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多環芳香烴受器與細胞色素 P4501B1 用於肺癌診斷及預防之研究(第 3 年)
研究成果報告(完整版)

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Introduction

Lung cancer is an important cause of cancer death worldwide [1, 2]. The tumor histology of lung cancer is clinically categorized into nonsmall cell carcinoma and small cell carcinoma [3]. About 80 to 85% of lung cancers belong to the nonsmall cell carcinoma, for which adenocarcinoma (AD) and squamous cell carcinoma (SQ) are two major types [4, 5]. Previously, we reported that cytochrome P450 1B1 (CYP1B1) was overexpressed in lung carcinomas, particularly AD, in comparison with that in normal lung tissues [6]. Some earlier studies also proved the higher expression of CYP1B1 for tumor cells, including lung cancer, than for corresponding non-tumor cells [7-9]. Furthermore, advanced lung cancers with CYP1B1 overexpression were more common than early lung cancers [10]. As our previous studies have shown regarding CYP1B1 being overexpressed in nonsmall cell carcinomas [11, 12], the underlying mechanisms appear to remain poor.

CYP1B1 is a well documented xenobiotic metabolizing enzyme and possesses the ability to biotransform some promutagens or procarcinogens that are involved in the cancer development via aryl hydrocarbon receptor (AhR)-aryl hydrocarbon receptor nuclear translocator (ARNT) pathway [13, 14]. AhR is a ligand-activated transcription factor. 2,3,7,8-Tetrachloro-dibenzo-p-dioxin (TCDD) is a potent AhR ligand, which has demonstrated to be one of etiologies in the development of lung cancer [15]. While ligand binding, AhR interacts with ARNT to form a heterodimer of AhR/ARNT which binds the sequence motifs containing dioxin response element (DRE) and then activates gene transcription including CYP1B1. Clearly, the gene expression is determined by the DNA-binding transcription factors that select specific DNA sequences for transcriptional activation or repression in its promoter region [16]. Thus, the factors may be proposed to affect CYP1B1 gene expression, including chromatin structure in transcription factor-bound forms, methylation level in promoter and/or DRE core sequence and the amounts of AhR/ARNT.

The expression of a gene is controlled through methylation of the cytosine phosphate quanosine (CpG)-rich regions of the chromosome, often linked to promoter regions of genes [17, 18]. Moreover, a body of researches showed the evidence that the promoter hypermethylation repressed the gene expression, particularly tumor suppressor genes, involved in lung carcinogenesis [19-21]. A series of animal studies and cell models for investigating the Ha-*ras* expression showed the evidence that hypomethylation of DNA may contribute the aberrant expression of protooncogenes involved in carcinogenesis [22-25]. Current researches showed the evidence that hypomethylation of CYP1B1 promoter might upregulate its expression in carcinomas of prostate and colorectum [26, 27]. Therefore, we suppose that CYP1B1 overexpression may be associated with aberrant methylation of CYP1B1 promoter in lung carcinomas.

In order to study the relationship between methylation levels of CYP1B1 promoter and CYP1B1 expression, we used five human lung cell lines including four lung carcinomas (Calu-1, H23, H226 and H1355) and one bronchial epithelial cell line (BEAS-2B) to investigate their methylation levels in the presence and absence of 5-AzaC and mutations of DRE core sequences in CYP1B1 promoter. In addition, we also used the 35 pairs of lung carcinoma cases, i.e., 35 lung carcinomas paired with their non-neoplastic lung tissues, to further investigate the methylation levels of CYP1B1 promoter region and CYP1B1 immunohistochemical expression. Based upon such information as generated from human tissues and cell models, we are able to further understand the relationship between molecular alterations and CYP1B1 expression in human lung carcinomas.

Material and Methods

Chemicals

5-Azacytidine (5-AzaC) and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). TCDD was purchased from AccuStandard Inc. (New Haven, CT). RPMI1640 medium, F12 medium, Laboratory of Human Carcinogenesis (LHC-9) medium and Dulbecco's MEM (DMEM) medium were purchased from Invitrogen (Carlsbad, CA).

Cell culture

Five human lung cell lines BEAS-2B, Calu-1, H23, H226 and H1355 were used in this study. H1355 cell was a gift from Dr. C-M Tsai (Veterans General Hospital-Taipei, Taiwan, ROC). Information for other cell lines was obtained from the American Cell Type Cell Collection (Manassas, VA). Calu-1 and H226 were lung squamous cell carcinoma. H1355 and H23 were lung adenocarcinomas. BEAS-2B was a SV-40 transformed, human bronchial epithelial cell line which was maintained in serum-free LHC-9 medium. Calu-1 cell was maintained in DMEM medium supplemented with 10 % heat-inactivated fetal bovine serum. H1355, H23 and H226 cells were maintained in RPMI 1640 supplemented with 5 % heat-inactivated fetal bovine serum. All cells were incubated in a 37°C incubator under a humidified atmosphere of 5% CO2 and 95% air.

5-azacytidine (5AzaC) and TCDD treatments of human cells

5-AzaC is a potent growth inhibitor which inhibits DNA methyltransferase, a regulatory epigenetic mechanism of gene expression, activation and silencing [28]. The effect of 5-AzaC was investigated on lung cell lines if CpG methylation was involved in the expression of CYP1B1 gene. When cells were grown to 70% confluence in 6-cm dishes, cells were treated with 10 μM 5-AzaC or not dissolved in culture medium for 7 days. On the 6th 5-AzaC-treated day, the culture medium was changed and cells treated with 1 nM TCDD or 0.01% DMSO (control). Our previous study [29] in human lung cell cultures showed that 1 nM TCDD is the minimal dose required to significantly increase CYP1B1 expression. After 24-h incubation, total RNA was extracted from harvested cells for reverse transcription (RT) and real-time polymerase chain reaction (PCR) assay, and genomic DNA for methylation-specific PCR (MSP) assay.

Reverse transcription and Quantitative real-time PCR assay

Total RNA was isolated from studied cell lines by using a TRIZOL reagent (Life Technologies, Rockville, MD) and a phenol-chloroform extraction method. In the step of reverse transcription, cDNA was reversely transcribed from 2 μg of total RNA sample using random primers of the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). cDNA samples were used to perform RT-PCR and guantitative real-time PCR assays. Quantitative real-time PCR was used by the TagMan Universal PCR Master Mix (Applied Biosystems) and analyzed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The PCR products were synthesized from cDNA samples using the PCR master mix. The specific primers and probes for CYP1B1 (Hs00164383) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs99999905) genes were purchased from Applied Biosystems. The PCR reactions consisted of an initial step for 2 min at 50°C, a polymerase activation step for 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Quantitative values were analyzed by the threshold cycle (C_T) number of *CYP1B1* gene expression levels in each sample normalized to *GAPDH* mRNA level. The relative mRNA levels of the *CYP1B1* gene = $2^{-\Delta Ct}$, $\Delta Ct = Ct_{CYP1B1} - Ct_{GAPDH.}$

Human lung tissues, microdissection and DNA extraction

Human lung tissue samples were collected in accordance with Institutional Review Board-approved protocols at Chung Shan Medical University Hospital (Taiwan). Thirty-five lung carcinomas paired with corresponding non-neoplastic lung tissues were used to microdissect the tumor and non-tumor cells on the sections. Three slices of 10 μm-thick lung tissues were deparaffinized and rehydrated, followed by a hematoxylin and eosin (H-E) staining. While observing the H-E sections through a microscope, the cell populations of tumor and non-tumor cells were gently scraped with a toothpick (sterilized before using). The dissected cells were detached from the slides and formed small dark clumps of tissues that were carefully collected into a clean 1.5-ml centrifuge tube. The DNA-containing fractions were digested with proteinase K (500 μg/ml) in a buffer containing 100 mM Tris-HCl and 10 mM EDTA (pH = 8.0) for overnight at 37°C. In addition, genomic DNA of studied cell lines was digested by a genoMarker reagent (Gunepure, Taiwan) for 30 min at room temperature. By following, all studied DNA samples were purified by phenol/chloroform extraction and ethanol precipitation. The concentration of total DNA in each sample was quantified by a spectrophotometry (OD 260/280). All genomic DNAs were stored at -20℃ until used.

MSP assay and DNA sequencing

The MSP assay enabled the rapid identification of genes that were methylated in cancers [30, 31]. DNA (300 ng to 500 ng) was modified with sodium bisulfite to convert unmethylated Cs to Us using a methylSEQr Bisulfite Conversion kit (Applied Biosystems), and subjected to nested PCR amplification. The CpG sites up to 900 bp upstream of CYP1B1 promoter divided into four fragments, each labeled as S2, S3 and S5, was amplified by nested PCR. Fragment S2 (nt -859 to -734), partially overlapped with fragment S3 (nt -877 to -734), contains two DRE core sequences and two specific protein 1 (SP1) sites. Fragment 5 (nt -160 to +12) contains two SP1 sites. In the first PCR, there were three sets of primers covering promoter region as follows: 5'-GGGTGGTGGTTTAAG/GTT-3' and 5'-ACTCCAATCATATCCCTAAAC-3' covering fragment S1; 5'-GTTTAGGGATATGATTGGAGT-3' and 5'-ACTAAAAAAACCTAAAAAAACTAAC-3' covering fragments S2/S3, and 5'-GATTGGAGGTGGTTGTGATGAAG-3' and 5'-CCACTCCCACTCCAAAATCAAAAC-3' covering fragment S5. The second PCRs in each region were performed by 2 sets of primers used to amplify methylated (M) and unmethylated (U) allele, respectively. The sequences of M and U primers and PCR conditions for *CYP1B1* promoter used have been reported by Tokizane and colleagues [26]. Genomic DNA treated with SssI methyltransferase (New England Biolabs, Beverly, MA, USA) was used as a positive control. Water instead of DNA template was used as protocol negative control.

PCR products were separated by 10% acrylamide gel electrophoresis and stained with ethidium bromide. The density of each band was calculated by Image J software (http//rsb.info.nih.gov/ij). The methylation level in each sample was determined using the following formula: MSP ratio (%) = density of M band x 100 / density of (M+U) bands. To avoid the interindividual variation, we used the methylation index (T/NT) calculated by the MSP ratio of lung carcinoma (T) over that of corresponding non-neoplastic lung tissue (NT) for 35 pairs of human lung tissues. When the methylation index was less than or equal to 1, the methylation status of the carcinoma case was referred as a hypomethylated state in CYP1B1 promoter; while that was greater than 1, the carcinoma case was referred as a hypermethylated state.

For confirming the DNA integrity and examining the occurrence of mutations in DRE core sequences and Sp1 sites, the M and U PCR products were loaded onto 1.5% low melting agarose gel in TBE buffer. The DNA

fragments in the low melting agarose gel were purified with a gel elution kit (GeneMark, Taichung, Taiwan) and directly sent for DNA sequencing using ABI 3100 sequencer (Applied Biosystems).

Immunohistochemistry

Thirty-five lung tumor tissues studied in the MSP assay were constructed as a tissue microarray (TMA) and also performed by CYP1B1 immunohistochemistry. Clinical features of the 35 samples, each composed of gender, tumor histology, smoking history and cancer staging at diagnosis [3], and were verified from the hospital medical records. Smoking history was determined as either yes or no. No smoking history was defined as subjects who had never smoked previously. Individuals who had currently or ever smoked regardless of smoking habit were defined as "yes" smoking history. Two major tumor types [32] of squamous cell carcinoma (SQ) and adenocarcinoma (AD) were only recruited because of rare samples in other tumor types.

TMA preparation and immunohistochemistry have been described previously [10]. 4 μ-thick sections were autoclaved (TM-327, Tomin Medical Equipment CO., LTD.) in a 0.01M citrate buffer (pH = 6.0) for 20 min for antigen retrieval. The subsequent procedures were performed in an immunohistochemical autostainer (DAKO, Produktionsvej, Denmark) including incubation of CYP1B1 antibodies (1:1500 dilution, Gentest) for 16 hour, of biotinylated secondary antibody (DAKO) for 30 min, of streptoavidin-peroxidase conjugate (DAKO) for 30 min and of 3'-3'-Diaminobenzidine (DAKO) for 2 min. Finally, hematoxylin (Merck, Darmstadt, Germany) was used for counterstain. The negative control was made by normal serum instead of primary antibody.

Assessment of CYP1B1 immunohistochemistry

The assessment of CYP1B1 immunostaining has been previously described [10]. The immunostaing of CYP1B1 revealed the immunoreactivity in cytoplasm of tumor cells. The stroma was CYP1B1-negative. The immunostaining intensity of CYP1B1 in a total of at least 300 stained cells per tissue core was quantified by the MetaMorph imaging software (Molecular Devices Corp., CA, USA). We used the average of CYP1B1 immunointensity of normal bronchiolar epithelium and vascular walls in lung tissues as a reference to define the intensity-strength of CYP1B1 expression, essentially, either high or low expressers. All representative images were captured by a Leica microscope (Leica microsystems GmbH, Wetzlar, Germany) equipped with a CoolSNAP CCD camera (Photometrics, Tucson, Arizona, USA) at a magnification of 200x for each tissue core.

Statistical analysis

Student t-test was used to compare the expression levels of *CYP1B1* mRNA in the various conditions. Mann-Whitney test was used to compare the MSP ratios of CYP1B1 promoter in each fragment between lung carcinoma groups and corresponding non-neoplastic lung groups. Pearson chi-square test was performed in order to examine the association between the methylation level of CYP1B1 promoter and clinical variables. For the analysis of overall survival, each patient's time started on the date of cancer diagnosis or surgery and censored on the date of the last date of follow-up or death. The univariate survival analysis was determined by the Kaplan-Meier test and the differences among the groups were accounted by the log-rank test. All statistics were conducted by a commercially available statistical software package, the Statistical Package for Social Sciences 14.0 (SPSS Taiwan Corp.). Differences in data were considered significant at a two-sided value of $p < 0.05$.

Results

The effect of 5-AzaC on expression of *CYP1B1* and *AhR* in human lung cell lines

5-AzaC inhibits the process of gene methylation to form a demethylated state. Using the quantitative real-time

PCR assay, the basal mRNA levels of CYP1B1 and AhR in BEAS-2B cells were 18×10^{-3} and 15×10^{-3}, respectively, normalized to the GAPDH mRNA level in the absence of 5-AzaC (Figure 1A and 1B). In the presence of 5-AzaC, BEAS-2B cells showed a significant 4-fold increase of *CYP1B1* mRNA level (109×10^{-3}) and 1.7-fold decrease in Calu-1 cells (24×10^{-3}), respectively (Figure 1A). The rest of cell lines including H23, H226 and H1355 cells showed no significant change in *CYP1B1* mRNA levels (Figure 1A). Furthermore, the effect of 5-AzaC on AhR mRNA levels was not detected in BEAS-2B cells and lung carcinoma cells (Figure 1B). The results suggested that the effect of 5-AzaC showed an upregulation for CYP1B1 expression in BEAS-2B cells in contrast to down-regulation or no change in lung carcinoma cells, indicating that DNA hypomethylation of *CYP1B1* might be associated with *CYP1B1* expression.

The effect of 5-AzaC on CYP1B1 induction in BEAS-2B cells and Calu-1 cells

To elucidate the relationship between 5-AzaC effect and AhR/CYP1B1 induction, BEAS-2B and Calu-1 cells used were treated with TCDD in the presence or absence of 5-AzaC. The *CYP1B1* mRNA levels were similar between cells cultivated in DMSO (TCDD solvent control)-supplemented medium (Figure 2A) and medium only (Figure 1A). In BEAS-2B cells, the *CYP1B1* mRNA level showed an increasing trend when cells treated with in order of DMSO, TCDD, DMSO plus 5-AzaC and TCDD plus 10 μM 5-AzaC. Accordingly, regardless of 5-AzaC treatment, the CYP1B1 mRNA levels responsive to TCDD showed about 2-fold increase (Figure 2A). It highlighted that the CYP1B1 mRNA level in cells with the co-treatment of DMSO and 5-AzaC as well as of TCDD and 5-AzaC showed 8-fold ($p = 0.005$) and 13-fold ($p = 0.006$) increase when compared with cells with DMSO treatment (Figure 2A). In Calu-1 cells, a condition similar to BEAS-2B cells was that CYP1B1 induction showed about 2-fold increase in the presence of TCDD (Figure 2A). However, in contrast to the result of 5-AzaC plus TCDD in BEAS-2B cells, there was no significant change of the CYP1B1 induction in Calu-1 cells with 5-AzaC treatment (Figure 2A). Calu-1 also showed about 2-fold increase of AhR mRNA expression after TCDD treatment regardless of 5-AzaC treatment. BEAS-2B cells showed no significant change of AhR mRNA levels with TCDD treatment or not; however, increased AhR mRNA expression was detected in BEAS-2B cells with co-treatment of DMSO or TCDD and 5-AzaC (Figure 2B), indicating that 5-AzaC exhibited a enhancing effect for AhR expression. Thus, BEAS-2B cells and Calu-1 cells showed TCDD-induced CYP1B1 expression; however, Calu-1 cells, but not BEAS-2B cells, showed TCDD-induced AhR expression. Interestingly, in the presence of 5-AzaC, BEAS-2B cells showed increased sensitivity for AhR and CYP1B1 expression.

MSP assay of *CYP1B1* promoter in lung cell lines

Since the 5-AzaC effect was associated with CYP1B1 expression regulated by gene promoter, the methylation level of CYP1B1 promoter in lung cell lines was investigated. The methylation ratios were proposed to reflect the effect of 5-AzaC. In the absence of 5-AzaC, the MSP ratios ranged from 13% to 31% in S2 fragment, 22% to 34% in S3 fragment and 25% to 41% in S5 fragment (Figure 3). In the presence of 5-AzaC, the MSP ratio of BEAS-2B cells in S2 fragment was 15% and it showed a significant reduction (Figure 3B); however, the MSP ratios of other lung carcinoma cells showed no significant change in any promoter regions (Figure 3). The results appeared to suggest that promoter hypomethylation of CYP1B1 in S2 fragment might correlate with the increased CYP1B1 mRNA expression in BEAS-2B cells.

Lung carcinomas with high levels of CYP1B1 expression associated with hypomethylation in S2 and S3 fragments of CYP1B1 promoter

Our previous papers have demonstrated the CYP1B1 overexpression with a high frequency in lung carcinomas as compared with corresponding non-neoplastic lung tissues [12]. In a total of 35 lung carcinoma cases, the average MSP ratio of S2, S3 and S5 regions in carcinoma cases was 37 ± 13, 35 ± 16 and 33 ± 19, respectively. In corresponding non-eoplastic lungs, the average MSP ratio of S2, S3 and S5 regions in carcinoma cases was 37 ± 15, 33 ± 15 and 38 ± 26, respectively. The methylation index (T/NT) indicated relative hypomethylation in the carcinoma to in corresponding lung tissue. In 35 lung nonsmall cell carcinomas, 21 low and 14 high expression of CYP1B1 were detected (Table 1). Representative immunohistochemical microphotos were presented as Figure 4 A and B. In 35 lung carcinomas paired with corresponding non-neoplastic lungs using Mann-Whitney testing, there was no association in any region of CYP1B1 promoter between carcinomas (T) and non-neoplastic lungs (NT; data not shown). In addition, the median and standard deviation of methylation index was 0.95 ± 0.70 in fragment S2, 0.97 ± 0.77 in fragment S3 and 0.71 ± 1.02 in fragment S5. The boxplot showed a significant reduction and a borderline significant reduction in fragment S2 and S3 of CYP1B1 promoter, respectively (Figure 4C). The number of methylation index less than 1 was 18 cases in fragment S2, 15 cases in fragment S3 and 19 cases in fragment S5 among 35 lung nonsmall cell carcinoma paired with corresponding non-neoplastic lung tissues (Table 1).

High CYP1B1 expression displayed 64.3% ($p = 0.048$) in fragment S3 and 71.4% ($p = 0.071$) in fragment S2 of CYP1B1, respectively (Table 1). No correlation between CYP1B1 expression and methylation status in fragment S5 of CYP1B1 promoter was detected (Table 1). The results confirmingcell model data showed hypomethylation of CYP1B1 promoter associated with its expression. The differential methylation in various regions of CYP1B1 promoter associated with CYP1B1 expression in lung carcinomas was also noted.

Methylation of CYP1B1 promoter weakly associated with cancer stage in lung nonsmall cell carcinomas

Lung carcinomas with hypomethylated S5 fragment of CYP1B1 promoter occurred more commonly in late stages (II-IV) than early stage (I) (Table 2). There was no association of CYP1B1 promoter hypomethylation with gender, tumor histology and smoking history. Using the Kaplan-Meier test and log-rank test, there was also no association between methylation status of CYP1B1 promoter and patient survival (data not shown).

Discussion

The results of this study demonstrate that in a demethylated state (in the presence of 5-AzaC), BEAS-2B cells exhibited a high level of *CYP1B1* mRNA as compared with lung carcinoma cells. Although the MSP assay of CYP1B1 promoter in normal bronchial epithelial cells was performed in only one cell line BEAS-2B, we observed a highlight reduction of MSP ratios in fragment S2 in the presence of 5-AzaC. Furthermore, in 35 lung carcinomas paired with corresponding non-neoplastic lungs, there was an association between high levels of CYP1B1 expression and hypomethylation in fragments S2 and S3 of CYP1B1 promoter in carcinomas. These results support our hypothesis that hypomethylation of CYP1B1 promoter might, at least partly, be associated with CYP1B1 overexpression, leading to the differential expression between lung nonsmall cell carcinomas and normal lung tissues.

In this study, in the presence of 5-AzaC, the demethylation effect can be obviously detected in BEAS-2B cells; however, such effect is barely detectable in lung carcinoma cells. The mechanism is not clearly understood. Cell models by Shehin and colleagues [33] also showed no significant change for CYP1B1 expression in breast and liver cancer cells in the presence of 5-AzaC. Clearly, methylation level in gene

promoter has been proved to be modified in cancer cells, either hypermethylated [34] or hypomethylated [24]. The hypermethylation of gene promoter is an important mechanism for silencing the gene expression [34]. In this regard, it is possible that the higher methylation of BEAS-2B cells than lung carcinoma cells in CYP1B1 promoter that much repression presented in the gene expression. Also, in a demethylated state, CYP1B1 in BEAS-2B cell appears to be upregulated throughout lack or reduction of the interference on transcription factors binding to DNA. In contrast, the interference on transcription factors binding to DNA is not obvious in lung carcinoma cell lines, leading to no significant change of CYP1B1 expression. Thus, we believe that methylation level of CYP1B1 promoter appears to be one of important determinants for CYP1B1 expression.

Interestingly in this study, we found that 5-AzaC treatment for AhR expression shown no significant change in lung carcinoma cells; however, 5-AzaC treatment enhanced the AhR expression in BEAS-2B, but not carcinoma (Calu-1) using real-time RT-PCR assay although such effect was not demonstrated in the western immunoblotting and RT-PCR methods (data not shown). The conflict results indicated that 5-AzaC effect was not strong and might weak in AhR expression. Not much data discuss the issue of AhR promoter methylation. Current *in-vitro* research by Mulero-Navarro [35] demonstrated that AhR expression was partly, at least, reduced by its promoter hypermethylation in Sp1 sites via interfering the Sp1 binding to AhR promoter. Thus, our data indirectly supported and provided a possible explanation was that 5-AzaC effect might show partly demethylation in unknown transcription sites of AhR promoter in which AhR expression enhanced regardless of TCDD treatment.

Not much data support the possibility of methylation in the regulation of CYP1B1 induction. The promoter methylation of CYP1A1 affecting gene induction has been found and it is mediated by CpG methylation of the recognition motifs containing DRE which interfere the liganded AhR/ARNT-DNA interaction observed by gel shift assays and inhibits the enhancer function of the DNA [36, 37]. However, conflict data in human cancer cell models displayed that the methylation level was not involved in the induction of CYP1A1 or CYP1B1 [33, 38]. Furthermore, mutations of DRE core sequences in CYP1B1 promoter identified were conferred to reduce the CYP1B1 induction [33]. In this study, no mutation of DRE core sequences and Sp1 sites in lung cell lines could be identified (data not shown). Although lung carcinoma cell lines showed no CYP1B1 induction in a demethylated state, CYP1B1 induction was undoubtedly observed in BEAS-2B cells. Our findings partly supported the connection between methylation and CYP1B1 induction; however, the factor of cell or tissue specificity for CYP1B1 induction should be considered.

Our data from clinical lung carcinoma samples also showed that lung carcinomas with DNA hypomethylation associated with high levels of CYP1B1 expression occurred in S2-S3 region of CYP1B1 promoter, however, with low levels of CYP1B1 expression in S5 region. The result seems to show the differential transcriptional activity of CYP1B1 promoter, higher in S2-S3 region than in S5 region, for gene expression. Wo and colleagues [39] used a series of heterogenous chloramphenicol acetyltransferase (CAT) reporter constructs to determine the transcriptional activity of CYP1B1 promoter. They found that the construct containing nt -1022 to +25 exhibited the highest CAT activity. They also suggested that enhancer elements may be located between nt -1022 and -835 which covered S2/S3 regions in this study as well as repressor elements between nt -835 and -164 which partly covered S5 region in this study. Clearly, environmental carcinogens including TCDD show increased risks for lung cancer [15]. Furthermore, the -1022/-835 region contained three

DREs and 2 Sp1 sites [39]. The presence of DREs contributes to the TCDD-induced CYP1B1 expression [33]. Sp1 site (5'-GGGCGG-3') has been demonstrated to activate a high level of transcription by binding the transcription factor Sp1 [40, 41]. In this regard, CYP1B1 expression has been proved to be promoter-dependent [39], the determinant for the level of CYP1B1 expression depends on the enhancement by positive transcription factors including Sp1 and/or dioxin exposure. It is a possible explanation is although most lung carcinomas with DNA hypomethylation occurred in both S2 and S5 regions, the enhanced strength were variable, higher transcriptional activity in S2 region than in S5 region.

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Legends

Figure 1. The effect of 5-azacytine (5-AzaC) on cytochrome P450 1B1 (CYP1B1) and aryl hydrocarbon receptor (AhR) expression in human lung cell lines. 5-AzaC is an inhibitor for DNA methyltransferase. Five lung cell lines included two squamous cell carcinomas (Calu-1 and H226), two adenocarcinomas (H26 and H1355) and one normal bronchial epithelial cells (BEAS-2B, 2B). The effect of 5-AzaC (10 μM) on the expression of of CYP1B1 (A), but not AhR (B), mRNA in BEAS-2B and Calu-1 cells was identified. The results are representative of three independent experiments. * $P < 0.05$ when compared with cells cultivated in 5-AzaC-free medium

Figure 2. The effect of TCDD on BEAS-2B cells and Calu-1 cells in the presence of 5-AzaC. TCDD is a potent ligand for AhR. *CYP1B1* is an AhR-linked downstream gene which is regulated by liganded AhR. A, BEAS-2B cells showed an increasing trend when cells treated in order of DMSO, TCDD, DMSO plus 5-AzaC and TCDD plus 5-AzaC, suggesting the synergistic effect of TCDD and 5-AzaC on BEAS-2B cells. TCDD-induced CYP1B1 expression was also present in Calu-1 cells; however, the 5-AzaC effect on CYP1B1 mRNA expression in Calu-1 cells was scarely. B, The 5-AzaC effect, but not TCDD, showed the significant change on AhR mRNA expression in BEAS-2B cells. In Calu-1 cells, increased AhR mRNA level was demonstrated when with TCDD treatment. 5-azacytidine, 5-AzaC; dimethylsulfoxide, DMSO; 2, 3, 7, 8-tetrachlorobenzo-p-dioxin,

TCDD. The results are representative of three independent experiments. * $P < 0.05$ when compared with cells exposed to 0.1% DMSO; ** $P < 0.05$ when compared with cells exposed to 1nM TCDD; *** $P < 0.05$ when compared with cells co-exposed to 10 μM 5-AzaC and 0.1% DMSO

Figure 3. Methylation analysis of *CYP1B1* promoter in lung cell lines. In the enhancer region (nucleotide - 897 ~ - 709), studied in 2 partially overlapped fragments, respectively labeled as S2 and S3, and core promoter region (nucleotide - 238 ~ + 18), labeled as S5 of CYP1B1. Mehtylation levels in each disulfite DNA fragment was analyzed by methylation-specific PCR (MSP) assay and represented as MSP ratios, described detailedly in "Material and Method". In cells cultivated in 5-AzaC-supplemented medium, the methylation level of BEAS-2B cells in S2 region showed a significant reduction (A); however, other lung carcinoma cells showed no significant change (B and C). The results are representative of three independent experiments. * $P < 0.05$ when compared with cells cultivated in 5-AzaC-free medium.

Figure 4. Association of high CYP1B1 expression with hypomethylation of CYP1B1 promoter in non-small cell carcinoma. CYP1B1 immunohistochemistry showed low (A) and high (B) expression in lung adenocarcinomas, respectively. C, The boxplot showed the distributions of three DNA fragments labeled as S2, S3 and S5 of CYP1B1 promoter. The T/NT represented the methylation index of carcinoma being the MSP ratio of carcinoma over the MSP ratio of corresponding non-neoplastic lung tissue. In carcinomas with high CYP1B1 expression, methylation index was significant reduced in S2 region when compared with low-CYP1B1-expressing carcinomas. Bar, 50 μm. ○, extreme values. * $P < 0.05$ and ** < 0.1 by a Mann-Whitney test.

Table 1. Association between methylation status of CYP1B1 promoter and CYP1B1 immunohistochemical expression

		Number (%) of T/NT ≤ 1		
	n	S2	S3	S5
CYP 1B1 expression				
Low	21	8 (38.1)	6 (28.6)	12 (57.1)
High	14	10 (71.4)	9 (64.3)	7 (50.0)
P value*		0.071	0.048	0.353

* Pearson chi-square test

Low expressers of CYP 1B1 represented as CYP1B1 immunostaining intensity lower than normal bronchial epithelium or vascular wall; inversely, high expressers were represented.

Table 2. Methylation status of CYP1B1 promoter of 35 lung carcinomas (T) corresponding with their non-neoplastic (NT) lung tissues

		Number (%) of T/NT ≤ 1					
	n	S2	P value*	S3	P value*	S5	P value*
Total	35	18 (51.4)		15 (42.9)		19 (54.3)	
Gender			0.880		0.519		0.418
Male	25	12 (48.0)		11 (24.4)		14 (16.6)	
Female	10	6 (60.0)		4 (40.0)		5 (50.0)	
Tumor histology			0.856		0.779		0.418
AD	23	13 (56.5)		11 (47.8)		14 (60.9)	
SQ	12	5 (41.7)		4 (33.3)		5 (41.7)	
Smoking history			0.431		0.732		0.409
Yes	19	8 (42.1)		7 (36.8)		11 (57.9)	
No	15	9 (60.0)		7 (46.7)		7 (46.7)	
Cancer stage			0.713		0.143		0.062
I	16	9 (56.2)		9 (56.2)		8 (50.0)	
II-IV	18	8 (44.4)		5 (27.8)		10 (55.6)	

Mehtylation index, which is less than 1, described as a MSP ratio of lung carcinoma (T) over that of its corresponding lung tissue (NT)

Smoking history and cancer stage showing 1 missing data, respectively.

* Pearson chi-square test

Figure 1.

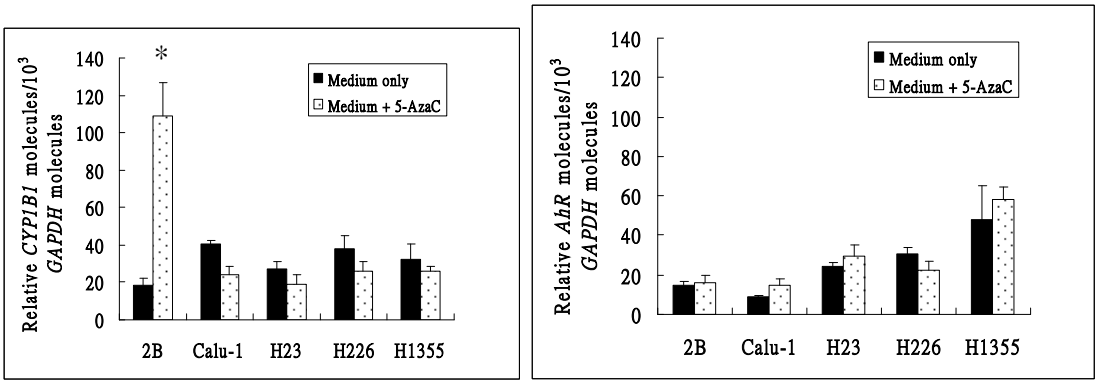


Figure 2

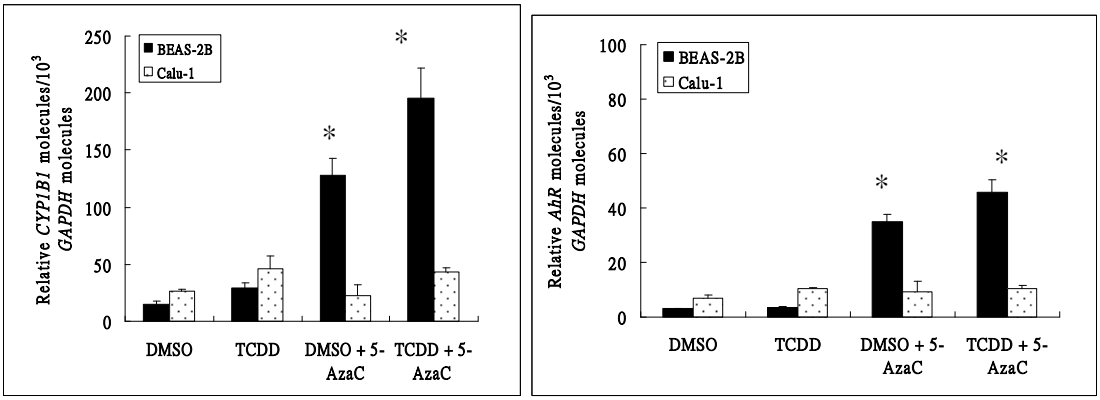


Figure 3

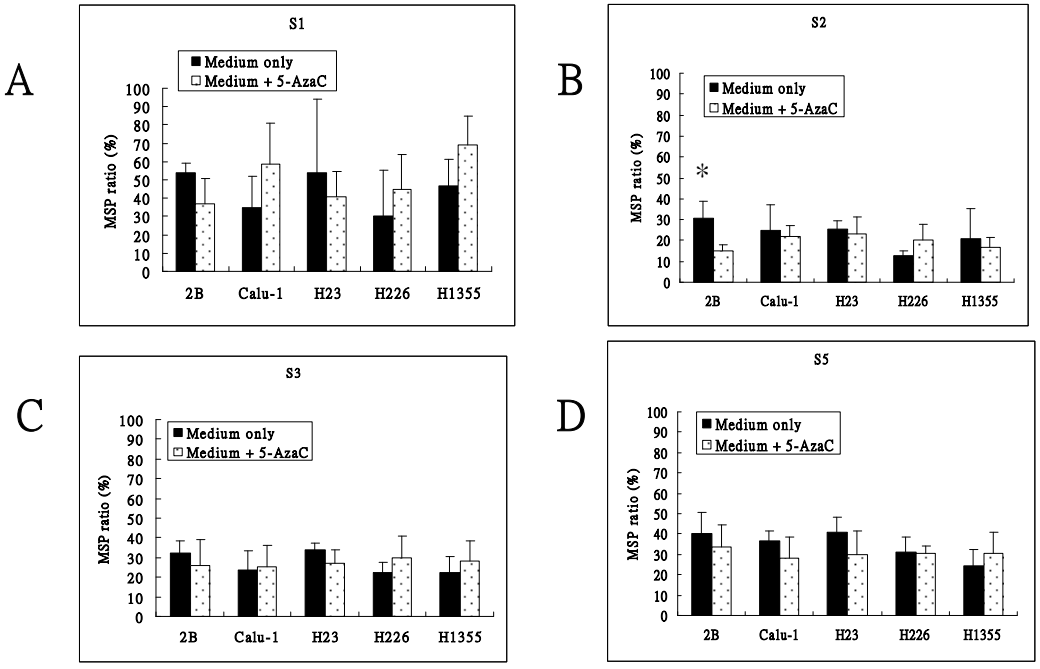
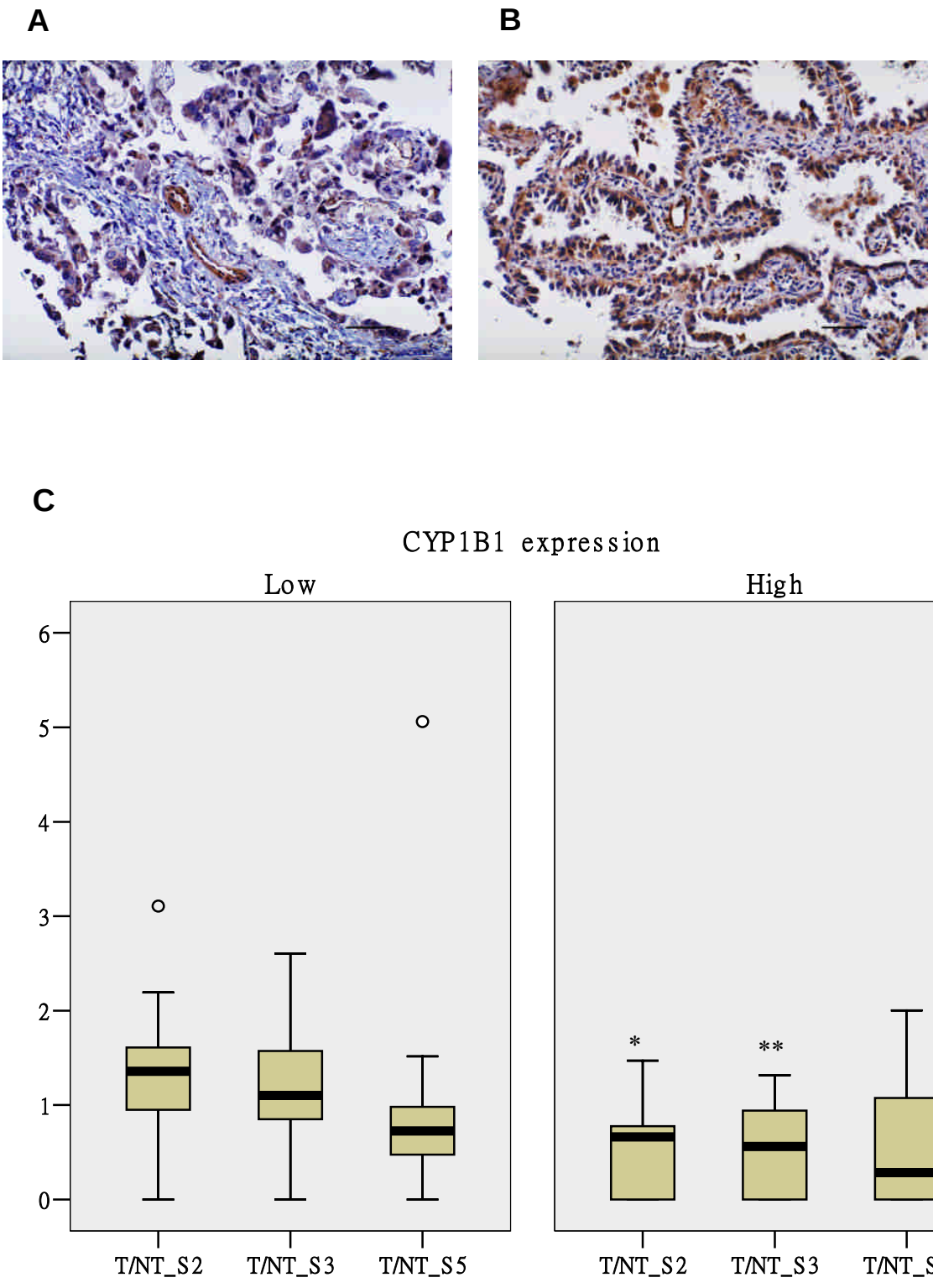


Figure 4


A
B
C
CYP1B1 expression
Low
High
6
5
4
3
2
1
0
*
**
T/NT_S2
T/NT_S3
T/NT_S5
T/NT_S2
T/NT_S3
T/NT_S5

無研發成果推廣資料

96 年度專題研究計畫研究成果彙整表

計畫主持人：張菡	計畫編號：96-2320-B-040-016-MY3
計畫名稱：多環芳香烴受器與細胞色素 P4501B1 用於肺癌診斷及預防之研究	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	1	1	100%		1. W-R Chao, H. Chang, J-M. Su, C-K Wang and P. Lin (2010) Nuclear AhR expression associated with expression of CYP1B1 and p53 in lung adenocarcinomas. 第二十五屆生物醫學聯合年會, 台北, March.
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	1	0	100%		

國外	論文著作	期刊論文	1	2	100%	篇	1. 7. Jang-Ming Su, Pinpin Lin, Chien-Kai Wang, Han Chang*. Overexpression of Cytochrome P450 1B1 in Advanced Non-small Cell Lung Cancer: a Potential Therapeutic Target. Anticancer Research, 2009, 29:509-516 2. Poor prognosis on lung adenocarcinoma patients with nuclear AhR expression (submit)
		研究報告/技術報告	0	0	100%		
		研討會論文	1	2	100%		2. H. Chang, J-M. Su, W-R Chao, C-K Wang and P. Lin (2010) Nuclear expression of aryl hydrocarbon receptor and prognosis for non-small cell lung cancer. 2010 ASCO Annual meeting, Chicago, USA, June.
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	1	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

研究結果僅初步結論，並將結論已寫成三篇論文，其中一篇已發表，一篇已送審修正中，有一部份有關甲基化的結論，因有新方法發展出來，因此想用新方法再確認，然後再發表。

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限)

1. 多環芳香烴受體與肺癌的關係，最近有學者已注意，利用多環芳香烴受體抑制劑，有潛力發展肺癌的治療藥物。

2. 利用細胞色素 CYP1B1 甲基化的程度做為肺癌早期診斷的工具，立意雖佳，但是，臨床實用性還很遙遠。