計畫編號：NHRI-EX93-9102BC

國家衛生研究院整合性醫藥衛生科技研究計畫

以精簡雜交和基因微矩陣技術探討肺腺癌致病機轉

計畫名稱

九十三年度成果報告

執 行 機 構：中山醫學大學

計 畫 主 持 人：蔡菁華

本年度執行期間： 93 年 1 月 1 日 至 93 年 12 月 31 日

＊＊本研究報告僅供參考用，不代表本院意見＊＊

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關鍵字：肺腺癌、抑癌基因、致癌基因、微矩陣分析、RNA 干擾、同步定量 PCR

壹、 九十三年度計畫研究成果摘要

中文摘要：

肺癌是世界上癌症死亡率最高的疾病，約 20%因癌症死亡者罹患肺癌。在台灣，肺癌的死亡率位居男性癌症死亡原因第二位，只少於肝癌 1.4 個百分點，但在女性的死亡率卻高居首位，綜合男性與女性的癌症死亡原因仍以肺癌為第一。抽煙與肺癌有密切的關係，大約有 90%的肺癌與抽煙有關。台灣的女性肺癌約佔肺癌罹患率之 1/3，其中 90%以上之女性肺癌患者從未吸過癌。肺癌可分為四大類：小細胞肺癌，大細胞肺癌，鱗狀細胞肺癌和肺顯癌。抽煙與肺癌、鱗狀細胞肺癌有密切的關係，與肺腺癌較無關。大約 70%的台灣女性肺癌為肺腺癌，值得一提的是男性罹患肺腺癌的機率也在攀昇中。過去的肺癌研究都注重於與抽煙有關之肺癌，因此對於非抽煙罹患肺腺癌的原因一直都不了解。

為了解肺腺癌致病過程必需更進一步了解與肺癌有關的細胞生物學與分子生物學。因此，過去幾年我們實驗室致力於尋找在肺腺癌與正常肺組織中表現有差異的基因。為了達到這個目標，我們利用抑制性的精簡雜交反應(suppressive subtractive hybridization)建立了兩個肺腺癌的精簡雜交基因庫；其中之一含有正常組織表現較高之基因(含可能的抑癌基因)，另

一個基因庫則含有肺腺癌表現較高的基因(含可能的致癌基因)。由於製備這些基因庫需要利用聚合酶反應(Polymerase chain reaction, PCR), 因此每一個基因的數目都會被放大好幾倍。為了進一步鑑定基因庫裡的基因是否在正常組織及肺癌組織中有差異的表現量, 我們發展了一個差異表現量基因的篩選法。此方法不僅可以初步篩選出於此二基因庫裡有差異表現量的基因, 並且可以將同一個基因重複多次的 clone 剔除掉。用此方法我們將好幾千個 clones, 精簡至將近三百個基因。這些 clones 帶有基因片段, 而有些片段可能來自同一個基因的不同位置。經過定序後, 我們可以進一步的刪掉一些來自同一個基因的 clones, 最後我們得到 113 個基因。

欲進一步了解這些基因是否與肺癌的形成有關, 我們將這些基因和幾個已知的資料庫進行比對, 這些資料庫包括 NCBI 的 EST database 和 SAGE database, 和由周成功教授利用國外之肺癌微矩陣整理出來的資料庫 http://61.218.37.60/cancer.html。分析的結果, 大約有 40 個基因在肺腺癌與肺正常組織中顯示出差異的表現量。

NCBI 及周成功教授之資料庫來自西方國家的病人資料。我們因而想知到這 40 個基因在台灣女性肺腺癌病人裡是否也有特殊的表現型態。正好, NHRI 之黃奇英博士進行了一組非常珍貴的微矩陣分析, 此分析包括 20 組女性肺腺癌和 5 組男性肺腺癌病人之腫瘤及鄰近之正常組織。透過黃博士之鼎力相助, 我們將 36 個基因進行本土肺腺癌病人基因表現的分析。其中有 20 個基因在本土之肺腺癌中有很顯著的差異表現量, 有 7 個基因不包括在所分析的晶片上。另外有 9 個基因沒有顯著的差異表現量, 這些可能是因為西方人和被測試之台灣人之間肺癌的差異性。那些沒有微矩陣分析資料的基因, 我們直接進行反轉錄-同步定量 PCR 反應(RT-realtime PCR)。

我們目前經過層層分析所得到的基因應該在台灣女性肺腺癌病人癌化上扮

演著重要的角色。所發現的這些基因數目已遠遠超過個別實驗室所能掌握，很幸運的有些研究者對這些基因感到興趣，因而挑選一些基因進行個別研究。希望這些基因能在多個實驗的努力之下很快的開化結果。

為了研究感興趣的個別基因之功能，我們想利用 RNA 干擾(RNA interference)技術，將癌細胞株裡的感興趣之基因表現量降低，再觀察這基因不表現之後對細胞的生長、存活、及轉移之影響。由於當時市面上尚未有可以用來構築穩定進行 RNA 干擾的載體，因此我們自己利用 pCDNA3.1 載體發展了一個穩定度很高的 RNA 干擾的載體。傳統上構築 RNAi 模板(template)時，利用兩條全長的引子(oligonucleotides, 約 65mer)，這全長的引子攜帶著模板全長的序列。這個方法雖然十分簡單，但因為需要很長的引子，因此合成引子時必須進行大規模的反應，然後再進行純化全長引子的步驟。大規模的合成及純化都需要較高的費用，除此之外越長的引子其合成過程很容易產生錯誤，因此當這些引子接入載體後需要進行相當多的定序反應。由於我們想利用 RNAi 技術進行功能性的篩選，因此我們發展了一個效率高又經濟的方法取代傳統的方法進行 RNAi 載體的構築。這個新的 RNAi 載體構築方法已發表並於多個實驗室成功的使用過。利用此法可以使整個過程所需費用降低至原來之 30%。

我們實驗室選了幾個基因正積極的進行基因功能的分析，主要的方法是利用 RNA 干擾將癌細胞中感興趣的基因表現量降低，或在癌細胞中過度表現此感興趣的基因。然後分析當這些基因過度表現或不表現時對癌細胞的影響。除此之外，我們正在構築一些感興趣的基因之片段縮氨酸(peptide)，經表現、純化之後再將其送入細胞內製造抗體。有了抗體之後，我們將可以進行更多的實驗，包括一系列的生化反應。

英文摘要：

Lung cancer has been the leading cause of cancer deaths worldwide, and it accounts for about 20% of total cancer deaths. The mortality rate of lung cancer has also been the highest in Taiwan. Cigarette smoking is attributed to about 90% of lung cancer. In Taiwan, about 30% of lung cancers are female and more than 90% of them have never smoked. Among four types of lung cancers (small cell carcinoma, large cell carcinoma, squamous cell carcinoma, and adenocarcinoma) small cell carcinoma and squamous cell carcinoma are strongly related with cigarette smoking, while adenocarcinoma and large cell carcinoma are less strongly related with cigarette smoking. About 70% of female lung cancers in Taiwan are adenocarcinoma. It is worth noting that the incidence rate of male adenocarcinoma has been increased lately. In the past, most of the lung cancer research is focused on smoking strongly related lung cancers. The mechanism of developing lung adenocarcinoma in non-smokers is still unknown.

To study carcinogenesis of lung adenocarcinoma requires advance knowledge in cell biology and molecular biology of lung cancers. Thus, the past few years we focused on identifying genes that have differential expression levels between lung adenocarcinoma and its adjacent normal counterpart. To reach the goal, we established two subtractive cDNA libraries via suppressive subtractive hybridization; one library contains enriched genes preferentially expressed in lung adenocarcinoma and the other library contains enriched genes preferentially expressed in normal lung tissue. Since these subtractive cDNA libraries were established through several rounds of PCR reaction, copy numbers of each interested genes were amplified. We developed a differential screening method not only to identify differentially expressed genes but also to rule out the redundant copies of individual gene in the libraries. After sequencing analysis, the genomic information of few hundred clones was obtained through database analyses and the redundant clones were eliminated. To further identify candidate genes, we utilized the available databases (EST

database and SAGE database of NCBI, and microarray database: http://61.218.37.60/cancer.html) to analyze the expression levels of each differentially expressed gene among lung cancers and normal lung. About 40 candidate genes were selected as a result of these prior analyses.

These candidate genes showed significant differential expression levels between lung cancers and their adjacent normal lung tissues from western lung cancer patients. We do not know whether the expression patterns of these genes are also significant among female lung adenocarcinomas in Taiwan. Dr. Chi-Yin Huang (NHRI) performed valuable microarray analyses on a set of 20- female lung adenocarcinomas and 5-male lung adenocarcinomas. With Dr. Huang's generous assistance, these candidate genes were further analyzed in their microarray database. Among 36 genes analyzed in the NHRI microarray, 20 of them showed significant differential expression patterns between lung cancers and normal lung tissues and 7 genes were not on the analyzed chip. The rest of clones selected from western database did not show significant differential expression patterns among Taiwanese female lung cancers tested. Genes that were not analyzed in this set of chip were directly analyzed by RT-realtime PCR. The number of identified candidate genes was far from we can handle in the individual laboratory. Fortunately, several researchers were interested in some of the genes and selected for further studies. Hopefully this study would have fruitful results in the near future.

To study the biological function of these candidate genes, we used RNA interference to knock down expression of target gene in cancer cell lines and study the effect of RNAi on cell growth, survival, and invasion. We constructed a vector prior any available commercialized vector that can stably express shRNA in cells. The traditional method of constructing RNAi vector for stable shRNA expression in cell is using two long oligonucleotides (65 mers) that encode full-length shRNA template. This method is straightforward

however long oligonucleotides requires large-scale synthesis and are relative error-prone thus very costly. Since we plane to interfere candidate gene's function by RNAi, it is important to reduce the cost of generating each shRNA template. Therefore, we developed an economic and efficient method for cloning shRNA template. This method was published and has been performed successfully by several laboratories. It would reduce the costs of oligonucleotide synthesis and subsequent sequence analysis to 30% of the regular costs.

We are vigorously studying biological functions of some candidate genes via RNAi and overexpression experiments in cancer cell lines. In addition, we are generating fusion proteins to inject rabbit for antibodies production. Antibodies would allow us carrying out biochemical studies of these candidate genes.

貳、九十三年度計畫著作一覽表

註：群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

若為群體計畫，請勾選本表屬於：□子計畫； 或 □總計畫(請自行整合)

1.列出貴計畫於本年度中之<u>所有計畫產出</u>於下表，包含已發表或已被接受發表之文獻、已取得或被接受之專利、擬投稿之手稿（manuscript）以及專著等

2.「計畫產出名稱」欄位：請依「臺灣醫誌」參考文獻方式撰寫

3.「產出型式」欄位：填寫該產出為國內期刊、國外期刊、專利、手稿或專著等

4.「SCI/SSCI」欄位：Social/Science Citation Index，若發表之期刊為 SCI/SSCI 所包含者，請在欄位上填寫該期刊當年度之 impact factor

5.「致謝與否」欄位：請註明該成果產出之致謝單位。若該成果產出有註明衛生署資助字樣者，請以 DOH 註明；若該成果產出有註明國家衛生研究院委託資助字樣者，請以 NHRI 註明；若該成果產出有註明衛生署及國家衛生研究院資助字樣者，請合併以 DOH&NHRI 註明；若該成果產出有註明非上述機構資助字樣者，請以機構全銜註明，舉例如下：

序號	計畫產出名稱	產出型式	SCI/SSCI	致謝與否
1.	Lin P, **<u>Chang JT</u>**, Ko J, Liao S, and Lo W. "Reduction of androgen receptor expression by benzo[a]pyrene and 7,8-dihydro-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene in human lung cells." 2004, Biochem Pharmacol. Apr 15;67(8):1523-30.	國外期刊	2.993	NHRI
2.	**<u>Jinghua Tsai Chang</u>** "An economic and efficient method of RNAi vector constructions" 2004, Analytical Biochemistry 334:199-200	國外期刊	2.174	NHRI
3.	Yu-Ying Lin and Jinghua Tsai Chang "Silencing of human Slit2 and N6 genes via RNA interference in cell lines" 2004 生醫年會	研討會論文		
4.	Jinghua Tsai Chang "An economic and efficient method of RNAi vector construction" 2004 生物學夏令營	研討會論文		
5.				
6.				
7.				
8.				
9.				
10.				

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參、九十三年度計畫重要研究成果產出統計表

註：群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料
若為群體計畫，請勾選本表屬於: □子計畫； 或 □總計畫(請自行整合)

(係指執行九十三年度計畫之所有研究產出成果)

科技論文篇數			技術移轉		
	國內	國外	類型	經費	項數
期刊論文	2篇	篇	技術輸入	千元	項
研討會論文	2篇	篇	技術輸出	千元	項
專著	篇	篇	技術擴散	千元	項

技術報告	技術創新	著作權	專利權
篇	項	(核准) 項	(核准) 項
		(申請中) 項	(申請中) 項

〔註〕:

期刊論文：指在學術性期刊上刊登之文章，其本文部份一般包含引言、方法、結果、及討論，並且一定有參考文獻部分，未在學術性期刊上刊登之文章（研究報告等）與博士或碩士論文，則不包括在內

研討會論文：指參加學術性會議所發表之論文，且尚未在學術性期刊上發表者

專　　著：為對某項學術進行專門性探討之純學術性作品

技術報告：指從事某項技術之創新、設計及製程等研究發展活動所獲致的技術性報告且未公開發表者

技術移轉：指技術由某個單位被另一個單位所擁有的過程。我國目前之技術轉移包括下列三項：一、技術輸入。二、技術輸出。三、技術擴散

技術輸入：藉僑外投資、與外國技術合作、投資國外高科技事業等方式取得先進之技術引進國內者

技術輸出：指直接供應國外買主具生產能力之應用技術、設計、顧問服務及專利等。我國技術輸出方包括整廠輸出、對外投資、對外技術合作及顧問服務等四種

技術擴散：指政府引導式的技術移轉方式，即由財團法人、國營事業或政府研究機構將

其開發之技術擴散至民間企業之一種單向移轉（政府移轉民間）

技術創新：指研究執行中產生的技術，且有詳實技術資料文件者

肆、九十三年度計畫重要研究成果

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※請依下列項目簡述計畫重要之研究成果※

一、 計畫之新發現、新發明或對學術界、產業界具衝擊性（impact）之研究成果。計畫之研究成果，請勾選下列項目並敘述其執行情形。

□ 1.研發或改良國人重要疾病及癌症的早期診斷方式及治療技術
□ 2.發展新的臨床治療方式
☑ 3.發展新生物製劑、篩檢試劑及新藥品
□ 4.瞭解常見疾病及癌症之分子遺傳機轉
□ 5.瞭解抗癌藥劑對癌細胞之作用機制
□ 6.提供有效的疾病預防策略
□ 7.利用生物統計與生物資訊研究，推動台灣生技醫藥研究，促進生物技術與基因體醫學之發展
□ 8.醫療保健政策相關研究
□ 9.瞭解環境毒理機制及重金屬對人體健康的影響
□ 10.研發適合臨床使用的人造器官及生醫材料
□ 11.縮短復健流程並增加復健效果的醫療輔助方式或器材之研究應用
□ 12.改進現有醫療器材的功能或增加檢驗影像的解析能力
□ 13.其他重要疾病或醫藥衛生問題研究_____

這年度我們實驗室研發了一個構築 RNA 干擾載體的新方法，這個方法可以節省至少 2/3 的費用。由於 RNA 干擾已被廣泛的運用於基因功能的研究，相信這個方法可以在研究界裡發揮一定的作用。

二、 計畫對民眾具教育宣導之研究成果（此部份將為規劃對一般民眾教育或宣導研究成果之依據，請以淺顯易懂之文字簡述研究成果，內容以不超過 300 字為原則）

我們實驗室歷經幾年的研究，終於從精簡基因庫裡找到二十幾個基因可能與台灣女性肺腺癌的形成有關。台灣女性肺腺癌約佔所有女性肺癌之 70%左右，由於多數女性肺癌病人不抽煙，其形成機制與抽煙引起之肺癌勢必不同。雖然女性肺癌的發生率不高但是其死亡率卻居所有癌症死亡之首，主要原因是早期發現困難，且肺癌細胞容易產生抗藥性。

如能找到與女性肺腺癌相關的基因，則可以幫助我們理解其形成的機制，將來可發展早期檢定、治療、甚至於預防的目的。

我們目前正致力於個別基因之功能研究，首先我們想要觀察的是，當這些基因在細胞裡大量表現或不表現時，對肺癌細胞的生長、存活、與轉移有何影響?

三、 簡述全程計畫成果之討論與結論，如有技術移轉、技術推廣或業界合作，請概述情形及成效

四、 成效評估（技術面、經濟面、社會面、整合綜效）

這年度我們突破了許多瓶頸，從精簡基因庫裡篩選到了許多基因可能與肺腺癌的形成有關。除此之外,我們研發了一個構築 RNA 干擾載體的新方法。此方法可以使得構築 RNA 干擾載體的費用降低至少 2/3。這個方法已經發表於期刊，已有許多實驗室利用這個方法成功的建立了 RNA 干擾載體。

五、 下年度工作構想及重點之妥適性

下年度的主要工作是對於這些基因進行功能性的探討。我們已將某些基因的全長複製出來，使其能在細胞中大量的表現，同時 RNA 干擾抑制基因的表現。除了觀察這些基因的表現與否和細胞的型態、生長、死亡、與轉移的關係之外，我們計劃進一步觀察這些基因與肺癌細胞在動物體內之形成與轉移的關係。

六、 檢討與展望

這一年的實驗有較突破性的發展是因為我們善加利用既有的資料庫從已建立之精簡雜交基因庫裡篩選出一些於肺癌可能有關的基因,在將這些基因與國衛院黃奇英博士所建立的女性肺腺癌微矩陣資料庫進行比對。因此所選出來的基因應該與台灣女性肺腺癌有密切的關係。

目前另有三個實驗室分別挑選一些基因進行進一步的研究，希望我們很快的能瀝青這些基因與肺腺癌的關係。

伍、九十三年度計畫所培訓之研究人員

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Reduction of androgen receptor expression by benzo[*a*]pyrene and 7,8-dihydro-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene in human lung cells

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Abstract

5α-Dihydrotestosterone significantly increased cell growth of lung adenocarcinoma cell line H1355. Benzo[*a*]pyrene (BaP) was a pulmonary carcinogen found in cigarette smoke. Treatment with 1 μM BaP tremendously reduced constitutive androgen receptor (AR) expression, as determined with Western immunoblotting and the real-time RT–PCR assay, as well as testosterone-induced AR protein levels in H1355 cells. Similarly, 1 μM BaP significantly reduced *AR* mRNA levels in human bronchial epithelial cells BEAS-2B. Although BaP, 2,3,7,8-tetrachlorodibenzo-*p*-dixin and polychlorinated biphenyl 126 activated aryl hydrocarbon receptor (AhR), which subsequently induced *cytochrome P4501A1* (*CYP1A1*) and *P4501B1* (*CYP1B1*) expression in H1355 cells, unexpectedly, neither TCDD nor PCB126 reduced AR expression. Antagonizing AhR activation and cytochrome P4501 activity with α-naphthoflavone, or inhibiting CYP1B1 activity with 2,4,3′,5′-tetramethoxystilbene, however, prevented BaP-induced AR reduction. Furthermore, 7,8-dihydro-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, a BaP carcinogenic metabolite catalyzed by CYP1A1 and CYP1B1, significantly reduced AR expression in H1355 cells and human lung fibroblasts WI-38. This was the first study that reports that BaP and BPDE reduced endogenous *AR* expression. These data suggest that metabolically activated BaP may disrupt androgen function by reducing AR levels in androgen-responsive organs.

Keywords: Androgen receptor; Benzo[*a*]pyrene; Cytochrome P4501A1; Cytochrome P4501B1; Lung cells

1. Introduction

Exposure to polycyclic aromatic hydrocarbons (PAH)-contaminated air pollutants has been associated with the occurrence of pulmonary diseases [1]. Benzo[*a*]pyrene (BaP), found in cigarette smoke and air pollutants, is one of the most widely studied PAH [2,3]. It is well recognized that BaP activates the aryl hydrocarbon receptor (AhR) which subsequently induces *cytochrome P4501A1* (*CYP1A1*) and *P4501B1* (*CYP1B1*) expression [4]. *CYP1A1* and *CYP1B1* are involved in the conversion of BaP into 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE), which forms DNA adducts [5,6]. CYP1B1 also converts BaP into other ultimate metabolites, such as 3-hydroxy benzo[*a*]pyrene(3OHBaP), which attack cellular DNA [7]. Several lines of evidences have shown that BaP-induced genotoxicity and carcinogenesis are AhR-dependent [8,9]. AhR belongs to one of the nuclear receptor superfamilies. While shared some regulatory proteins with steroid hormone receptors [10,11], AhR was reported to interact with several steroid hormone receptors, including estrogen receptor, androgen receptor (AR), glucocorticoid receptor and progesterone receptor, to alter their functions [12–14]. Therefore, BaP may activate AhR and subsequently interfere with steroid hormone receptors function to elicit toxicological responses.

Most studies have investigated effects of sex hormones on steroidogenic and steroid hormone-responsive organs, such as the reproductive system. However, growing evi-

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Abbreviations: BaP, benzo[*a*]pyrene; AR, androgen receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dixin; PCB126, polychlorinated biphenyl 126; AhR, aryl hydrocarbon receptor; CYP1A1, cytochrome P4501A1; CYP1B1, cytochrome P4501B1; BPDE, 7,8-dihydro-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; PAH, polycyclic aromatic hydrocarbons; DHT, 5α-dihydrotestosterone; ANF, α-naphthoflavone; TMS, 2,4,3′,5′-tetramethoxystilbene; RT–PCR, real-time reverse transcription–polymerase chain reaction.

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dences show that sex hormones also target non-genital organs. Several studies reported that AR is expressed in normal fetal and adult lungs and human lung tumors [15,16]. Testosterone and 5α-dihydrotestosterone (DHT) have been shown to affect pulmonary surfactant production during fetal lung development [17,18]. More recently, Ojeda *et al.* [19] demonstrated that surgical and pharmacological castration significantly modified the components of phospholipids in lung surfactant of adult male rats. This alteration was similar to that observed in human lung emphysema. These information suggests that testosterone is required to maintain lung morphology. Therefore, exposure to antiandrogens may have adverse effects on normal lung morphology and function.

The effects of PAH on androgen function are rarely reported. It is well-known that the androgenic effect is dependent on AR activation. Vinggaard *et al.* [20] showed that PAH, including BaP, antagonized recombinant human AR activity in a the reporter gene assay system. Kizu *et al.* [21] reported that PAH antagonized the DHT-induced expression of prostate-specific antigen in LNCaP human prostate carcinoma cells. Both Vinggaard and Kizu's studies demonstrated that AhR was required for the anti-androgenic effect of PAH [20,21]. In our present study, we examined effect of BaP in AR protein and gene expression in human lung cells. We further investigated how the AhR signaling pathway is involved in the effect of BaP on AR expression.

2. Materials and methods

2.1. Chemicals

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and polychlorinated biphenyl 126 (PCB126) were purchased from ULTRA Scientific. α-Naphthoflavone (ANF), BaP, dimethylsulfoxide, DHT and testosterone were purchased from Sigma. BPDE was purchased from Midwest Research Institute. 2,4,3′,5′-Tetramethoxystilbene (TMS) was ordered from Tocris Cookson Inc. RPMI 1640, Eagle's basal medium, and fetal bovine serum were purchased from Gibco™ Invitrogen Inc.

2.2. Cell culture

The human lung adenocarcinoma cell lines NCI-H1355 were gifts from Dr. C.-M. Tsai (Veterans General Hospital, Taipei, Taiwan, ROC). Information for H1355 was available in the American Cell Type Cell Collection. H1355 cells were maintained in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum. Human lung fibroblasts WI-38 were purchased from the American Type Culture Collection and maintained in Eagle's basal medium containing 10% fetal bovine serum. BEAS-2B cells were human bronchial epithelial cell lines immortalized with SV40 (American Type Culture Collection) and maintained in LHC-9 medium (BioSource International Inc.).

2.3. Cell viability assay

H1355 cells were seeded to 12-well plates. Twenty-four hours later, the medium was changed to phenol red-free medium containing 5–10% charcoal-stripped fetal bovine serum and incubated for another 48 hr. After treatment with DHT for 6 days, cell viability was determined with trypan blue exclusion assay.

2.4. Quantitative real-time reverse transcription–polymerase chain reaction (RT–PCR) assay

The total RNA of the cells was isolated using the TRIZOL® Reagent (Life Technologies) and the phenol–chloroform extraction method. Synthesis of cDNA was done with 2 μg total RNA, 1 μg oligo dT primer and 20 nmol deoxynucleotide triphosphates using M-MLV Reverse Transcriptase (Promega). Quantitative PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen) and analyzed on an ABI PRISM 7700 Sequence Detector System (Perkin-Elmer Applied Biosystem). Primers were chosen with the assistance of the computer program—Primer Express (Perkin-Elmer Applied Biosystem). The primer sequences and optimal concentrations of *CYP1A1*, *CYP1B1*, *AhR*, *AR* and *β-actin* were previously described [22,23]. Each data point was repeated three times. Quantitative values were obtained from the threshold PCR cycle number (Ct), where the increase in signal associated with an exponential growth for PCR product becomes detectable. The relative mRNA levels in each sample were normalized to its *β-actin* content. The relative expression levels of target gene $= 2^{-\Delta Ct}$, $\Delta Ct = Ct_{\text{target gene}} - Ct_{\beta\text{-actin}}$.

2.5. Western immunoblotting

Cells were sonicated in 20 mM Tris pH 7.4, 0.5 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 50 μg/mL leupeptin, 50 μg/mL aprotinin, 2.5 μg/mL pepstatin A, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitors cocktail (Roche Applied Science). Cell homogenates were centrifuged at 10,000 *g*, 4°. The cytosol supernatants were dissolved in sample buffer for electrophoresis. The cytosol in sample buffer were boiled, resolved by a denaturing electrophoresis on discontinuous polyacrylamide gel, electrotransferred to a PVDF membrane and immunostained with AR antibody (Upstate Co). The bands were visualized with the enhanced chemoluminescence kit according to the manufacturer's instructions (Amersham Biosciences). Band intensity was quantified with the ChemiImager™ System (Alpha Innotech Corp.). The relative expression of AR protein was calculated by normalizing to the band intensity of β-actin.

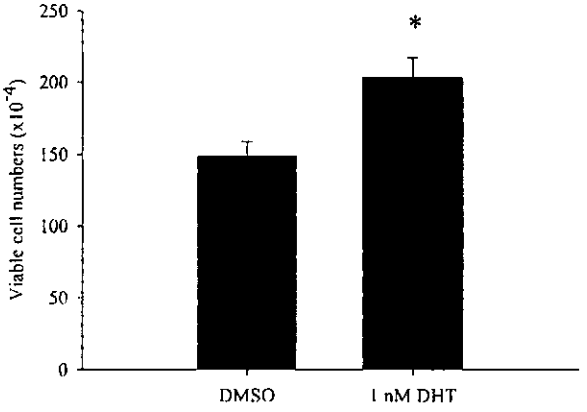


Fig. 1. Effect of DHT on the cell growth of H1355 cells. H1355 cells were treated with 0.1% DMSO or 1 nM DHT for 6 days. Viable cell numbers were determined with the trypan blue exclusion assay. Each data is the mean of four replicates. The symbol (*) indicates $P < 0.05$ as comparing with DMSO-treated cells.

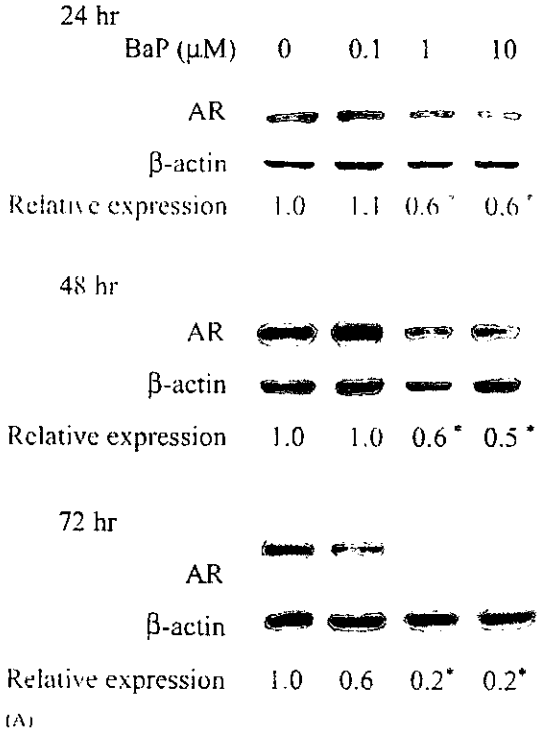
2.6. Statistical analysis

Comparisons of the cell numbers, quantification of Western immunoblotting or gene expression levels between groups were done by Student's t test.

3. Results

3.1. Effects of BaP on constitutive or testosterone-induced AR expression in lung cells

When human lung adenocarcinoma cells H1355 were treated with 1 nM DHT, viable cell numbers were significantly increased 6 days later (Fig. 1). This result indicated that the H1355 cells were responsive to androgens. Treatment with 1 or 10 μM BaP slightly but significantly reduced AR protein levels in H1355 cells 24 hr later (Fig. 2A). The reduction became more dramatic 72 hr later (Fig. 2A). Furthermore, *AR* mRNA levels in H1355 and BEAS-2B cells were significantly reduced by 1 μM BaP 48 or 72 hr later (Fig. 2B and C). Therefore, the decrease in *AR* mRNA levels might partially count for the decrease in AR protein levels by 1 μM BaP treatment. While AR protein levels were significantly increased by 10 nM testosterone in H1355 cells (Fig. 3), co-treatment with BaP prevented the increase in AR levels by testosterone (Fig. 3). These results indicated that BaP reduced the AR expression in H1355 and BEAS-2B cells at the transcription and translation levels.



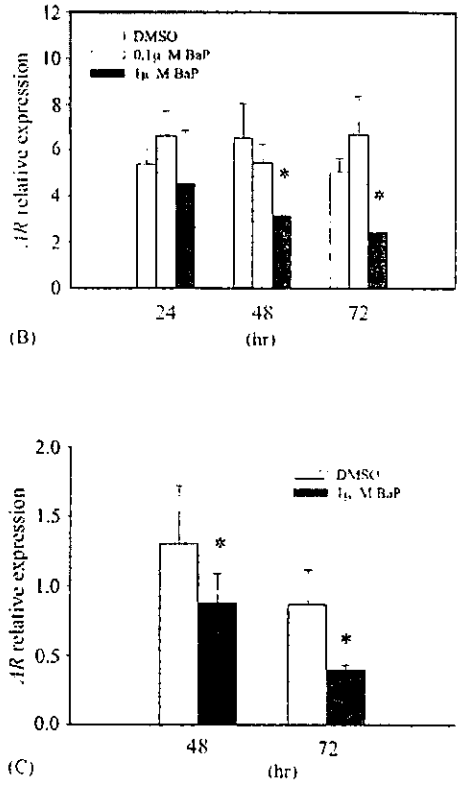


Fig. 2. Effects of BaP on AR expression in lung cells. (A) H1355 cells were treated with 0.1% DMSO, 0.1, 1 or 10 μM BaP for 24, 48 or 72 hr. AR protein was detected with Western immunoblotting. Each experiment was repeated four times. Relative expression levels were the mean of four replicates. (B) H1355 cells were treated with 0.1% DMSO, 0.1 or 1 μM BaP for 24, 48 or 72 hr and (C) BEAS-2B cells were treated with 0.1% DMSO or 1 μM BaP for 48 or 72 hr. *AR* mRNA levels were quantified with the real-time RT–PCR assay. Each experiment was repeated twice with eight replicates. The symbol (*) indicates $P < 0.05$ as comparing with DMSO-treated cells.

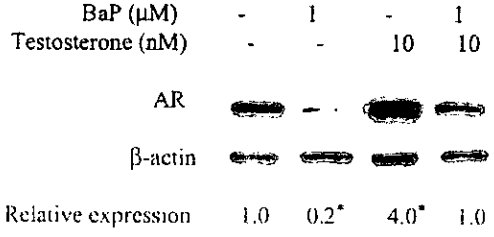


Fig. 3. Effect of BaP on testosterone-induced AR expression in H1355 cells. H1355 cells were treated with 0.1% DMSO, 1 μM BaP, 10 nM testosterone or testosterone with BaP for 72 hr. AR protein was detected with Western immunoblotting. The experiments were repeated four times. The symbol (*) indicated $P < 0.05$ as comparing with DMSO-treated cells.

3.2. Effects of TCDD and PCB126 on AR expression in H1355 cells

BaP, TCDD and PCB126 are potent AhR agonists [24]. We further investigated whether TCDD and PCB126 had a similar effect on AR expression. Treatment with 1 μM BaP, 10 nM TCDD or 1 μM PCB126, respectively, increased *CYP1A1* expression levels to 100-, 1600- or 600-fold of the levels in DMSO-treated H1355 cells (Fig. 4A). Similarly, BaP, TCDD and PCB126 increased *CYP1B1* expression levels to approximately 6-fold of the levels in DMSO-treated H1355 cells (Fig. 4B). However, only BaP reduced AR expression (Fig. 4C). Neither TCDD nor PCB126 had significant effects on AR protein levels (Fig. 4C). Therefore, the induction of *CYP1A1* and *CYP1B1* by AhR agonists was not necessarily accompanied by a reduction in AR expression.

3.3. Involvement of CYP1A1/CYP1B1 induction and BPDE in BaP-induced AR reduction

To understand whether BaP-induced AR reduction was dependent on *CYP1A1* or *CYP1B1* induction, H1355 cells were co-treated with BaP and AhR antagonist/cytochrome P4501 (CYP1) inhibitor (ANF) or CYP1B1-specific inhibitor (TMS) [25] for 48 hr. Treatment with either ANF, TMS alone slightly but not significantly increased AR protein levels (Fig. 5A and B). Co-treatment with 1.5 μM ANF or 5 μM TMS partially and significantly prevented the BaP-induced AR reduction in H1355 cells (Fig. 5A and B). These data suggested that AhR activation and CYP1A1/CYP1B1 activities might be required for BaP-induced AR reduction.

Since AhR activation is not sufficient to decrease AR levels, as demonstrated by TCDD and PCB126 treatment (Fig. 4), it is reasonable to speculate that BaP metabolites generated by AhR pathway might participate in BaP-induced AR reduction. When H1355 cells were directly treated with a BaP metabolite, 1 μM BPDE, for 48 hr, both AR protein and mRNA levels were significantly reduced (Fig. 5C and D).

3.4. Effects of BaP on CYP1A1, CYP1B1 and AR expression in fibroblasts WI-38

Treatment with 1 μM BaP significantly induced *CYP1A1* and *CYP1B1* mRNA levels in H1355 and BEAS-2B cells, but not in WI-38 cells (Fig. 6A and B). Comparing with H1355 and BEAS-2B cells, AhR protein levels were extremely low in WI-38 cells (Fig. 6C). Con-

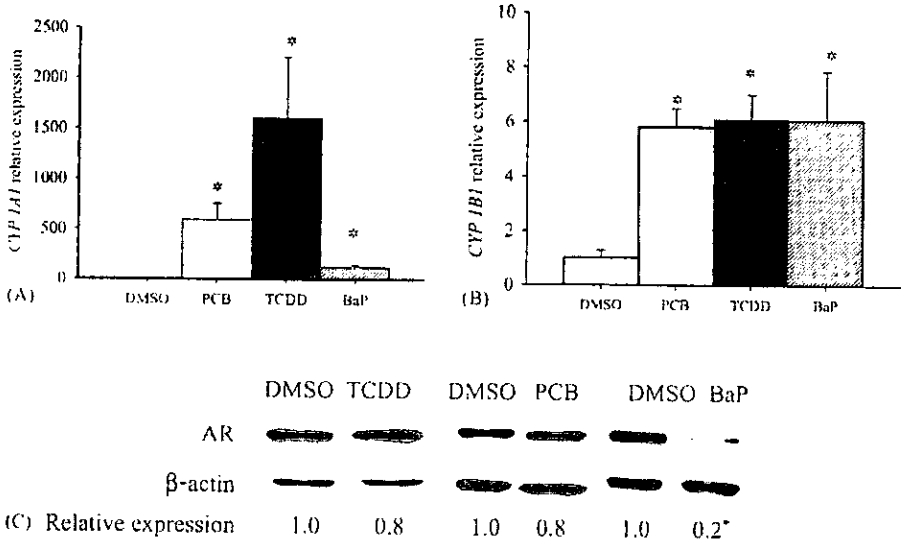


Fig. 4. Effects of BaP, TCDD and PCB126 on *CYP1A1*, *CYP1B1* and AR expression in H1355 cells. H1355 cells were treated with 0.1% DMSO, 1 μM BaP, 10 nM TCDD or 1 μM PCB126 for 24 hr. (A) *CYP1A1* and (B) *CYP1B1* mRNA levels were quantified with the real-time RT–PCR assay. Each experiment was repeated twice with six replicates. (C) H1355 cells were treated with 0.1% DMSO, 1 μM BaP, 10 nM TCDD or 1 μM PCB126 for 72 hr. AR protein was detected with Western immunoblotting. Each experiment was repeated four times. Relative expression levels were the mean of four replicates. The symbol (*) indicated $P < 0.05$ as comparing with DMSO-treated cells.

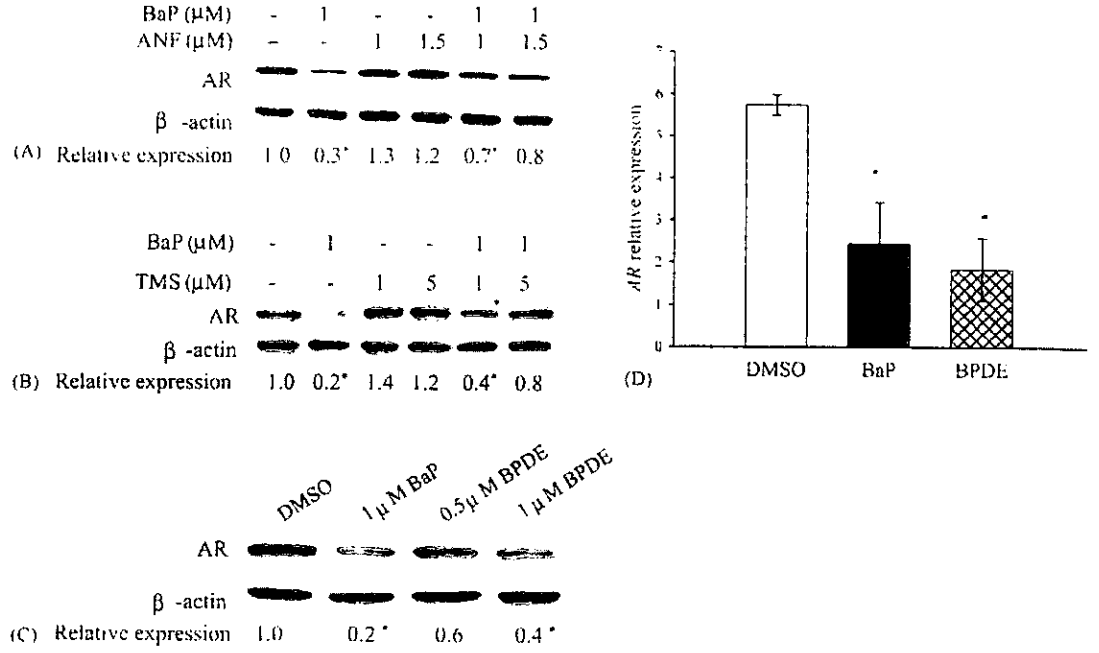


Fig. 5 Effects of BaP metabolism on BaP-induced AR reduction in H1355 cells. One micromolar BaP-treated H1355 cells were co-treated with (A) 1 or 1.5 μM ANF or (B) 1 or 5 μM TMS for 72 hr. H1355 cells were treated with 0.1% DMSO, 1 μM BaP, (C) 0.5 and 1 μM BPDE for 48 hr. AR protein was detected with Western immunoblotting. Each experiment was repeated four times. Relative expression levels were the mean of four replicates. (D) H1355 cells were treated with 0.1% DMSO, 1 μM BaP, or 1 μM BPDE for 48 hr. *AR* mRNA levels were quantified with the real-time RT–PCR assay. Each experiment was repeated twice with six replicates. The symbol (*) indicated $P < 0.05$ as comparing with DMSO-treated cells.

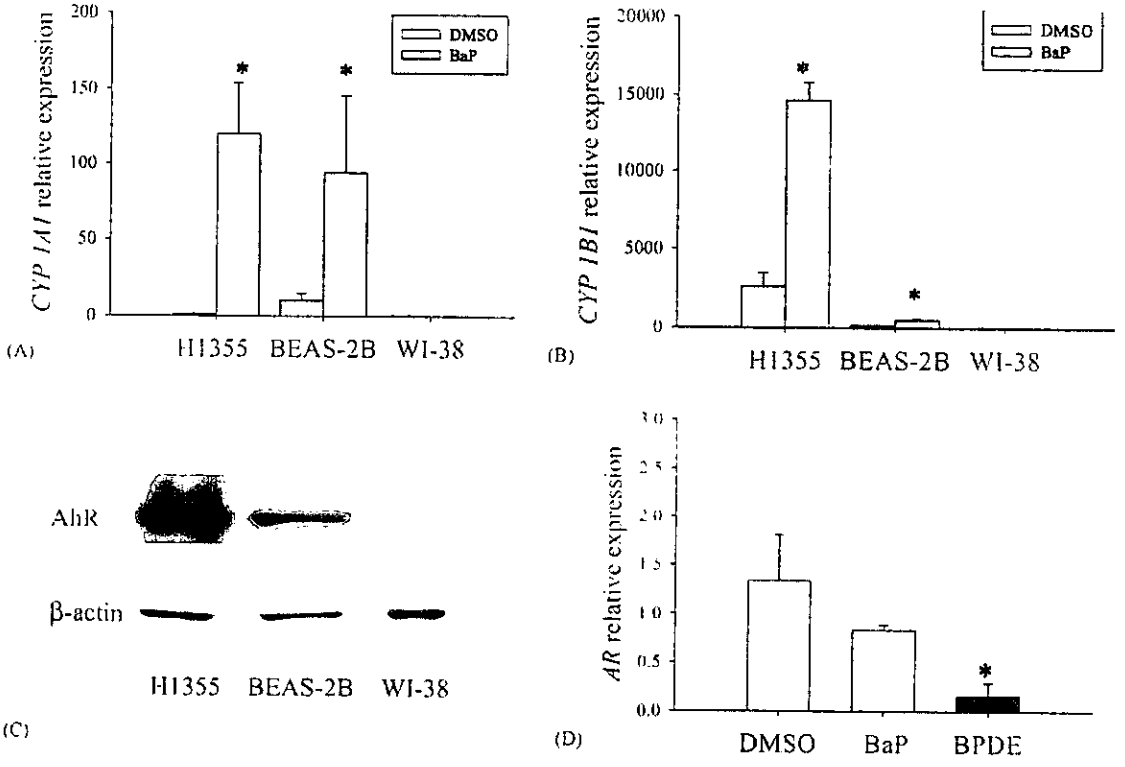


Fig. 6. Effects of BaP or BPDE on *CYP1A1*, *CYP1B1* and *AR* expression in WI-38 cells. H1355, BEAS-2B and WI-38 cells were treated with 0.1% DMSO or 1 μM BaP for 24 hr. (A) *CYP1A1* and (B) *CYP1B1* mRNA levels were quantified with the real-time RT–PCR assay. (C) AhR protein in H1355, BEAS-2B and WI-38 cells was detected with Western immunoblot. (D) WI-38 cells were treated with 0.1% DMSO, 1 μM BaP or 1 μM BPDE for 48 hr. AR mRNA levels were quantified with the real-time RT–PCR assay. Each experiment was repeated twice with six replicates. The symbol (*) indicated $P < 0.05$ as comparing with DMSO-treated cells.

sistent with the absence of *CYP1A1* and *CYP1B1* induction and low AhR expression, 1 μM BaP failed to reduce *AR* levels in WI-38 cells (Fig. 6D). Treatment with 1 μM BPDE, however, significantly reduced *AR* mRNA levels in WI-38 cells (Fig. 6D). These data demonstrated that BPDE could compensate the low induction of *CYP1A1/CYP1B1* by BaP and reduce *AR* levels in WI-38 cells.

4. Discussion

Several environmental chemicals have been reported to possess antiandrogenic properties *in vitro* and *in vivo* [26–29]. In the present study, our data represent the first observation that BaP significantly reduced AR expression in human lung cells (H1355) at both transcriptional and translational levels.

It has been reported that the AhR agonist-induced toxicity was dependent on AhR activation, which was indicated by *CYP1A1* and *CYP1B1* induction. We performed a series of experiments to investigate whether and how AhR signaling pathway would involve in BaP-induced AR reduction. First, we compared effects of different AhR agonists, TCDD, PCB126 and BaP, on AR levels. Our data showed that only BaP, but not TCDD or PCB126, reduced AR levels. It appears that either AhR signaling pathway was not involved, or AhR signaling pathway stimulated BaP metabolites production involved in AR reduction. To clarify whether AhR signaling pathway or AhR-mediated CYP1 enzyme activities play a role in BaP-induced AR reduction, BaP-treated cells were co-incubated with either AhR antagonist/CYP1 inhibitor (ANF) or CYP1B1 inhibitor (TMS). Preventive effect of ANF and TMS on BaP-induced AR reduction implied that AhR-mediated CYP1A1 and CYP1B1 activities were required for this effect. Furthermore, BaP failed to reduce AR levels in AhR non-responsive fibroblasts WI-38. These data suggested that AhR-mediated cytochrome P450 activities were specifically involved in BaP-induced AR reduction. Therefore, we proposed that BaP metabolites converted by CYP1A1 and/or CYP1B1, such as BPDE, might participate in the mechanism of AR reduction. Finally, this hypothesis was supported by our observation that BPDE directly reduced AR levels in H1355 cells and AhR non-responsive fibroblasts WI-38.

An interesting finding in our time course study was that BaP-induced AR protein reduction occurred prior to *AR* mRNA reduction. AR protein levels were slightly but significantly reduced after treatment with BaP for 24 hr. But *AR* mRNA levels were not significantly reduced until 48 hr after treatment. Furthermore, the extent of reduction in AR protein levels was greater than that in mRNA levels after 72 hr treatment. It is likely that BaP-induced AR reduction occurred through at least two different mechanisms that reflected at post-transcriptional and transcriptional levels. It has been shown that AR protein was degraded rapidly, unless bound to agonists, in cells [30]. Manin *et al.* [31] also demonstrated that DHT increased AR protein levels, but not mRNA levels in prostate cancer cells. Similar results were observed in testosterone-treated H1355 cells in our present study (data not shown). Therefore, it is possible that either BaP increased the turnover rate of AR protein or reduced the binding of androgens to AR within 24 hr. A secondary mechanism might be triggered 48 hr later, such as the conversion of BaP into BPDE, to further reduce *AR* mRNA levels.

Our present data showed that both BaP and BPDE reduced AR levels in H1355 cells. However, BaP was more effective than BPDE at the same dose (1 μM) and 1 μM BPDE was too high for a biological dose. Furthermore, ANF and TMS only partially prevented BaP-induced AR reduction. Therefore, it is likely that other mechanisms, in addition to BPDE formation, might participate in BaP-induced AR reduction. We also notice that ANF or TMS alone slightly increased AR protein levels, although the increase was not significant. In the absence of AhR agonists, CYP1A1 expression and activity was barely detectable in lung cells [9]. Nevertheless, *CYP1B1* mRNA was constitutively expressed in lung cells [9]. Treatment with ANF and TMS were expected to lower CYP1B1 expression and activity in the cells. Our data implied that a linkage between CYP1B1 activity and AR expression might exist, but more evidences are needed to establish their relationship.

BaP is shown to be converted into BPDE, which elicits genetic toxicity by forming DNA adducts and results in DNA damage [32]. Recently, BaP and BPDE were also reported to activate several signaling pathways that triggered epigenetic effects. Jyonouchi *et al.* [33] demonstrated that inhibiting PI-3 kinase activity abolished the BPDE-induced de-differentiation in airway epithelial cells. Jyonouchi *et al.* [34] also reported that BPDE increased the cytosolic calcium of airway epithelial cells. In human B cells, BPDE was shown to increase tyrosine phosphorylation [35]. Similarly, BaP was shown to increase PI-3 kinase activity, tyrosine phosphorylation or cytosolic calcium in human mammary epithelial cells and rat liver cells [36,37]. We will study the role of the PI-3 kinase, tyrosine phosphoryation and calcium in the BaP- and BPDE-induced AR reduction in the future.

BaP and BPDE reduced both the AR protein and mRNA levels in H1355 cells. From our data, it is unclear whether these chemicals decreased the rates of AR transcription or translation, or the stability of AR mRNA or protein. A few studies proposed different mechanisms for the regulation of the steady state levels of AR mRNA and protein. First, Kinoshita *et al.* [38] demonstrated that methylation of several CpG sites in the AR promoter correlated with the loss of AR gene expression in the prostate cancer cells and tissues. BPDE has been demonstrated to induce CpG methylation in mammalian cells [39], therefore, BPDE might induce the methylation of the AR promoter to

suppress AR gene transcription in H1355 cells. Second, Lin *et al.* [40] revealed that Akt mediated AR ubiquitylation and degradation. Since Akt activity was up-regulated by the PI-3 kinase signaling pathway [41], BPDE might increase AR protein degradation through the Akt signaling pathway. We will further investigate the mechanisms of AR reduction by BaP and BPDE in the future.

In utero exposure to BaP caused sterility in the male offspring of mice [42], suggesting that BaP might disturb androgen function in androgen-responsive organs. Our present study showed that H1355 cells were responsive to DHT and showed increased cell growth, nevertheless, BaP reduced AR expression in H1355 cells. Thus, BaP might interfere with lung development or surfactant production in the lungs by antagonizing AR function. Cigarette smoking and exposure to environmental tobacco smoke have been associated with higher risks of pulmonary diseases, such as lung cancer, chronic obstructive pulmonary diseases and asthma [45–47]. *In utero* passive tobacco smoke exposure was reported to affect lung function of infants [48]. BaP levels and BPDE DNA adducts were found to be significantly higher in the lung tissues of smokers than in non-smokers [43,44]. Therefore, it is plausible that BaP-induced AR reduction may interfere with AR function in the lungs and involve in the occurrence of cigarette smoking associated pulmonary diseases.

BaP was proposed to increase hormone metabolism and to disturb endocrine functions by inducing cytochrome the P4501 family. The novel finding in our present study was that BaP was metabolically activated to reduce AR expression. The reduction in AR expression may disturb androgen functions in androgen-responsive organs, such as the prostate.

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Notes & Tips

An economic and efficient method of RNAi vector constructions

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RNA interference (RNAi)[1] is a powerful technique that is used to block gene expression in eukaryotes (reviewed in [1]). Because of its speed and ease, it is suitable not only for functional studies of individual genes but also for genome-wide screening. Application of this system to mammals has been hampered by a potential interferon response to long double-strand RNA (dsRNA). A groundbreaking experiment showed that 21-bp synthetic dsRNA introduced into mammalian cells was able to block the expression of a target gene efficiently [2]. However, RNAi by synthetic dsRNA is transient only due to dilution in sibling cells. To exert a long-term effect, expression vectors have been developed to express short hairpin RNAs (shRNA) in vivo that mimic the effect of such dsRNA and trigger the RNAi effect [3–7]. The loop sequence in these shRNAs is typically 9 nt long.

The common practice of constructing an shRNA expression system is to synthesize a full-length DNA (about 60 bp long) that encodes the shRNA sequence and insert it into an expression vector under the control of a specific promoter. This method is straightforward and consists of only limited molecular manipulations. However, there are two disadvantages to the synthesis of long oligonucleotides. First, it is relatively error-prone, thus requiring screening of more clones to obtain an error-free final product. In our hands, an error rate of over 50% was not uncommon among shRNAs constructed with long oligonucleotides. Second, the synthesis of long oligos produces relatively low yields of full-length products and, thus, in most cases, the length of oligos over 40-mers requires scaling up synthesis (100 nm vs 25 nm synthesis scale) and an additional purification step (HPLC vs standard desalting).

To overcome these problems, I have developed a cost-effective and time-efficient technique to generate RNAi constructs. Instead of synthesizing full-length shRNA-coding DNA sequences, two oligonucleotides (shRNA-F and shRNA-R) containing *partial* complementary sequences of the shRNA with an overlapping loop region are synthesized (Fig. 1). This shRNA cassette is inserted into an expression vector and introduced into *Escherichia coli*, in which the desired circular plasmid constructs are produced by the host enzymes.

In practice, I used an expression vector pCDNA-HU6 [8], a derivative of pCDNA3.1/Myc-His(-) with a human U6 promoter, for expression of such a shRNA. Two oligonucleotides, shRNA-F (33 nt) and shRNA-R (38 nt), were synthesized, each consisting of a 19-nt stem sequence, a 9-nt loop sequence, and an additional nt in the complementary stem (Fig. 1), the last 11 nt of which can form a 11-bp duplex with each other. An additional T_5 was present in shRNA-R, to serve as a transcription termination signal in the final construct. The 5′ ends of these oligonucleotides contain 4 nt that are complementary to the overhangs produced by *Bam*HI and *Hin*dIII, respectively (Fig. 1). To construct the shRNA vector, pCDNA-HU6 DNA was digested with *Bam*HI and *Hin*dIII and treated with alkaline phosphatase (CIP; New England Biolab). The two oligonucleotides were treated with T4 polynucleotide kinase (New England Biolab) to add 5′ phosphates and ligated to the *Bam*HI and *Hin*dIII-cleaved pCDNA-HU6 DNA by T4 DNA ligase (New England Biolab) at 25 °C for 30 min. The ligation mixture was used to transform *E. coli* XL-10. Ampicillin-resistant transformants were isolated at frequencies of approximately 2×10^3 transformants/μg ligated DNA (transformation efficiency of about 10^6 per microgram of uncut vector). A PCR test on the transformed colonies using a vector forward primer and the shRNA-R reverse primer showed that the designed shRNA expression

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[1] *Abbreviations used:* RNAi, RNA interference; dsRNA, double-strand RNA; shRNA, short hairpin RNA; nt, nucleotide.

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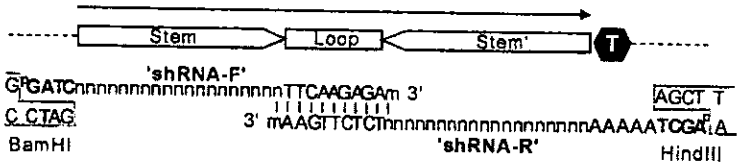

Fig. 1. Construction of shRNA templates using short overlapping oligonucleotides. The designed stem-and-loop template with its direction of transcription is outlined at the top. The transcription stop signal is indicated by T in the hexagon, and the restriction site attachment sequences are indicated by the dashed lines. Nt in the stem sequence and its complement are represented by n's and m's, respectively. The two oligonucleotides, shRNA-F and shRNA-R, are shown with an 11-bp duplex formed between the complementary loop sequences and the two flanking nt. The four nt (in boldface) at the 5′ ends are complementary to the 5′ overhang of *Bam*HI and *Hin*dIII sites of the vector (bracketed). The phosphates added to the 5′ end of the oligonucleotides by polynucleotide kinase are represented by the superscript p's. Details of the construction are presented in the text.

plasmid was present in essentially all the transformants. Restriction digestion analysis of the isolated plasmid DNA showed that the structure was as expected from the design. The inserted shRNA template sequence was confirmed by nucleotide sequencing. These results indicated that the ligated product was circularized and the gaps were filled efficiently in the *E. coli* host. Although the gap-filling error rate in *E. coli* is not known, occurrence of error-containing RNAi constructs has been less than 10% in our experience.

The purified shRNA vector DNA was introduced into human lung adenocarcinoma cell line A549 by transfection. The presence of the shRNA vector in the stable transfected clones was confirmed by PCR using the same set of primers (vector forward primer and shRNA-R primer). The interference efficiency of the shRNA on the target mRNA in these clones was detected by real-time reverse transcriptase-PCR with a set of primers flanking the siRNA target site (data not shown).

The selection of the restriction enzymes for cleavage of the vector and the design of the matching sequence at the 5′ ends of the oligonucleotides are noteworthy. Two different enzymes that produce distinct overhang sequences were chosen to ensure the correct attachment of the two oligomers. This not only avoids attachment of two identical oligomers but also forces the correct orientation of the inserts. In addition, the use of restriction enzymes that produce 3′ overhangs would render the subsequent CIP-kinase ligation scheme impossible and is thus to be generally avoided. Without the CIP treatment of the vector DNA, a significant proportion of the final *E. coli* transformants would contain recircularized vector DNA without the inserted shRNA templates.

This method of constructing shRNA templates has been routinely used in our laboratory with success. It significantly reduces the cost, effort, and potential errors that accompany the use of long oligonucleotides and this approach is particularly useful when used in conjunction with mass screening.

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拾、九十三年度計畫執行情形

註：群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料
若為群體計畫，請勾選本表屬於: □子計畫； 或 □總計畫(請自行整合)

一、請簡述原計畫書中，九十三年預計達成之研究內容

1. 製造抗原用來打入兔子體內以產生抗體。
2. 利用免疫組織染色法鑑定與肺癌相關的基因。
3. 確認肺腺癌特有的膜蛋白。
4. 篩選經由藥物處理產生之精簡雜交基因庫。

二、 請詳述九十三年度計畫執行情形，並評估是否已達到原預期目標（請註明達成率）

我們今年執行的情形與計劃內容有一些出入，因為我們今年最大的突破是一口氣篩選出將近 20 個基因可能與女性肺腺癌的形成有關，因此我們調整了研究內容，針對這些基因，今年度及明年度優先執行第五年的計劃內容。第五年的計劃內容為基因之功能研究，我們針對六個基因分別構築可表現全長基因的載體，及 RNA 干擾載體。這些載體有些已經送入細胞內表現，有一個基因的 RNA 干擾已有初步的結果。

我們一邊執行第五年的計劃，原計劃內容也在執行中，例如有四個基因的部分蛋白片段(peptide)已被構築於能在細菌表現的載體。目前有一個融合蛋白(fusion peptide)已經在純化的步驟，有一個融合蛋白無法於細菌裡表現，另有兩個融合蛋白雖然在細菌裡表現量很高但都不溶於水。如果無法解決這些問題，我們可能得花一大筆經費化學合成蛋白打入兔子以製備抗體。

我們所篩選出來的基因裡面，有好幾格是膜蛋白，有已知的膜蛋白，也有未知的膜蛋白，但是這些蛋白大部分在肺正常組織中表現量較

高。我們正在研究這些膜蛋白和肺腺癌的關係。由於工作量的關係，我們為了全力的研究個別基因的功能及與肺腺癌的關係，目前第四點研究內容暫不施行(於第二年的研究我們已經將這實驗內容刪除)。但如果這些基因與藥物代謝有關，或可能牽涉到藥效的途徑，我們可能針對這些基因的表現量與藥效的關係進行研究。因此綜合今年的研究，我們約達成第三年級及第五年之研究內容約 75%左右。