation of CYP1B1 promoter in lung cancer tissues when compared with non-cancerous tissues. Because sample size was small, no conclusive results could be explored. However, the result showed the clue that the aberrant methylation of CYP1B1 promoter was evident.

Mutations in AhR ligand-binding domains in lung tumors (Table 2).

As shown in Table 3, the 28 human lung tumors included 1 benign tumor and 27 malignant carcinomas. 7% (2/28) of studied tumors contained mutations in AhR ligand binding domain. Only one mutation type was found: 36275 ins C, however, it seemed to show no association with histology type of lung tumor. Because sample size was small, this result might represent a clue that mutations of AhR ligand binding domain were not common and controversy significance in human lung tumors.

Correlation between mutation status of AhR ligand binding domain and AhR overexpression in lung tumors (Table 3, figure 2)

As shown in Table 4, 14 of 18 (78%) lung carcinomas showed no mutation of AhR ligand binding domain but AhR overexpressions. 2 lung carcinomas contained AhR mutations but showed low levels of AhR expression. Moreover, our previous reports have demonstrated that the frequency of AhR overexpression is about 60% to 70% in lung carcinoma, especially in adenocarcinomas; AhR overexpression was considered to be associated with lung cancer development [29]. Therefore, the levels of AhR expressions were not correlated with mutations of AhR ligand-binding domains, although one report has suggested that mutation of AhR ligand-binding domain affected the binding between ligands and AhR, resulting in AhR overexpression in hepatoma cells [28]. In this study, our results cannot support such mechanism affecting AhR overexpression in lung carcinomas. However, the other possibility for AhR overexpression has been proved that the methylation status of AhR promoter was associated with the levels of AhR expression in leukemia cells [30] Thus, epigenetic alteration of AhR promoter would be the other possible mechanism for AhR overexpression. Detailed mechanism needs to be further investigated.

Table 1. Primer sequences in this study

Primer labeling	Sense primer	Antisense primer	Anneal temp., °C (cycle no.)	Product size (bp)
For AhR ligand-binding domains				
PAS1-1	TGGCTGTGTTTGTGAAAT GT	AATGGCCAACTGTGTCCTA A	57 (35)	299
PAS1-2	TCATCACCCTAGCAAGC AC	CAGCCTCCTTTCACAGACT C	57 (35)	296
PAS2-1	ACTTCCACCTCAGTTGGC TT	AGGCTACACTGGAAGAATG T	57 (35)	275
PAS2-2	TGCAGAACTAGCGTAAA AC	ATGCCGTTTTCTTCTGACAT	59 (35)	229
For CYP1B1 promoter				
Pan5	GATTGGAGGTGGTTGTGA TGAAG	CCACTCCCCTCCAAAATC AAAAC	50 (45)	256
MS5	AGTTTCGAGGTCGGTCGG TGC	AACAACGCGACCTAACAAA ACG	59 (38)	106
US5	GTAGAGTTTTGAGGTTGG TTGGTGT	AAAACAACACAACCTAACA AAACAA	61 (32)	112

Table 2. Frequency of AhR mutation in human lung tumors

	Number	Mutation number (%)	Mutation Type
Total	28	2 (7)	
Tumor type			
AD	18	1 (5)	36275 insC
SQ	7	1 (14)	36275 insC
ADSQ	1	0 (0)	
Large CC	1	0 (0)	
Benign	1	0 (0)	

Table 3. AhR overexpression and AhR mutation type in 18 nonsmall cell lung cancers

AhR-LBD Mutation/AhR Expression	Number (%)
Positive/High	0 (0)
Positive/Low	2 (11)
Negative/High	14 (78)
Negative/Low	2 (11)

Figure 1. Methylation frequency of the CYP1B1 promoter region. Methylation frequency was significantly lower in lung tumor sample as compared with non-tumor samples (n = 10, p < 0.001).

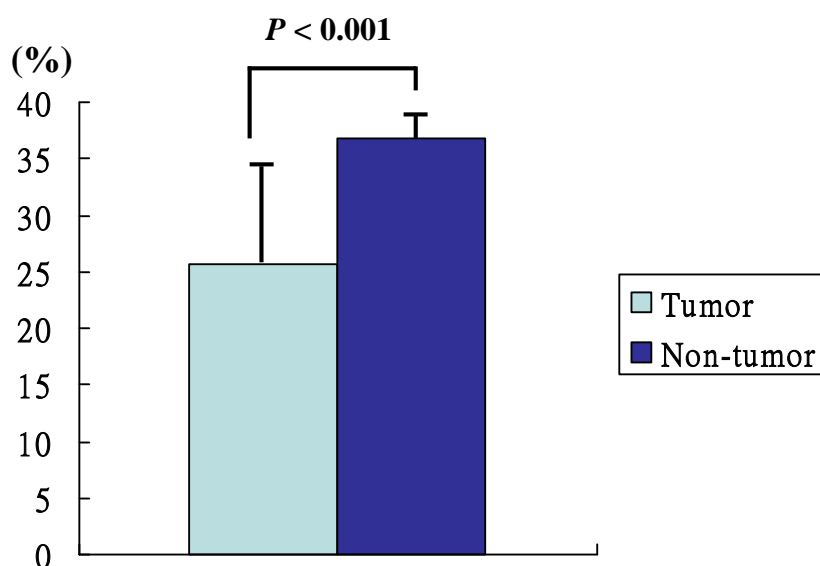
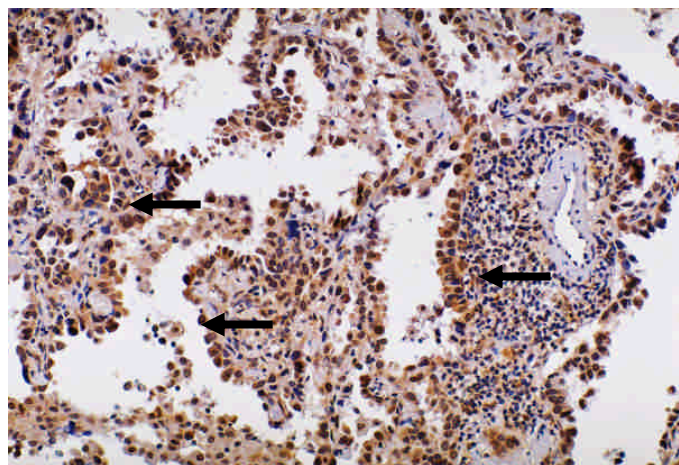


Figure 2. AhR overexpression in human lung adenocarcinoma (arrow). Immunohistochemistry, X200.



References

1. Chen, C.J., et al., *Cancer epidemiology and control in Taiwan: a brief review*. Jpn J Clin Oncol, 2002. **32 Suppl**: p. S66-81.
2. Statistics, H., *Republic of China: Health Statistics. Vol. II. Vital statistics, 1981-1992.*, D.o.H. Taipei, Editor. 2002.
3. Ger, L.P., et al., [*Risk factors of lung cancer*]. J Formos Med Assoc, 1992. **91 Suppl 3**: p. S222-31.
4. Rowlands, J.C. and J.A. Gustafsson, *Aryl hydrocarbon receptor-mediated signal transduction*. Crit Rev Toxicol, 1997. **27**(2): p. 109-34.
5. van Agen, B., et al., *B[a]P-DNA adduct formation and induction of human epithelial lung cell transformation*. Environ Mol Mutagen, 1997. **30**(3): p. 287-92.
6. Hecht, S.S., *Tobacco smoke carcinogens and lung cancer*. J Natl Cancer Inst, 1999. **91**(14): p. 1194-210.
7. Shimizu, Y., et al., *Benzo[a]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor*. Proc Natl Acad Sci U S A, 2000. **97**(2): p. 779-82.
8. Bodner, K., et al., *Cancer risk for chemical workers exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin*. Occup Environ Med, 2003. **60**: p. 672-675.
9. Mori, M., et al., *Comparative ultrastructural study of atypical adenomatous hyperplasia and adenocarcinoma of the human lung*. Ultrastruct Pathol, 1998. **22**(6): p. 459-66.
10. Kitamura, H., et al., *Cytodifferentiation of atypical adenomatous hyperplasia and bronchioloalveolar lung carcinoma: immunohistochemical and ultrastructural studies*. Virchows Arch, 1997. **431**(6): p. 415-24.
11. Terasaki, H., et al., *Lung adenocarcinoma with mixed bronchioloalveolar and invasive components: clinicopathological features, subclassification by extent of invasive foci, and immunohistochemical characterization*. Am J Surg Pathol, 2003. **27**(7): p. 937-51.
12. Henschke, C.I., et al., *Computerized tomography screening for lung cancer: new findings and diagnostic work-up*. Semin Thorac Cardiovasc Surg, 2003. **15**(4): p. 397-404.
13. Manser, R., *Screening for lung cancer: a review*. Curr Opin Pulm Med, 2004. **10**(4): p. 266-71.
14. Brambilla, C., et al., *Early detection of lung cancer: role of biomarkers*. Eur Respir J Suppl, 2003. **39**: p. 36s-44s.
15. Sozzi, G., et al., *Analysis of circulating tumor DNA in plasma at diagnosis and during follow-up of lung cancer patients*. Cancer Res, 2001. **61**(12): p. 4675-8.
16. Paulsen, M. and A.C. Ferguson-Smith, *DNA methylation in genomic imprinting, development, and disease*. J Pathol, 2001. **195**(1): p. 97-110.
17. Antequera, F. and A. Bird, *Number of CpG islands and genes in human and mouse*. Proc Natl Acad Sci U S A, 1993. **90**(24): p. 11995-9.
18. Ribeiro-Filho, L.A., et al., *CpG hypermethylation of promoter region and inactivation of*

- E-cadherin gene in human bladder cancer.* Mol Carcinog, 2002. **34**(4): p. 187-98.
19. Li, L.C., et al., *Methylation of the E-cadherin gene promoter correlates with progression of prostate cancer.* J Urol, 2001. **166**(2): p. 705-9.
 20. Li, L.C., et al., *Frequent methylation of estrogen receptor in prostate cancer: correlation with tumor progression.* Cancer Res, 2000. **60**(3): p. 702-6.
 21. Toyota, M., et al., *Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype.* Cancer Res, 1999. **59**(21): p. 5438-42.
 22. Toyota, M., et al., *Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification.* Cancer Res, 1999. **59**(10): p. 2307-12.
 23. Tokizane, T., et al., *Cytochrome P450 1B1 is overexpressed and regulated by hypomethylation in prostate cancer.* Clin Cancer Res, 2005. **11**(16): p. 5793-801.
 24. Belinsky, S.A., *Gene-promoter hypermethylation as a biomarker in lung cancer.* Nat Rev Cancer, 2004. **4**(9): p. 707-17.
 25. Soria, J.C., et al., *Aberrant promoter methylation of multiple genes in bronchial brush samples from former cigarette smokers.* Cancer Res, 2002. **62**(2): p. 351-5.
 26. Lin, P., et al., *Association of aryl hydrocarbon receptor and cytochrome P4501B1 expressions in human non-small cell lung cancers.* Lung Cancer, 2003. **42**: p. 255-261.
 27. Chang, J.T., et al., *Requirement of aryl hydrocarbon receptor overexpression for CYP1B1 up-regulation and cell growth in human lung adenocarcinomas.* Clin Cancer Res, 2007. **13**(1): p. 38-45.
 28. Sun, W., J. Zhang, and O. Hankinson, *A mutation in the aryl hydrocarbon receptor (AHR) in a cultured mammalian cell line identifies a novel region of AHR that affects DNA binding.* J Biol Chem, 1997. **272**(50): p. 31845-54.
 29. Lin, P., et al., *Overexpression of aryl hydrocarbon receptor in human lung carcinomas.* Toxicologic Pathology, 2003. **31**: p. 22-30.
 30. Mulero-Navarro, S., et al., *The dioxin receptor is silenced by promoter hypermethylation in human acute lymphoblastic leukemia through inhibition of Sp1 binding.* Carcinogenesis, 2006. **27**(5): p. 1099-104.