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國家衛生研究院整合性醫藥衛生科技研究計畫

人類乳突病毒和台灣女性肺癌之相關性研究

九十三年度成果報告

執 行 機 構：中山醫學大學醫學分子毒理學研究所

計 畫 主 持 人：李 輝

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

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關鍵字：人類乳突病毒、女性肺癌、基因不穩定性

自民國七十一年開始，肺癌一直是台灣婦女和男性癌症的第一和第二大死亡原因。已知抽煙是肺癌發生的最重要病因。歐美國家的肺癌發生有 90% 以上可用抽菸行為來解釋。但是台灣地區則有一半的肺癌發生無法以抽菸來說明，尤其台灣女性有 90% 以上是不抽菸者，因此不抽者罹患肺癌之病因學研究，是當前我國重要的醫藥衛生研究課題。

過去本研究室發現不抽煙之女性肺癌患者有高達 60% 和 73% 的高危險性人類乳突瘤病毒 16和 18型 (human papillomarvirus 16/18) 的感染率。此感染率遠高於男性肺癌患者（HPV 16, 24%; HPV 18, 26%）。因此推測 HPV 16/18 的高感染率可能與不抽菸的台灣婦女罹患肺癌有相關。而由過去兩年之研究結果發現同時感染 HPV 16/18 者比未感染者罹患肺癌之危險性高 38.52 倍 ($P = 0.0004$)，而在 HPV 16 及 HPV 18 單獨感染方面，感染者比未感染者分別有 6.51 倍 ($P < 0.0001$) 及 9.23 倍 ($P < 0.0001$) 罹患肺癌之危險性 (Chiou et al., 2003)，顯示血球中有 HPV 16/18 DNA 的感染可做為罹患肺癌的危險指標。為了進一步了解女性肺腫瘤組織中之 HPV 的感染路徑，本研究假設女性肺癌患者肺臟組織中之 HPV，可能是由子宮頸感染，再經由血液循環而感染至肺組織。在分析 15 位患者之子宮頸抹片及血球中 HPV 的感染情形後發現有 86.7% 的女性肺癌患者之血球及子宮頸抹片 HPV 感染具有一致性 ($P = 0.022$)。其中沒有任何一個子宮頸抹片檢體偵測到 HPV 18 感染。

因此推測女性肺癌患者血球中 HPV 16 的感染，可能是經由子宮頸而感染至血球。除了證明 HPV 的感染途徑，此外本計畫進一步分析 HPV DNA 是否會嵌入到肺腫瘤組織細胞之染色體中，以證明 HPV 確實參與肺腫瘤的的形成。已知 HPV 嵌入宿主染色體後會表現出 E6 及 E7 等致癌蛋白造成細胞的腫瘤化，因此本研究首先以 *in situ* RT-PCR 方法分析 E6 mRNA 的表現，以篩檢出可能有 HPV integration 之肺癌患者，發現在 46 位 HPV16 感染之肺癌患者中，有 27 位可偵測到 E6 mRNA 的表現(58.7%)，而在 54 位 HPV18 感染之肺癌患者中 HPV 18E6 mRNA 有 46.3% (25/54) 的表現率，因此進一步以 RS-PCR 確定具有 E6 mRNA 表現之肺腫瘤是否確實有 HPV DNA integration 的現象，至今共完成 22 個檢體，其中有 18 個是 E6 mRNA positive 檢體，另外有三個是 E6 mRNA negative 作為控制組，結果發現 E6 mRNA positive 檢體，RS-PCR 分析都是 positive, 而 E6 mRNA negative 者，則 RS-PCR 為 negative 結果。這結果亦再次確定 in situ RT-PCR 的結果之正確性。因此有 50%的 HPV 感染之肺腫瘤具有 E6 mRNA 的表現和 HPV DNA 嵌入到肺腫瘤細胞之染色體中。

為了解 HPV 主要嵌入的基因體位置，本計畫利用 DNA 自動定序分析 RS-PCR 所獲得之 PCR 產物。經由比對 DNA 自動定序之結果後，共有 8 位肺癌患者之 RS-PCR 產物可比對出 HPV 嵌入之基因體的位置，包含 5 位女性及 3 位男性，這些位置包括：Xp11.3-11.4 (PAC95C20)，Chromosome 12 (BACRP11-478H3)，Chromosome 1 (RP11-477H21)，Chromosome 1 (RP11-147C23)，Xp22.31-22.13 PHKA2 (phosphorylase kinase alpha2)，Chromosome 18 (RP11-138E9)，Chromosome 17 (953)。而其中有兩位女性肺癌患者所比對出來之位置相同都在 Xp11.3-11.4 (PAC95C20)，而在此 8 個比對到的位置中，共有 3 個位置在 X 染色體上占 42.9%，2 個位置在 Chromosome 1 占 28.6%，其餘位置均不相同，此部分結果顯示 HPV 嵌入宿主 DNA 之位置似乎

有些特異性與過去在 cervical cancer 之研究結果有些不同。

過去的研究發現 90%以上的子宮頸癌患者有高危險型 HPV 感染，且會造成 FHIT 基因發生 LOH (基因座異質性缺失) 或缺失。又知抽煙患者肺腫瘤組織中之 FHIT LOH 頻率顯著高於不抽煙之肺癌患者。因此擬了解不抽煙之女性肺腫瘤組織中之 FHIT 基因發生 LOH 之頻率，是否會因 HPV 感染而增高，就如抽煙之肺癌患者一樣有高頻率之 FHIT LOH？本實驗共收集了 176 位肺癌檢體，利用序列片段分析方法配合 DNA 自動定序儀分析 FHIT 之基因座異質性缺失，共分析 5 個微衛星序列標記，包括：D3S1300、D3S1234、D3S1313、D3S1312 及 D3S1285。結果顯示不抽煙之女性肺癌患者之 D3S1300 位置發生 LOH 與 HPV16 感染有關，而此現象並沒有在抽煙與不抽煙男性患者發現。因此 HPV16 感染可能如子宮頸癌一樣，會經由 FHIT 發生 LOH 而參與女性肺腫瘤之形成。

為了進一步篩檢 HPV 可能參與肺癌形成之重要基因，本年度計劃利用有及沒有轉染 HPV E6 之肺癌細胞株進行 cDNA microarray 分析研究，本研究之初步成果主要將表現差異較大之基因依據 Nees et al., 2001 的研究區分為四類，分別是：Cluster I: INF-related gene；Cluster II：NF-κB related gene；Cluster III：Cell cycle related gene and DNA synthesis related gene；Cluster IV：Other。Cluster I: INF-related gene，在 microarray 的分析結果中此類基因在 HPV+與 HPV- 的腫瘤組織中表現量隨 HPV 16/18 E6/E7 之表現而有抑制之現象；而在過去的研究中也發現高危險型 HPV 確實會抑制 INFs 及 INF-response 基因的表現 (Nees et al., 2001; Chang et al., 2000)。Cluster II：NF-κB related gene，已知 NF-κB 基因受 TNF-α 所調控，TNF-α 可活化 NF-κB 及 AP1 等轉錄因子，並進而刺激許多與發炎反應有關之基因表現，如 IL-6 及 IL-8。過去有研究指出 HPV 16 E6 會抑制 NF-κB 的活性進而抑制免疫反應 (Patel et al., 1999)。而在本研究之結果中此類基因表現差異較大者，範

圍從 0.18- 4.00，此部份基因有 58.8% 在 HPV 致癌蛋白表現者有被抑制的現象，因此肺腫瘤組織中 HPV 16/18 E6/E7 致癌蛋白的表現確實會影響細胞之免疫功能。Cluster III：Cell cycle related gene and DNA synthesis related gene，過去的許多研究結果發現高危險型之 HPV 所表現之 E6 及 E7 蛋白會使細胞中兩個重要的細胞週期調控因子 p53 及 Rb 去活化，進而促使細胞的分化及增生而終致細胞癌化 (EL Deiry et al., 1998; Foster et al., 1996)。本研究結果顯示，在有 HPV 感染之肺腫瘤之表現量較沒有感染之肺腫瘤有增加的現象，這結果顯示 HPV 感染確實會影響細胞週期之調控。而 Cluster IV：Other，此類主要包括膜蛋白及結構蛋白等而此部份選取之基因亦有 66.7%被抑制，主要影響細胞間物質交換之通道及細胞之結構，但詳細之機轉仍需進一步研究。

HPV 16/18 引起人類腫瘤之主要分子機轉是透過研究子宮頸癌而獲得。一是經由 E6 和 E7 致癌蛋白將 p53 與 Rb 蛋白分解，使細胞增生而腫瘤化。另一是 HPV DNA 會嵌入宿主染色體之 common fragile sites (CFS)，而引起染色體不穩定而發生 LOH，終致引起腫瘤。在先前計畫之 cDNA microarray 的結果已獲知，HPV 感染之肺腫瘤中參與細胞週期與 DNA 合成以及免疫相關之基因表現與圖與 E6 與 E7 轉形之子宮頸角質細胞(transformed cervical keritinocytes)以及肺癌細胞轉染 E6, E7 之表現與圖相似。這顯示 HPV 感染確實會改變肺腫瘤基因之表現，而與子宮頸角質細胞感染 HPV 引起轉形之過程相似。本研究室過去以 RS-PCR 結果發現 HPV 16/18 DNA 會嵌入到染色體 1, 12, 17, 18 和 X 中，顯示 HPV DNA 嵌入到肺腫瘤細胞可能是隨機性的，因此欲找到 HPV 嵌入引起之染色體 LOH，可能須要以 genome-wide 全面篩檢方式，較能找到參與 HPV 感染引起之肺腫瘤化的抑癌基因(tumor suppressor genes, TSGs)。本計畫進一步以 genomic-wide 分析人類乳突病毒感染之不抽菸女性肺腺癌主要發生異質性基因座缺失 (loss of heterozygosity, LOH)之染色體區域，並希望能經由發生 LOH 位置，找到並確定主要參與之已

知與未知之 TSGs。本計畫分析檢體將由病理學專家確定 HPV 有或沒有感染之女性肺腺癌做研究標本，以 LCM 將腫瘤細胞取下萃取純化出 DNA，另外以該病患之週邊血液淋巴球做正常組 DNA，用來進行 genome-wide 分析以找出 HPV 感染之肺腺癌較常發生 LOH 之染色體區域，並推測可能之已知 TSGs。

本計劃目前已完成 Chromosome 1-10 的基因不穩定分析，結果發現 HPV 嵌入宿主細胞染色體中的可能造成下列位置發生基因不穩定，分析結果如下：chromosome1: D1S484, D1S2878, D1S196, D1S2800, D1S2785, D1S2842, D1S2836, D1S2667, D1S199, D1S207 及 D1S2726 這些位置包含的基因包括 TRK, PBX1, PCaP, TP73 及 GSTM1 等。Chromosome3: D3S1311, D3S1263, D3S2338, D3S1266, D3S1289, D3S 1300 等，這些位置包含的基因包括 OPA1, VHL, RAPB, MLH1, FHIT 等。Chromosome4: D4S2935, D4S391，這些位置包含的基因包括 FGFR3 及 SLT2。Chromosome5: D5S424 及 D5S436，這些位置包含的基因包括 MSH3 等。Chromosome6: D6S1574, D6S309, D6S1581，這些位置包含的基因包括 PEC1, PRP4, TUBB, P16, ELANH2, NMOR2, BPHL, FKHL6, FKH7, HLA class 及 IGF2F 等。Chromosome9: D9S286, D9S290, D9S1817, D9S293 等，這些位置包含的基因包括 JAK2, DBCCR1 及 TGFBR1 等。chromosome10: D10S597 及 D10S1693 等，這些位置包含的基因包括 RET, PTEN, MX11, DMBT1, FGFR2 等。因此 HPV16/18 感染可能如子宮頸癌一樣，會經由嵌入宿主染色體造成基因不穩定而參與肺腫瘤之形成。

本計畫之研究結果除了發現肺組織確實可偵測到 HPV 病毒外，並由血液中之檢測推測 HPV 之感染可能來自血液循環，且肺組織中感染之 HPV 病毒確實會嵌入宿主 DNA 中產生 E6 致癌蛋白，並使宿主發生基因不穩定，而造成肺腫瘤化。本年度計畫之研究結果，將有助於了解 HPV 感染與肺細胞腫瘤化之相關性。

英文摘要

Lung cancer is the leading and second cause of cancer death in women and men in Taiwan, respectively. Cigarette smoking has been considered to be the most risk factor of lung cancer. In western countries, 90% of lung cancer can be explained by cigarette smoking. However, about 50% of lung cancer was considered to cause by cigarette smoking in Taiwan, especially 90% of Taiwanese women are nonsmokers. Therefore, aetiological studies of lung cancer is one of important medical issues in Taiwan.

Our previous report have indicated that extremely higher frequencies of HPV 16 (60%) and 18 (73%) infections were found in female lung cancer patients as compared with male cases (24% for HPV 16, 26% for HPV 18) (Cheng et al., 2001). Therefore, we suggested that HPV 16/18 infection may be associated with female lung cancer development in Taiwan. Our recent case-control study showed that subject with HPV 16/18 infections in blood circulation had 38.52-fold of lung cancer risk of subject without HPV 16/18 DNA in blood circulation (P = 0.0004, Chiou et al., 2003). Thus, the presence of HPV 16/18 DNA in blood circulation may act as a risk marker of lung cancer in Taiwan. To elucidate the HPV transmission route in female lung cancer patient, palp smear and blood samples were collected from 15 female lung cancer patients for HPV 16/18 DNA detection. Our data showed that HPV 16 infection was correlated between palp smear and blood cells (p = 0.022), but the correlation was not observed in HPV 18. This result seems to support the speculation that HPV 16 may be originated from cervix and transmitted into lung through blood circulation.

To understand whether HPV infection was involved in lung tumorigenesis, LCM-cDNA microarray was performed to compare differently expressed genes including INF-, NF-kB-, cell cycle and DNA synthesis-related genes. These results suggested that HPV was indeed involved in lung tumoringenesis through alteration of immune response- and cell proliferation-related gene expression and the altered expression profile

was similar with those of HPV 16/E6/E7 transfected keritinocytes. HPV DNA integration in HPV infected-lung tumors were determined by RS-PCR. E6 mRNA expression in HPV infected lung tumors was first examined by in situ RT-PCR to screen the possible lung tumors for HPV integration analyses. Our data showed that 58.7 (27 of 46) and 46.3% (25 of 54) of HPV 16 and 18 infected-lung tumors had positive E6 mRNA expressions. All of HPV infected-lung tumors with E6 mRNA expression had positive RS-PCR result. This result strongly indicated that HPV 16/18 DNA can be integrated into the chromosomes of lung tumors. To further identify the integration site, the PCR products were eluted from RS-PCR gels of 22 lung tumors for direct sequencing. HPV DNA integration site from 8 of 22 lung tumors were successfully identified. Seven integration sites were found in Xp11.3-11.4 (PAC95C20), Chromosome 12 (BACRP11-478H3), Chromosome 1 (RP11-477H21), chromosome 1 (RP11-147C23), Xp22.31-22.13 PHKA2 (phosphorylase kinase alpha2), Chromosome 18 (RP11-138E9) and Chromosome 17 (953). Among these, the most prevalence site was observed in X chromosome (3 of 7, 42.9%), followed by Chromosome 1 (2 of 7, 28.6%). Our preliminary data seemed to reveal that HPV integration in lung cancer was not random. This phenomenon was not consistent to HPV associated-cervical cancer reported previously.

A higher FHIT LOH frequency was found in HPV infected cervical cancer and this due to HPV integrated into FRB fragile site in FHIT to cause LOH. On the other hand, a higher FHIT LOH frequency was also observed in smoking lung cancer as compared to nonsmoking cases. Thus, we hypothesized that HPV infected-nonsmoking female lung cancer may have high FHIT LOH frequency as same as that of non-HPV infected-smoking male lung cancer. Our results from LOH analysis using D3S1300 marker showed that FHIT LOH frequency in female lung tumors with HPV 16 infection was significantly higher than those without HPV 16 infection, not in HPV 18. Additionally, the finding was not observed in smoking and

nonsmoking male lung cancer patients. This result suggested that HPV 16 involved in female lung tumorigenesis was at least in part mediated through the increase of FHIT LOH.

The genome-wide experiment was investigated in HPV-infected lung tumor in this project. Until now, chromosome 1 to 10 has been completed and our preliminary data seemed to reveal that the genome instability locus found in HPV infected lung cancer was similar to that of HPV associated cervical cancer. This result indicated that HPV was indeed involved in lung tumorigenesis through genome instability by HPV DNA integration.

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)

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2.「計畫產出名稱」欄位：請依「臺灣醫誌」參考文獻方式撰寫

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序號	計畫產出名稱	產出型式	SCI/ SSCI	致謝與否
例	Kao CF, Chen SY, Lee YHW. Activation of RNA polymerase I transcription by hepatitis C virus core protein. J. Biomed. Sci. 2004 Jan-Feb;11(1):72-94.	國外期刊	2.322	NHRI
1.	Cheng YW, Chiou HL, Chen JT, Chou MC, Lin TS, Lai WW, Chen CY, Tsai YY, **Lee H**. (2004) Gender difference in human papillomarvirus infection for non-small cell lung cancer in Taiwan. Lung Cancer, 46(2):165-70.	國外期刊	2.451	NHRI &NSC
2.	Wu MF, Cheng YW, Lai JC, Hsu MC, Chen JT, Liu WS, Chiou MC, Chen CY, **Lee H**. (2004) Frequent p16INK4a promoter hypermethylation in human papillomavirus-infected female lung cancer in Taiwan. Int J Cancer., in press.	國外期刊	4.056	NHRI &NSC
3.	Lin TS, **Lee H**, Chen RA, Ho ML, Lin CY, Chen YH, Tsai YY, Chou MC, Cheng YW. An Association of DNMT3b Prptein Expression with P16INK4a Promoter Hypermethylation in Non-smoking Female Lung Cancer with Human Papillomavirus Infection. Cancer Lett., revised.	國外期刊	2.61	NHRI &NSC
4.	Shigematsu H, Lin L., Takahashi T, Normura M., Suzuki M, Fong WKM, **Lee H**, Toyooka S, Shimizu N, Fujisawa T, Feng Z, Roth JA, Her J, Minna JD, Gazdar AF. Clinical and Biological features of epiderman growth factor receptor mutation of lung cancers. J Natl Cancer Inst, in press.	國外期刊	13.844	NHRI &NSC
5.	Cheng YW, Chen PL, Chiou HL, Chen JT, Chen CY, Chou MC, **Lee H.** Involvement of HPV 16/18 E6 Status in Taiwanese Lung Cancer with P53 Protein Expression. Submitted (Int J Cancer).	國外期刊	4.056	NHRI &NSC
6.	Cheng YW, Wu DW, Chen JT, Chen CY, Chou MC, **Lee H**. Frequent *FHIT* Gene Loss of Heterozygosity in Human Papillomavirus-infected Nonsmoking Female Lung Cancer in Taiwan. Submitted (Int J Cancer).	國外期刊	4.056	NHRI &NSC

*本表如不敷使用，請自行影印。

參、九十三年度計畫重要研究成果產出統計表

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(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	篇	6篇	技術輸入	千元	項
研討會論文	篇	6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 (申請中) 項	(核准) 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

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
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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

【補充說明】過去有關台灣女性肺癌之研究大多著重於二手煙與炒菜油煙暴露之流行病學研究。而本研究之結果，提出有力證據不僅證明 HPV 感染與女性肺癌的相關性，同時亦證明 HPV DNA 存在於肺組織中，會經由表現 E6 致癌基因以及 HPV DNA 嵌入到宿主之細胞之染色體中。同時還會增加基因不穩定性而導致細胞腫瘤化。這些結果都可說明 HPV 感染很有可能參與台灣女性肺癌之發生。

□ 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＿＿＿＿＿＿＿＿＿＿

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）

過去有關台灣女性肺癌之研究大多著重於二手煙與炒菜油煙暴露之流行病學研究。但都無法有效說服民眾，因此在預防不抽煙女性肺癌之發生一直成效不彰，而本研究之結果，提出有力證據不僅證明 HPV 感染與女性肺癌的相關性，同時亦證明 HPV DNA 存在於肺組織中，會經由表現 E6 致癌基因以及 HPV DNA 嵌入到宿主之細胞之染色體中。同時還會增加基因不穩定性而導致細胞腫瘤化。這些結果都可說明 HPV 感染很有可能參與台灣女性肺癌之發生。因此未來在國內應進行大規模之人群研究，並推展預防子宮頸癌之疫苗施打時，

可同時了解 HPV 感染之女性肺癌患者是否有減少之趨勢，若以上之研究結果可證實，則更能確定 HPV 與女性肺癌之相關性。

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

本計畫之全程結果將有助於了解 HPV 感染與女性肺癌之相關性，若本結果能證明期間之相關性，則未來台灣可全面進行實施 HPV 疫苗防治，以減少 HPV 引起之女性肺癌。

四、 成效評估（技術面、經濟面、社會面、整合綜效）

計畫結果將提出 HPV 感染可能是台灣不抽煙女性引起肺癌之重要致病因子。至今台灣女性癌症死亡之首要殺手即是肺癌，但由於九成以上之女性又不抽煙，因此又無法以抽煙來解釋其病因，因此本研究結果似乎可用來解釋其病因，這對未來減少國內女性肺癌之發生有莫大之貢獻。

五、 下年度工作構想及重點之妥適性

本計畫之完成將有助於了解 HPV 感染與肺癌形成之相關性，對於未來應用於肺癌之預防、診斷及治療有其一定的重要性，但除找出相關受影響之基因外，礙於計畫年限與經費無法針對有意義之基因做進一步深入之探討，為計畫執行之唯一缺憾。

六、 檢討與展望

本計畫之執行在以初級培養建立本國人 HPV 感染之肺癌細胞株有些困難，因此在第三年之計畫執行上遭遇一些困難。其他分子生物技術等研究技術發展與克服技術瓶頸在本研室都可以如期完成，因此本

計畫改以基因轉染方式進行，同時亦增加以 genomic-wide 分析人類乳突病毒感染之不抽菸女性肺腺癌主要發生異質性基因座缺失 (loss of heterozygosity, LOH)之染色體區域，並希望能藉由兩部分實驗找到並確定主要參與之已知與未知之 TSGs，以順利達成本計畫之研究目標。

伍、九十三年度計畫所培訓之研究人員

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
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			已結訓	2	
	2.	碩士級研究人員	訓練中	4	
			已結訓	2	
	3.	學士級研究人員	訓練中	0	
			已結訓	0	
	4.	其他	訓練中	3	
			已結訓	2	
兼任人員	1.	博士班研究生	訓練中	6	
			已結訓	0	
	2.	碩士班研究生	訓練中	3	
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醫師			訓練中	3	
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陸、參與九十三年度計畫所有人力之職級分析

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職級	所含職級類別	參與人次
第一級	研究員、教授、主治醫師	3人
第二級	副研究員、副教授、總醫師、助教授	5人
第三級	助理研究員、講師、住院醫師	1人
第四級	研究助理、助教、實習醫師	5人
第五級	技術人員	2人
第六級	支援人員	1人
合計		17人

〔註〕

第一級：研究員、教授、主治醫師、簡任技正，若非以上職稱則相當於博士滿三年、碩士滿六年、或學士滿九年之研究經驗者

第二級：副研究員、副教授、助研究員、助教授、總醫師、薦任技正，若非以上職稱則相當於博士、碩士滿三年、學士滿六年以上之研究經驗者

第三級：助理研究員、講師、住院醫師、技士，若非以上職稱則相當於碩士、或學士滿三年以上之研究經驗者

第四級：研究助理、助教、實習醫師，若非以上職稱則相當於學士、或專科滿三年以上之研究經驗者

第五級：指目前在研究人員之監督下從事與研究發展有關之技術性工作，且具備下列資格之一者屬之：具初（國）中、高中（職）、大專以上畢業者，或專科畢業目前從事研究發展，經驗未滿三年者

第六級：指在研究發展執行部門參與研究發展有關之事務性及雜項工作者，如人事，會計、秘書、事務人員及維修、機電人員等

柒、參與九十三年度計畫所有人力之學歷分析

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類別	學歷別	參與人次
1	博士	5人
2	碩士	6人
3	學士	0人
4	專科	3人
5	博士班研究生	6人
6	碩士班研究生	8人
7	其他	0人
合計		28人

捌、參與九十三年度計畫之所有協同合作之研究室

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機構	研究室名稱	研究室負責人
中山醫學大學	病毒研究室	邱慧玲
中山醫學大學	基礎分子醫學研究室	鄭雅文

玖、九十三年度之著作抽印本或手稿

依「貳、九十三年度計畫著作一覽表」所列順序附上文獻抽印本或手稿

如附件。

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
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一、請簡述原計畫書中，九十三年預計達成之研究內容

本計劃第三年原預定由新鮮肺腫瘤組之中以初級細胞培養技術，建立本國人 HPV 感染之肺癌細胞株，再以 cDNA microarray 分析法，篩檢 HPV 可能參與肺癌形成之重要基因，以作為將來進行基礎研究之重要依據，同時亦可由比對有感染及沒有感染 HPV 之肺腫瘤細胞株之基因表現與圖，找出 HPV 引起之肺癌形成，和子宮頸癌細胞株之異同? 以得知何種基因可能是 HPV 感染而造成肺癌形成之重要基因。

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）

本計畫之研究結果除了第一年發現肺腫瘤組織中之 HPV 可能來自血液循環且已發表於國際期刊 Cancer 中，第二年之研究成果以 in situ RT-PCR、RS-PCR 及 DNA 自動定序之研究方法發現肺組織中感染之 HPV 病毒確實會嵌入宿主 DNA 中產生 E6 致癌蛋白，並使 FHIT 基因發生 LOH，而造成肺細胞的腫瘤化，此部分之研究成果亦投稿中。第三年原預定由新鮮肺腫瘤組之中以初級細胞培養技術，建立本國人 HPV 感染之肺癌細胞株，但因在初級培養發生困難因此改以基因轉染方式完成此部分實驗，同時亦進一步以 genomic-wide 分析人類乳突病毒感染之不抽菸女性肺腺癌主要發生異質性基因座缺失 (loss of heterozygosity, LOH)之染色體區域，並希望能經由發生 LOH 位置，找到並確定主要參與之已知與未知之 TSGs。但礙於計畫年限與經費無法針對有意義之基因做進一步深入之探討，為計畫執行之唯一缺憾。

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Gender difference in human papillomarvirus infection for non-small cell lung cancer in Taiwan

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KEYWORDS
Human papillomavirus;
Non-small cell lung cancer

Summary Our previous reports have indicated that high risk human papillomarvirus (HPV) 16/18 were much more frequently detected in lung tumors of female patients as compared to that of male patients and HPV 16/18 in lung tumors were evolutionally correlated with those in blood circulation. In the other hand, it is well known that HPV 6/11 are frequently associated with upper aerodigestive and respiratory diseases. HPV 6/11 DNA were detected in lung tumors by nested PCR and in situ hybridization to investigate if any difference in prevalent types of HPV exists between genders. Our data showed that HPV 6 infection was detected in 28.4% (40 of 141) lung tumors, which was significantly higher than that in non-cancer controls (1.7%, 1 of 60; $P < 0.0001$), however, such high prevalence was not observed for HPV 11. Among studied clinico-pathological parameters, HPV 6 infection was significantly related with gender ($P = 0.002$) and smoking status ($P = 0.014$). After being stratified by gender and smoking status, HPV 6 infection rate in lung tumors of non-smoking male patients was much higher than that in non-smoking female patients (33.3% versus 11.1%; $P = 0.023$), but no difference between smoking and non-smoking male patients (38.1% versus 33.3%). With adjustments for age, tumor type, and tumor stage, smoking male lung cancer patients had a much higher OR value (OR, 7.35; 95%CI, 2.11-25.58) for HPV 6 infection compared with 3.93 (95% CI, 1.17-13.12) of non-smoking male patients. Moreover, a higher prevalence of HPV 6 was detected in lung tumors of smoking male patients with early tumor stage than those with advanced stages ($P = 0.008$), but not in non-smoking male and female patients. A higher prevalence of HPV 6 in male lung

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cancer patients, as compared with female lung cancer patients, indicating not only different HPV infection routes for different genders, but also that HPV 6 infections may act as a prospective early risk marker of lung cancer for smoking male patients in Taiwan.
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1. Introduction

Over 100 types of human papillomarvirus (HPV) have been detected to date, and the genomes of nearly 70 types have been sequenced completely [1]. Based on the tumorigenic potential, HPV may be classified into two groups: one group consists of high risk HPVs, such as HPV 16 and 18, which are known to be tumorigenic in human epithelial tissues; the other group consists of low risk HPVs, such as HPV 6 and 11 [2]. Our previous report have indicated that high risk of HPV 16 and 18 types were much more frequently detected in lung tumors of female patients as compared with that of male patients [3]. Therefore, we hypothesized that HPV 16 and/or 18 infections may be originated from cervix and then through blood circulation into lung organ. This possible transmission route was partly confirmed by our recent study showing that the presence of HPV DNA in blood circulation was significantly correlated with the same viral infection in lung tumors [4]. Additionally, our preliminary autosequencing data have revealed identical sequences of L1 and E6 of HPV 16/18 in lung tumors and blood cells. Moreover, the presence of HPV 16/18 in cervical pap smears of female lung cancer patients was correlated with those present in their lung tumors.

Low risk HPV 6 and 11 infections were predominately associated with diseases in upper aerodigestive and respiratory tract, such as carcinomas of oral cavity and pharnx, laryngeal papillomas, respiratory papillomatosis, etc. [5-8]. HPV 6/11 DNA were also found in primary lung neoplasms [9]. Therefore, we hypothesized that gender difference in HPV infections may exist in Taiwanese lung cancer. If a high prevalence of HPV 6/11 infections was found in lung tumors of male patients as compared with that in lung tumors of female patients, the infection route of HPV 6/11 in male lung cancer patients may be predominately mediated through upper aerodigestive and respiratory tracts, not through blood circulation. This result may support the possibility that the infection route of HPV 6/11 in male lung cancer patients was quite different from that of HPV 16/18 type infection in female lung cancer patients that was spread from anogential organs, such as vulva, vagina, and cervix.

2. Patients and methods

2.1. Study subjects

One hundred and forty-one primary lung cancer patients including 45 female and 96 male, who had undergone thoracic surgery at Veterans General Hospital, Taichung, were enrolled. Sixty non-cancer patients with different lung diseases, including pneumothorax, tuberculosis, chest wall deformity, cryptococcal infection, fibrosis, and tuberculosis who had undergone thoracic surgery at Chen-Kung University Hospital, Tainan or Changhua Christian Hospital, Changhua, served as control subjects. None of the subjects had received radiation therapy or chemotherapy prior to surgery. Information on smoking history was obtained from the patients by interview with informed consent. Smokers and non-smokers were defined as current smokers who smoked up to the day of pulmonary surgery and life-time non-smokers, respectively.

2.2. Nested polymerase chain reaction (nested PCR)

Genomic DNA was prepared from a tissue section and isolated by a conventional phenol-chloroform extraction, ethanol precipitation and finally dissolved in 20 μl of sterile distilled water. HPV viral DNA was first amplified with type consensus primers MY09 and MY11 followed by a second round of amplification with type specific primers flanking the L1 region to identify the subtype [10]. The final PCR product of 10 μl was loaded onto a 2% agarose gel, stained with ethidium bromide and visualized under UV illumination. Appropriate negative and positive controls were included in each PCR reaction. A part of the β-actin gene was amplified to exclude false-negative results while DNA preparations from cervical cancer patients containing HPV 6 or HPV 11 were used as positive controls.

2.3. In situ hybridization (ISH)

In situ hybridization (ISH) for the detection of HPV type 6 and 11 DNA was performed by using digenoxenin-labeled (DIG-labeled) oligonucleotide probes and a commercially available hybridiza-

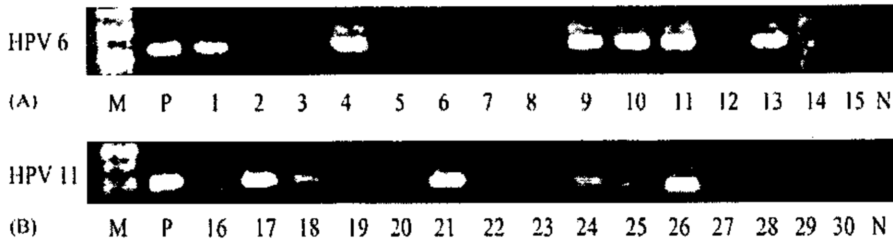

Fig. 1 Representatives of positive and negative HPV 6 (A) and HPV 11 (B) DNA detected by nested PCR in lung tumors of lung cancer patients (Lane 1-30). M, 100 bp ladder DNA marker. P, positive control, DNA of known HPV 6 or HPV HPV 11 positive cervical cancer tumor tissues were served as positive control for HPV 6 and HPV 11, respectively. N, negative control, DNA template was replaced with distilled water.

tion kit (Boehringer Mannheim, Indianapolis, IN). Briefly, the hybridizing probes were prepared by PCR amplification using HPV 6 or 11 type specific primers with DIG-ddUTP as substrate following the manufacturer's instructions [10]. The deparaffinised and rehydrated sections of 5 μm in thickness were digested with proteinase K, rinsed with PBS and dehydrated. The hybridization was performed in a humidified chamber at 48 °C for 16 h followed by washing with sodium chloride-sodium citrate (SSC). Thereafter, the detection reagent (anti-DIG antibody conjugated with alkaline phosphatase) was applied to the sections and then the sections were incubated with the NBT/BCIP solution to allow the signals to develop. After the signal development, the sections were counterstained with methylgreen, rinsed briefly in absolute ethanol, mounted, and observed for signals under a microscope.

2.4. Statistical analysis

Statistical analysis was performed using the SPSS statistical software program (SPSS Inc., Chicago, IL). The difference in HPV 6 or 11 infections between gender, smoking status, tumor type, tumor stage, grade, and TMN value were calculated by χ^2-test. Logistic regression analysis was used to assess which variable was important for HPV infection and which factor may act as a risk factor of lung cancer.

3. Results and discussion

HPV 6 and 11 DNAs were detected by nested PCR in 141 lung tumors and 60 normal lung tissues from non-cancer patients and the representative nested PCR results were shown in Fig. 1. Among these 141 lung tumors, 40 and 14 lung tumors were positive for HPV 6 and 11 DNA (28.4% for HPV 6; 10.0% for HPV 11), respectively (Table 1). However, only 1 of 60 non-cancer cases (1.7%) had HPV 6 DNA, and 8 of 60 (13.3%) non-cancer cases were detected with HPV 11 DNA. A statistical difference between lung cancer and non-cancer group was observed for HPV 6 ($P < 0.0001$), but not for HPV 11 ($P = 0.469$). To confirm these nested PCR data, in situ hybridization was used to detect HPV 6 DNA in 20 lung tumors which were randomly selected from cases. The concordant detection of HPV 6 DNA by nested PCR and in situ hybridization were seen in 86.7% of cases, which were similar to those of HPV 16/18 reported previously [3]. The HPV 6 DNA signals present in lung tumor cells, but not in adjacent normal lung cells, were shown in Fig. 2. Among studied clinico-pathological parameters, HPV 6 infection was significantly related with gender ($P = 0.002$), and smoking status ($P = 0.014$), but not with tumor stage, tumor type, T and N values (Table 2). After being stratified by gender and smoking status, we found a similar prevalence of HPV 6 for smoking and non-smoking male lung cancer patients, which was much higher than that of non-smoking female lung

Table 1 The prevalence of HPV 6/11 in lung tumors and non-cancer controls

HPV type	Lung tumors ($n = 141$) (%)	Non-cancer control ($n = 60$) (%)	P
HPV 6			
Negative ($n = 160$)	101 (71.6)	59 (98.3)	
Positive ($n = 4$)	40 (28.4)	1 (1.7)	<0.0001
HPV 11			
Negative ($n = 179$)	127 (90)	52 (86.7)	
Positive ($n = 22$)	14 (10)	8 (13.3)	0.469

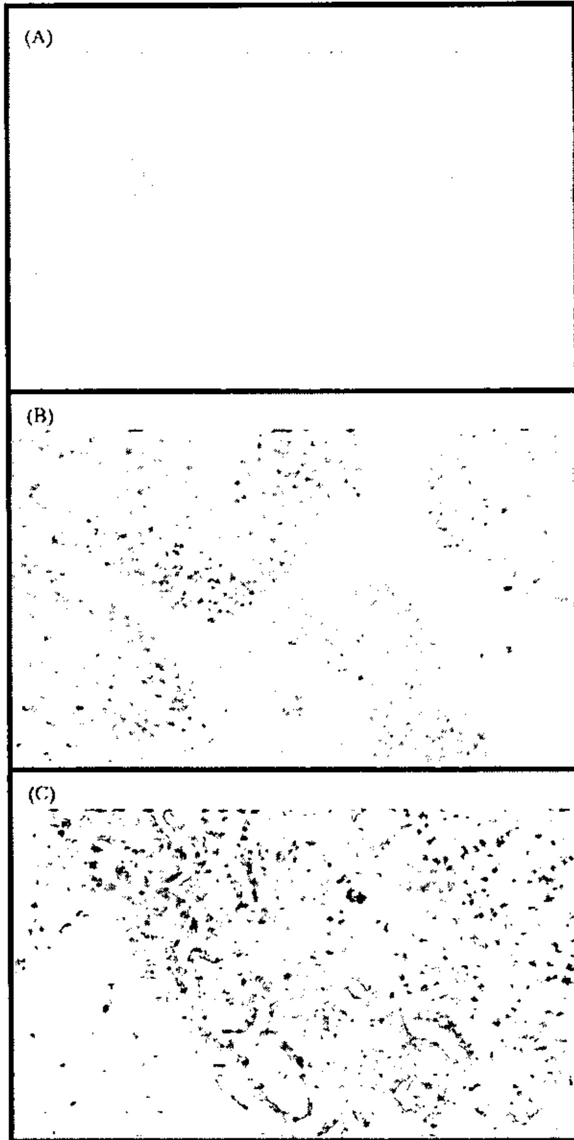

Fig. 2 Representative negative and positive HPV 6 DNA detection by in situ hybridization in paraffin sections of lung tumors, (A) negative detection, (B) positive detection in squamous cell lung carcinoma, (C) positive detection in lung adenocarcinoma.

Table 2 Relationships between HPV 6 infection and clinico-pathological parameters in lung tumors

Parameter	HPV 6		P
	Negative ($n = 101$)	Positive ($n = 40$)	
Gender			
Female ($n = 45$)	40	5	
Male ($n = 96$)	61	35	0.002
Smoking status			
− ($n = 77$)	62	15	
+ ($n = 64$)	39	25	0.014
Tumor type			
AD ($n = 83$)	60	23	
SQ ($n = 58$)	41	17	0.851
Tumor stage			
I ($n = 46$)	29	17	
II ($n = 42$)	28	14	
III ($n = 53$)	44	9	0.062
T factor			
1 ($n = 10$)	7	3	
2 ($n = 91$)	65	26	
3 ($n = 30$)	22	8	
4 ($n = 10$)	7	3	0.995
N factor			
0 ($n = 65$)	41	24	
1 ($n = 34$)	24	10	
2 ($n = 41$)	35	6	
3 ($n = 1$)	1	0	0.065
Grade			
1 ($n = 3$)	2	1	
2 ($n = 87$)	61	26	
3 ($n = 51$)	38	13	0.842

AD, lung adenocarcinomas; SQ, squamous cell lung carcinomas.

cancer patients (Table 3). To avoid the influence of gender imbalance in study subjects, female lung cancer cases were continuously collected and the current case number has reached 98. The infection ratio of HPV 6 in these female lung cancer patients was also lower than that of male (12.24%, 12 of 98 versus 36.5%, 35 of 96). These results strongly suggested that HPV 6 infection may be associated with lung cancer of males, but not with female cases. The OR of HPV 6 infection in lung tumors with various genders and smoking status was statistically analyzed. As showing in Table 4, smoking male lung cancer patients had a similarly significant OR of HPV 6 infection as high as 4.92 (95% CI, 1.71-14.21), which was similarly significant as compared with 4.00 (95% CI, 1.23-12.99) of non-smoking male lung cancer patients. With adjustments for age, tumor type, and tumor stage, smoking male lung cancer patients had a much higher OR value (OR, 7.35; 95%CI, 2.11-25.58) compared with 3.93 (95% CI, 1.17-13.12) of non-smoking male lung cancer patients. Nevertheless, these results suggested that HPV 6 infection is strongly associated with lung cancers of smoking and non-smoking males, but not with that of females in Taiwan.

Our previous reports have revealed a similar infection rate of HPV 16 in lung tumor tissues and blood circulation of female lung cancer patients

Table 3 HPV 6 infection in lung tumors of patients with different genders and smoking status

HPV 6	Non-smoking		Smoking male ($n = 63$) (%)	P
	Female ($n = 45$) (%)	Male ($n = 33$) (%)		
Negative ($n = 101$)	40 (88.9)	22 (66.7)	39 (61.9)	
Positive ($n = 40$)	5 (11.1)	11 (33.3)	24 (38.1)	0.007
P	0.023		0.824	

χ^2-test was used for statistical analysis.

Table 4 Odds ratio of the presence of HPV 6 in lung tumors according to gender and smoking status

Gender	Smoking	HPV 6	
		OR (95% CI)	AOR (95% CI)
Female	No	1	1
Male	No	4.0 (1.23-12.99)	3.93 (1.17-13.12)
Male	Yes	4.92 (1.71-14.21)	7.35 (2.11-25.58)

AOR, odds ratio after adjustment for age, tumor type and tumor stage.

(60.0% in lung tumors versus 57.6% in blood circulation) [4]. However, our preliminary data also showed that HPV 6 infection was detected in blood circulation of only 7.1% (20 of 282) lung cancer cases (unpublished data). The infection rate of HPV 6 in blood circulation was much lower than that in lung tumors, as determined in this study (28.4%, 40 of 141 lung tumors, Table 1). This result suggested that HPV 6 infection may be mediated through upper aerodigestive and respiratory tracts, instead of blood circulation.

Since HPV 6 infection was prevalent in lung cancers of smoking males, to understand whether HPV 6 infection was associated with lung cancer development of smoking males, the correlation between HPV 6 infection and tumor stage was statistically analyzed. Surprisingly, HPV 6 infection was significantly correlated with tumor stage only in smoking male lung cancer cases ($P = 0.008$, Table 5), but not in entire studied cases ($P = 0.065$, Table 2), non-smoking male, or female cases (Table 5). The highest infection rate was from stage I lung tumors of smoking males (11 of 17, 64.7%) followed by stage II (10 of 26, 38.5%) and stage III (3 of 20, 15.0%). This suggested that the presence of HPV 6 DNA in lung tumors may act as an early risk marker for smoking male lung cancer patients.

In the present study, a high prevalence of HPV 6 lung cancers of males compared with that of females seemed to reflect the possibility of different infection routes between genders. Our preliminary data showed that sequences of HPV 16/18 E6, E7 and L1 DNA was identical among various types of samples, including blood lymphocytes, Pap smear and tumors, of female lung cancer patients. Therefore, we suggest that HPV 16/18 infection in female lung tumors may be transmitted from cervix through blood circulation, but HPV 6 infection in male lung cancer patients may be initiated at upper

Table 5 Correlation of HPV 6 infection with tumor stages among three categories of lung tumors

Tumor stage	HPV 6 infection					
	Non-smoking female		Non-smoking male		Smoking male	
	Negative	Positive	Negative	Positive	Negative	Positive
I ($n = 46$)	13	2	10	4	6	11
II ($n = 42$)	7	0	5	4	16	10
III ($n = 53$)	20	3	7	3	17	3
P	0.406		0.713		0.008	

The three categories of lung tumors were grouped based on gender and smoking status.

aerodigestive and respiratory tracts. These different infection routes for different types of HPV between genders may be used to explain why female lung cancer patients had a much higher prevalence of HPV 16/18 infection, but not HPV 6/11. However, more data are needed to evidence the possibility of different HPV infection route of female and male lung cancer patients and various studies including animal model experiment are under investigation in this laboratory.

Acknowledgements

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Clinical and Biological Features of Epidermal Growth Factor Receptor Mutations in Lung Cancers

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ABSTRACT

Background: Recent reports of mutations in the tyrosine kinase (TK) domain of the epidermal growth factor receptor gene (EGFR) in lung cancers have generated considerable interest because they predict for sensitivity to EGFR kinase inhibitors. ***Methods:*** To further understand the role of EGFR mutations in the pathogenesis of lung cancers we sequenced exons 18-21 of the TK domain in 635 lung cancers collected from four different countries as well as 243 other epithelial cancers and compared the mutational status with clinico-pathological features and the presence of KRAS mutations. All statistical tests were two-sided. ***Results:*** EGFR mutations were present in 20% of non-small cell lung cancers and were absent in other types of carcinomas. EGFR mutations were somatic in origin and were significantly more frequent in never smokers, adenocarcinoma histology, Pacific rim countries and female gender. Mutations were not related to patient age, clinical stage, bronchioloalveolar subtype or patient survival. The 134 mutations were of three common types (in-frame deletions of exon 19, a missense mutation in exon 21, and in-frame duplications/insertions of exon 20). Mutations of KRAS were present in 8% of lung cancers, and while they also targeted adenocarcinoma histology, mutations in both genes were never present in individual tumors. ***Conclusions:*** Our findings demonstrate that geographic origin, absence of smoke exposure, gender and histologic type influence the frequencies of EGFR mutations in lung cancers, and indicate that the pathogenesis of EGFR mutant and KRAS mutant adenocarcinomas are different and that unidentified carcinogen(s) other than environmental tobacco smoke contribute to the origin of lung cancers arising in never smokers.

INTRODUCTION

Despite improvements in diagnostic and therapeutic approaches, lung cancer is the major cause of cancer related deaths (*1,2*). Non- small cell lung cancer (NSCLC), the major form of lung cancer, is divided into adenocarcinoma, squamous, and large cell carcinoma types and adenocarcinoma is the most frequent histological type in both genders in many parts of the world. Although smoking is the major cause of all lung cancers, adenocarcinoma is relatively less strongly associated with smoking (*3*). Lung cancer is also characterized by an accumulation of multiple genetic and/or epigenetic alterations including activation of oncogenes and inactivation of tumor suppressor genes (*4-6*). Increased understanding of molecular mechanism may provide new and more effective strategies for chemoprevention, early diagnosis, and targeted treatment for lung cancers (*7*).

Deregulation of protein kinases is common in malignancies (*8,9*) and has led to the development of therapies that target these oncogenes (*10*). Gefitinib (Iressa, ZD1839) is a small molecule tyrosine kinase (TK) inhibitor that targets the epidermal growth factor receptor (EGFR) which is highly expressed in many epithelial cancers and has been widely utilized for the treatment of NSCLC (*11*). Several preclinical and clinical trials showed that gefitinib treatment appeared promising for advanced NSCLC (*12-15*), however, the mechanism of antitumor effect or drug sensitivity has not been fully established because neither EGFR expression nor phosphorylation status correlate with the response to gefitinib (*16*). Although the factors predicting for clinical benefits from gefinitib treatment were not understood, some patients had dramatic and durable

responses (*14*). Recent reports of EGFR mutations in the TK domain have generated considerable interest because they predict for sensitivity to gefitinib therapy (*17,18*). All mutations occurred within the TK domain where gefitinib competes with adenosine triphosphate (ATP) for binding and these mutants retained response to ligand and showed increased sensitivity to gefitinib *in vitro* (*17*). While only relatively small numbers of tumors were analyzed, the mutations were more frequent in patients with adenocarcinoma histology, female gender and Japanese origin (*18*), which are the same subpopulations having the highest response rates to gefitinib (*14,15,19,20*).

In this report, we searched for mutations of EGFR gene in a large number of primary lung tumors from four countries (Japan, Taiwan, USA, and Australia) to determine whether there was a relationship between EGFR mutation and gender, age, histology, clinical stage, smoking history, and geography. In addition, to understand the relationship between EGFR and KRAS, which is one of the important downstream genes of the EGFR signaling pathway and is frequently mutated in lung cancers (*21*), we also examined mutations of KRAS codons 12 and 13.

PATIENTS AND METHODS

Tumor Samples

A total of 617 selected and unselected NSCLC tumors were obtained from patients undergoing curative intent surgical resections. While most samples were unselected (Table 1), additional cases with well documented smoking histories were collected from the USA and Australia. The samples were from Japan (Chiba University, Chiba, and Okayama University, Okayama, n = 263), Taiwan (Veterans General Hospital, Taichung,

n = 92), USA (MD Anderson Cancer Center, Houston, n = 161), and Australia (Prince Charles Hospital, Brisbane, n = 101). Corresponding non-malignant tissues located far from the tumor site were also obtained from cases from Japan, USA and Australia. Six primary small cell lung cancers (SCLCs) were obtained from Chiba University and 12 bronchial carcinoids were obtained from MD Anderson Cancer Center. Two hundred and forty three carcinomas arising at other sites were obtained from the USA (prostate, bladder, breast, and colorectal cancer) or Chile (gallbladder cancer). Institutional Review Board permission and informed consent were obtained at each collection site. Clinical information including gender, age, histology, clinical stage, and smoking history were available for all samples and survival for sites other than Taiwan. Clinical staging was based on the revised International System for Staging Lung Cancer (*22,23*).

DNA Extraction and Sequencing

Genomic DNA was obtained from primary tumors by digestion with sodium dodecyl sulfate and proteinase K (Life Technologies Inc., Rockville, MD) at 37°C overnight followed by standard phenol-chloroform (1:1) extraction and ethanol precipitation.

The intron-based polymerase chain reaction (PCR) primer sequences for four examined exons of EGFR were as follows (forward and reverse, respectively): Exon 18 (AGC ATG GTG AGG GCT GAG GTG AC and ATA TAC AGC TTG CAA GGA CTC TGG), Exon 19 (CCA GAT CAC TGG GCA GCA TGT GGC ACC and AGC AGG GTC TAG AGC AGA GCA GCT GCC), Exon 20 (GAT CGC ATT CAT GCG TCT TCA CC and TTG CTA TCC CAG GAG CGC AGA CC), Exon 21 (TCA GAG CCT GGC ATG AAC ATG ACC CTG and GGT CCC TGG TGT CAG GAA AAT GCT GG), Exon 22

(AAT TAG GTC CAG AGT GAG TTA AC and ACT TGC ATG TCA GAG GAT ATA ATG), Exon 23 (CAT CAA GAA ACA GTA ACC AGT AAT G and AAG GCC TCA GCT GTT TGG CTA AG), and Exon 24 (TTG ACT GGA AGT GTC GCA TCA CC and CAT GTG ACA GAA CAC AGT GAC ATG). All PCRs were carried out in 25μl volume containing 100 ng of genomic DNA using HotStarTaq DNA polymerase (QIAGEN Inc., Valencia, CA). DNA was amplified for 33 cycles at 95 °C for 30 seconds, 65 °C for 30 seconds, and 72 °C for 45 seconds, followed by 7 min extension at 72 °C. The intron-based PCR primer sequences for exon 2 of *KRAS* were as follows (forward and reverse, respectively): (GTA TTA ACC TTA TGT GTG ACA and GTC CTG CAC CAG TAA TAT GC). DNA was amplified for 33 cycles at 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds, followed by 7 minutes extension at 72 °C. All PCR products were incubated using exonuclease I and shrimp alkaline phosphatase (Amersham Biosciences Corp., Piscataway, NJ) and sequenced directly using Applied Biosystems PRISM dye terminator cycle sequencing method (Perkin-Elmer Corp., Foster City, CA). All sequence variants were confirmed by independent PCR amplifications and sequenced in both directions.

Statistical Analyses

Unconditional Chi-square test was used to assess the relation between EGFR mutations and each factor. Effects of geographic region, gender, histology type, and smoking status were also examined after being adjusted for each other using logistic regression models with EGFR mutations as the outcome. Kaplan-Meier curves were drawn for the two groups with and without the mutations. The agreement between EGFR

and KRAS mutations was tested and the Kappa coefficient was determined. The agreement between the four studied exons of the TK domain was tested among those unselected cases with EGFR mutations. All statistical tests were two-sided and *P* values less than 0.05 were considered statistically significant.

RESULTS

Preliminary sequencing of the EGFR TK domain (exons 18-24) indicated that mutations were limited to the first four exons (exons 18 – 21) coding for the N lobe and part of the C lobe (Fig. 1). Further analyses were limited to these exons. Mutations were limited to NSCLC and were absent in neuroendocrine lung tumors (SCLC and bronchial carcinoids) and carcinomas from other sites (Table 1). A total of 120 mutations were detected in 519 unselected cases (23%) (Table 1). They preferentially targeted Pacific rim countries (Japan and Taiwan) (86%), female gender (60%), never smokers (71%), and adenocarcinoma histology (95%). Logistic regression models confirmed that these variables were independent. The variables were examined after adjustment for each other with EGFR mutations as the outcome, and all the factors as explanatory variables and no interactions were found. Even when the analysis was confined to adenocarcinomas in never smokers (utilizing selected and unselected cases, n = 157), EGFR mutations were found in 64% of patients from the Pacific rim, and 36% from "Western" countries (USA and Australia). The difference was significant before and after adjustment for gender (*P* = 0.003 and 0.004 respectively). For 160 USA cases with detailed smoking data, 3.4% of current smokers, 8.1% of former smokers and 20.0% of never smokers had *EGFR* mutations. There was a significant trend effect of smoking status with an adjusted *P*

value of 0.018. There were no significant differences noted in mutational patterns relative to gender, smoking status or geographic origin.

Because EGFR mutations have been reported to target the bronchioloalveolar (BAC) subtype of adenocarcinoma, two pathologists (IIW and AFG) familiar with the World Health Organization (WHO) classification of lung cancers (which states that BAC is a true non-invasive cancer without stromal or pleural invasion) (23,24) reviewed 99 adenocarcinomas from the USA. BAC subtype was present in 7 of 85 (8%) of adenocarcinomas without EGFR mutations and in none of 14 tumors with mutations.

For all NSCLC, 134 mutations were found in 130 tumors, and in the 95 cases in which adjacent non-malignant lung tissue was available, mutations were absent, indicating that they were somatic in origin. The mutations consisted of three very different types and were located in or near functionally important sites (Table 2; Fig. 1). In-frame deletions of exon 19 (Δ1-11), involving 3 to 7 codons centered around the uniformly deleted codons 747 to 749 (Leu-Arg-Glu sequence) accounted for 62 (46%) of the mutations, and were occasionally accompanied by missense mutations at the carboxyl terminal amino acid position flanking the deletion. Missense mutations (n = 60, 45%) in exons 18, 20 or 21 were the second most common mutation, especially mutation L858R (M1) in exon 21 (n = 52, 39%). In-frame duplications/insertions of one to three codons in exon 20 (D1-8) involving amino acids 770 to 776 accounted for 12 (9%) of the mutations. In addition, a silent mutation was detected in codon 718 in exon 18. Except for three tumors each having two or three rare mutations, the mutations were totally exclusionary of each other with a negative Kappa coefficient indicating that the agreement was even worse than the random assumption under the null hypothesis.

While only semi-quantitative, the sequence electropherograms provide clues to relative ratios of the mutant and wild type alleles. These are easier to interpret with the in frame deletions and duplication/insertions as the entire sequences subsequent to the mutations differ from the wild type. Because tumor specimens invariably contain a considerable percentage of non-malignant cells, we interpreted electropherograms with mutant form equal to or greater than the corresponding wild type sequence to indicate amplification of the former. Using these criteria, 51 of 134 (38%) electropherograms suggested amplification of the mutant form (Fig. 2).

Because KRAS mutations in lung cancers are also almost exclusively limited to adenocarcinomas (*21*), we analyzed the adenocarcinomas for KRAS mutations in codons 12 and 13 (the codons involved in the vast majority of lung cancers mutations). KRAS mutations were present in 12% of adenocarcinomas and were more frequent in ever smokers ($P = 0.029$) and in "Western" countries ($P = 0.066$), but there was no significant relation with gender ($P = 0.6$). Mutations in EGFR and KRAS were completely mutually exclusionary, with a negative Kappa coefficient. For the unselected cases, there were no effects of age or bronchioloalveolar subtype of adenocarcinoma on EGFR mutational rate. For American adenocarcinoma cases, mutation rates in early (stages I and II) (10 of 67, 15%) and advanced (stages III and IV) (5 of 28, 18%) were similar ($P = 0.76$). Survival analyses also indicated no differences between those with or without mutations ($P = 0.46$, log-rank test; Fig. 3).

Of the 93 Taiwanese cases, 58 had been previously studied for the presence of high risk strains 16 and 18 of human papillomavirus (HPV) (*25*). Of these cases, 32 (55%) tumors tested positive for HPV DNA, and has been previously reported, they

targeted women (75%) and never smokers (67%). Of the 58 cases, 12 cases had an EGFR mutation. The concordance between the two tests was 53% ($P = 0.42$).

DISCUSSION

Because preliminary data suggest that lung cancer patients with EGFR mutations respond to drugs that target the TK domain of the gene (*17,18*), a detailed study of the mutational patterns and their relationship to clinico-pathological features is essential. In this study, we tested for EGFR mutations in a large number of primary lung and other cancers and explored the relationship between EGFR mutation status and multiple parameters including KRAS mutations and the presence of HPV DNA. Mutations were limited to lung cancers of the NSCLC type, and absent in neuroendocrine lung cancers and in cancers (mainly adenocarcinomas) arising in the other cancer sites, including those of breast, colorectal and prostate origins. The mutations were limited to the first four exons (exons 18-21) of the TK domain. They were of three types – and 94% (the major mutations) consisted of deletions in exon 19, duplication/insertions in exon 20 and a single point mutation in exon 21. The remaining 6% consisted of rare missense mutations, mainly in exon 18, but also in exons 20 and 21. A total of 28 varieties of mutations were identified and designated according to type. What do these very different mutation types have in common? They all target structures around the ATP binding cleft (which is also the docking site of the small molecule EGFR inhibitors) (*26*), including the αC-helix, the activation loop (A-loop) and the phosphate binding loop (P-loop). The L858R mutation located on near the conserved Asp-Phe-Gly sequence stabilizes the A-loop (*27*). The other two common mutations occur around the critical αC-helix in the N lobe (Fig. 1). We hypothesize that both mutations result in similar configurational changes causing a shift of the helical axis, narrowing the ATP binding cleft and resulting in both increased gene activation and TK inhibitor sensitivity. While the necessity for

ligand binding remains, at least two of the common mutations (deletions in exon 19 and the L858R missense mutation) are known to increase the amount and duration of ligand dependent activation and explain the much greater sensitivity of mutant cells to EGFR inhibitors (*18,20*). Most of the rare mutations target the P-loop. Of interest, the major mutations always occurred as single mutations in individual tumors, while the rare mutations usually occurred as multiple mutations in individual tumors. These results suggest that the tumorigenic effects of the minor mutations may not be as powerful as those of the major mutation types. Additional investigations are required to elucidate the relationship between each mutation type and TK inhibitor sensitivity and to tumorigenesis.

Examination of the electropherograms indicated that mutations often occurred in a setting of allelic imbalance, with the mutant allele in excess of the wild type allele. Polysomy or amplification of the EGFR gene occurs in many cancers including NSCLC, and may occur relatively early during multistage pathogenesis (*28,29*). Our findings suggest that a combination of mutation and increased copy number of the mutant allele may play a role in lung cancer pathogenesis (and perhaps to response to EGFR targeted therapy).

For unselected cases there were significantly higher mutation frequencies for adenocarcinoma histology, never smoker status, Pacific rim country origin (Taiwan and Japan) and female gender. Multivariate analyses confirmed that these factors were independent. These findings are consistent with the preliminary results previously published (*17,18*) and with the features associated with response to gefitinib (*14*). There was no relationship between mutational status and patient survival (in the absence of

EGFR inhibitor therapy), with patient age or with tumor stage. While responses to gefitinib have been reported to be higher in tumors having BAC features (*14*), we found no association with the BAC subtype of adenocarcinoma, using the strict criteria as stated by the WHO Classification of lung tumors (*23*). This classification defines BAC as "an adenocarcinoma with a pure bronchioloalveolar growth pattern and no evidence of stromal, vascular or pleural invasion". This strict definition implies that BAC is at a non-invasive stage of growth and perhaps the precursor form of most invasive peripheral adenocarcinomas. Unfortunately these strict criteria for diagnosing the BAC subtype are not applied uniformly by many pathologists, leading to considerable confusion in the literature.

DNA sequences of the high risk HPV strains 16 and 18 have been detected in lung cancers in Taiwan (*25*), and less frequently in other parts of the world. In Taiwan these sequences are more frequent in women never smokers who develop lung cancer, which usually are of the adenocarcinoma type. Thus, the distribution of HPV sequences in lung cancers shows similarities with the pattern of EGFR mutations and with response to gefitinib therapy. However, we found no relationship between these HPV sequences and EGFR mutations in our Taiwanese cases.

With the possible exception of HPV sequences, EGFR mutations are the first known mutations to target lung cancers arising in never smokers, and are more frequent in Pacific rim countries and women. They are almost entirely limited to the adenocarcinoma type of lung cancer. KRAS mutations are also relatively frequent in NSCLC, especially in adenocarcinomas (*30*). However KRAS mutations target smokers and "Western" countries although a male gender bias was not detected, contrary to prior

reports. While one or the other of the two forms of gene mutations were present in nearly 50% of lung adenocarcinomas, they were completely exclusionary. Mutations of EGFR activate multiple downstream signaling pathways including RAS, JAK-STAT and AKT (*31,32*). Our findings suggest that activation of either EGFR or RAS signaling pathways have similar effects on lung carcinogenesis, obviating the necessity for both forms of mutations. In contrast to other cancers, most KRAS and TP53 mutations in lung cancers are G:T transversions, molecular events that are believed to be linked to exposure to tobacco smoke carcinogens (*33,34*). Lung cancers arising in never smokers rarely have KRAS mutations, and their TP53 mutations are seldom G:T transversions, suggesting that these cancers arose after exposure to carcinogens other than those present in tobacco smoke. Our findings suggest that at least two distinct molecular pathways are involved in the pathogenesis of lung adenocarcinomas, one involving EGFR mutations and the other involving KRAS mutations. The very different pattern of mutations in KRAS, EGFR and TP53 genes and the presence of HPV DNA in ever and never smokers suggest that exposure to environmental tobacco smoke carcinogens may not be the major pathogenetic factor involved in the origin of lung cancers in never smokers, but that as yet unidentified carcinogen(s) play(s) an important role (*35*). Because mutations of the TK domain of EGFR are more frequent in Pacific rim countries and have not been identified in other human carcinomas, exposure to the hypothetical carcinogen(s) may be more common in certain geographic regions and may not play a role in the pathogenesis of other human carcinomas.

In summary, our findings suggest that activating mutations of EGFR target a subset of lung adenocarcinoma while KRAS mutations target a different subset. EGFR mutations are the first molecular change known to specifically target never smokers.

NOTES

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Additional PCR primers sequences and conditions used in this study for analysis of the entire EGFR gene are available on request from A. F. Gazdar and have been deposited with the Editorial office of the Journal. We thank Dr. Margaret Spitz for obtaining some of the detailed smoke exposure histories.

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TABLES

(Note: these are the Table legends, while actual tables are submitted as an Excel worksheet).

Table 1. Frequencies of EGFR mutations in unselected resected NSCLC and their relationship to gender, smoking status and histologic subtype. A total of 123 mutations were found in 120 of 519 tumors (23%). Significantly higher rates of mutations were present in never smokers, adenocarcinoma histology, female gender and in Pacific rim countries (Japan and Taiwan).

Table 2. Types of mutations in tyrosine kinase domain of EGFR (n=134, plus one silent mutation) in all cases studied, both selected and unselected (n = 635) (also see Fig. 1). The mutations consisted of three major forms accounting for 94% of the mutations: 1) in frame deletions in exon 19 with a common deleted region consisting of codons 747-749 (11 varieties designated Δ1 to Δ11 identified); 2) a single missense mutation, L858R, designated M1; and 3) in frame duplication/insertions 1-3 codons in length involving codons 770-776 (8 varieties identified, designated D1 to D8). In addition, 6% of the mutations were relatively rare or minor missense mutations mainly in exon 18, but also in exons 20 and 21 (8 varieties identified, designated M2 to M9). The 134 mutations were present in 130 tumors. Single mutations were present in 127 cases and multiple mutations in three cases. One tumor, arising in an Australian never smoker, had three minor mutations. One tumor, arising in a Japanese smoker with a 38 year pack year exposure history had two minor mutations. A third case arising in an Australian never

smoker with adenosquamous carcinoma, had two minor mutations and in addition a silent mutation, L718L.

FIGURE LEGENDS

Fig. 1. Locations and types of EGFR mutations in lung cancers (also see Table 2). The 134 mutations were limited to non-small cell lung cancer and were located in the first four exons (exons 18-21) of the tyrosine kinase (TK) domain. The mutations consisted of three major types that compromised 94% of the mutations. These three major types were in frame deletions in exon 19 (Δ1-11) 3-7 codons in length invoving a common deleted region of codons 747-749, in frame duplications/insertions (D1-8) involving a small region of exon 20 (one to three codons involving codons 770-776), and a single missense point mutation, L858R (M1) in exon 21. These major mutations always occurred as single mutations. In addition, rare missense mutations (M2 to M9) mainly in exon 18 but also in exons 20 and 21, constituted the remaining 6% of mutations. The mutations targeted critical structures surrounding the ATP binding cleft (which lies between the N and C lobes). The major deletions and insertions form an interesting tandem around the critical αC-helix in the N lobe. Both mutations are hypothesized to result in similar configurational changes causing a shift of the helical axis, narrowing the adenosine triphosphate (ATP) binding cleft and resulting in both increased gene activation and TK inhibitor sensitivity. The L858R (M1) mutation is located in the Activation loop (A-loop). Most of the minor missense mutations (M2 to M7) occurred in or near the Phosphate binding loop (P-loop) while M8 occurred in the αC-helix and M9 in the region of the A-loop. In contrast to the major mutations, the minor mutations often occurred as multiple mutations (two or three) in individual tumors.

Fig. 2. Electropherograms demonstrating mutational patterns (see text). The three major forms of mutations are illustrated. While only semi-quantitative, they suggest allelic imbalance, with the mutant form in excess over the wild type form. Panel A, 15 bp deletion (Δ2) in exon 19. Panel B, 9 bp duplication/insertion (D1) in exon 20. Panel C, L858R (M1) missense mutation in exon 21.

Fig. 3. Kaplan-Meier survival curves of non-small cell lung cancer patients undergoing curative intent resections who did not receive gefitinib or other EGFR targeted therapy. There are no significant differences in the survival curves of patients with and without EGFR mutations.

Table 1. The univariate analysis on the relation between EGFR mutation and gender, smoking status, and histologic subtype within and across all 4 countries (519 unselected cases)

Country (No.)*	Mutation (%)	Gender (No.)	Mutation (%)	*P* value	Smoking status (No.)	Mutation (%)	*P* value	Histologic subtype (No.)	Mutation (%)	*P* value
Japan (263)	71 (27)	Male (183)	30 (16)	< 001	Never smoker (78)	47 (60)	< 001	Adenocarcinoma (154)	67 (44)	<.001
		Female (80)	41 (51)		Smoker (185)	24 (13)		Others[†] (109)	4 (4)	
Taiwan (93)	32 (34)	Male (64)	14 (22)	< .001	Neve smoker (55)	27 (49)	< 001	Adenocarcinoma (55)	31 (56)	<.001
		Female (29)	18 (62)		Smoker (38)	5 (13)		Others (38)	1 (3)	
USA (80)	11 (14)	Male (43)	3 (7)	116	Never smoker (26)	7 (27)	.043	Adenocarcinoma (44)	11 (25)	<.001
		Female(37)	8 (22)		Smoker (54)	4 (7)		Others (36)	0 (0)	
Australia (83)	6 (7)	Male (58)	1 (2)	013	Never smoker (7)	4 (57)	<.001	Adenocarcinoma (36)	5 (14)	081
		Female (25)	5 (6)		Smoker (76)	2 (3)		Others (47)	1 (2)	
Total (519)	120 (23)	Male (348)	48 (14)	< 001	Never smoker (166)	85 (51)	<.001	Adenocarcinoma (289)	114 (40)	<.001
		Female (171)	72 (42)		Smoker (353)	35 (10)		Others (230)	6 (3)	

**P* value for testing the relation between EGFR mutation and country is less than 0.001 (Japan and Taiwan versus USA and Australia).

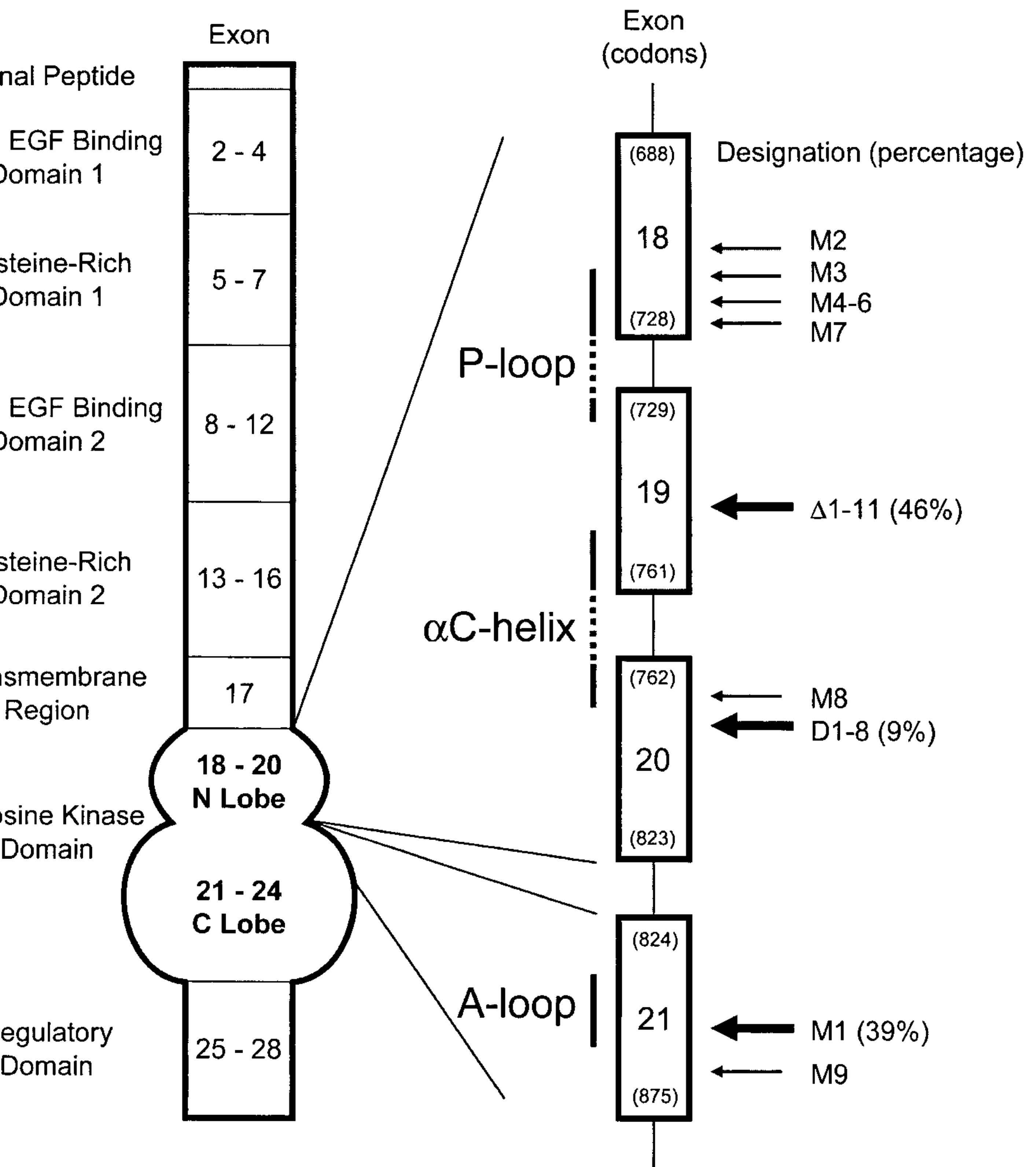
[†]Others include squamous cell, adeno-squamous cell, and large cell carcinoma.

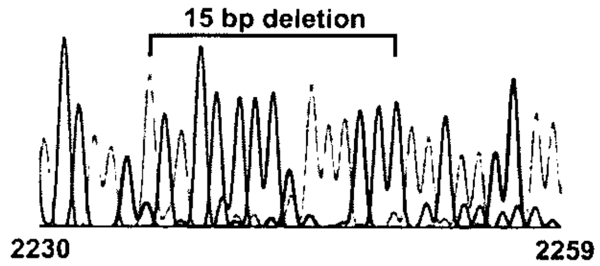
Table 2. Analysis of 134 mutations in kinase domain of EGFR

Type of mutation	Exon	Designation	Nucleotide	Amino acid	No. of mutation (%
In-frame deletions	19	Δ1	2235-2249 del GGAATTAAGAGAAGC	E746-A750 del	27 (20)
		Δ2	2236-2250 del GAATTAAGAGAAGCA	E746-A750 del	15 (11)
		Δ3	2240-2254 del TAAGAGAAGCAACAT	L747-T751 del	4 (3)
		Δ4	2239-2247 del TTAAGAGAA, 2248 G>C	L747-E749 del, A750P	3 (2)
		Δ5	2239-2250 del TTAAGAGAAGCA, 2251 A>C	L747-T750 del, T751P	3 (2)
		Δ6	2240-2257 del TAAGAGAAGCAACATCTC	L747-S752 del, P753S	3 (2)
		Δ7	2237-2254 del AATTAAGAGAAGCAACAT, 2255 C>T	E746-T751 del, S752V	2 (1.5)
		Δ8	2239-2256 del TTAAGAGAAGCAACATCT	L747-S752 del	2 (1.5)
		Δ9	2235-2252 del GGAATTAAGAGAAGCAAC, 2254 T>A, 2255 C>	E746-T751 del, S752I	1 (1)
		Δ10	2237-2251 del AATTAAGAGAAGCAA, 2252 C>T	E746-A750 del, T751V	1 (1)
		Δ11	2239-2256 del TTAAGAGAAGCAACATCT, 2258 C>A	L747-S752 del, P753Q	1 (1)
Total					62 (46)
Substitution of single nucleotide*	21	M1	2573 T>G	L858R	52 (39)
	18	M2	2126 A>T	E709V	1 (1)
	18	M3	2144 T>G	I715S	1 (1)
	18	M4	2155 G>T	G719C	1 (1)
	18	M5	2155 G>A	G719S	1 (1)
	18	M6	2156 G>C	G719A	1 (1)
	18	M7	2159 C>T	S720F	1 (1)
	20	M8	2303 G>T	S768I	1 (1)
	21	M9	2582 T>G	L861Q	1 (1)
Total					60 (45)
In-frame duplications / insertions	20	D1	2308-2316 ins GCCAGCGTG	ASV770-772 ins	4 (3)
		D2	2320-2322 ins CAC	H774 ins	2 (1.5)
		D3	2311-2313 ins GGT	G771 ins	1 (1)
		D4	2308-2313 ins TGCGTG	CV770-771 ins	1 (1)
		D5	2317-2222 ins AACCCC, 2223 C>T	NP773-774 ins, H775Y	1 (1)
		D6	2320-2325 ins CCCCAC	PH774-775 ins	1 (1)
		D7	2320-2328 ins AACCCCCAC	NPH774-776 ins	1 (1)
		D8	2323-2328 ins CACGTG, 2322 G>C	HV775-776 ins	1 (1)
Total					12 (9)

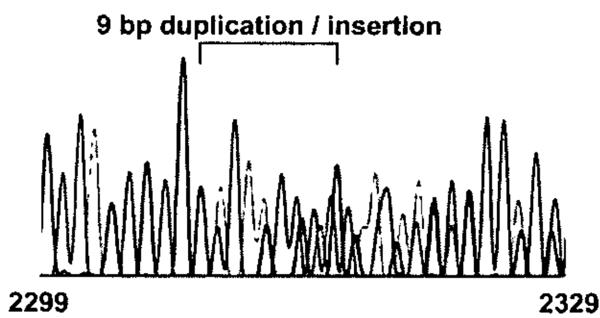
*Patients had multiple mutations as follows; Japan # 316: G719S and S768I; Australia # 108: I715S, S720F,and L861Q;
Australia # 364: E709, L718L (silent), and G719C.

g. 1

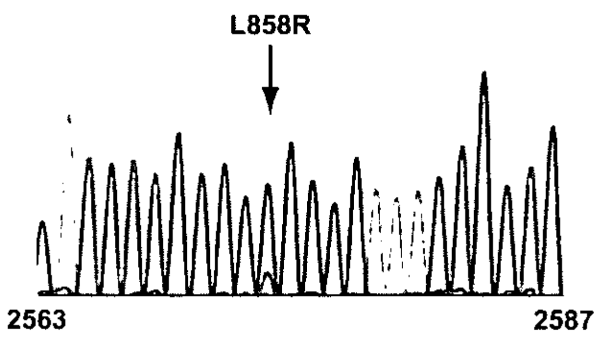




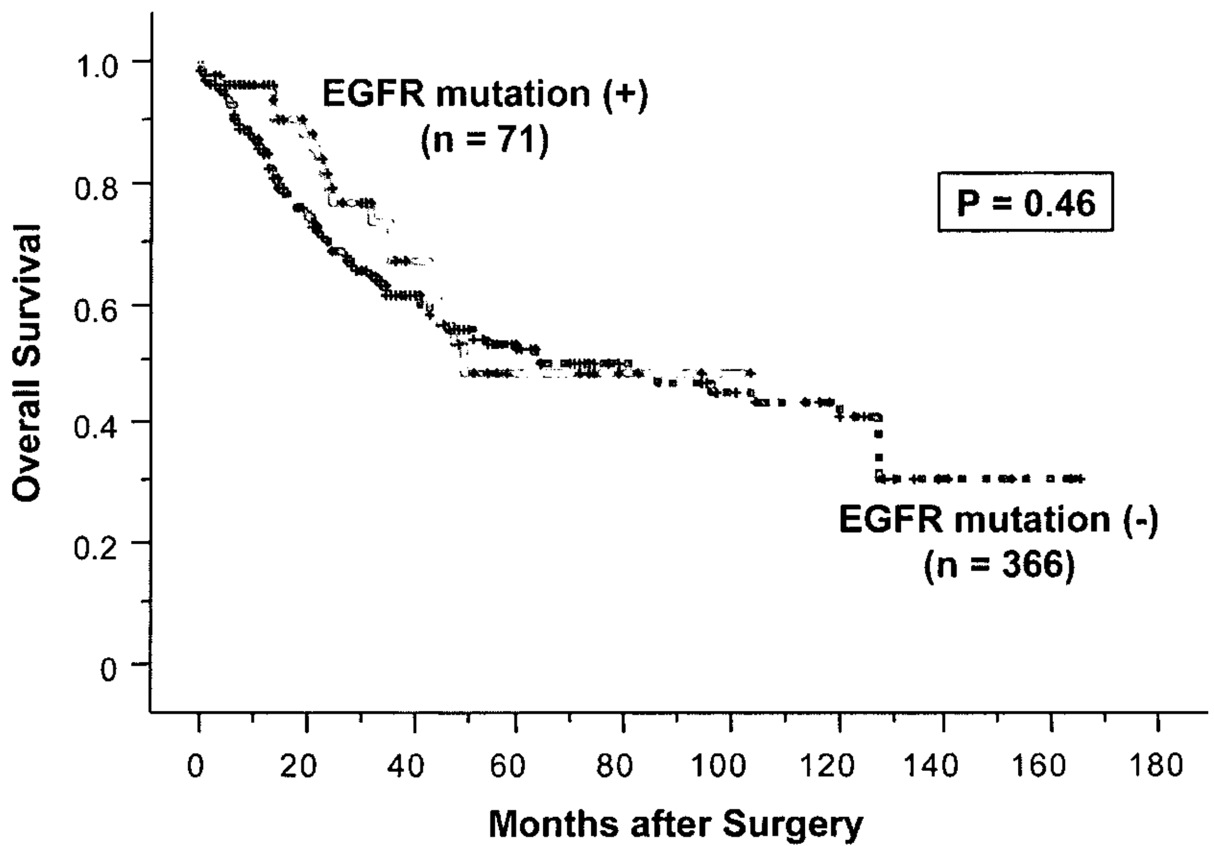
A
15 bp deletion
2230
2259
2230
2259
Exon19
A
G
C



B
Lung
Japan #87
Tumor
9 bp duplication / insertion
2299
2329
2299
2329
Exon20



C
Lung
USA #493
Tumor
L858R
2563
2587
2563
2587
Exon21


EGFR mutation (+)
(n = 71)
P = 0.46
EGFR mutation (-)
(n = 366)
Overall Survival
1.0
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
140
160
180
Months after Surgery

FREQUENT p16INK4a PROMOTER HYPERMETHYLATION IN HUMAN PAPILLOMAVIRUS-INFECTED FEMALE LUNG CANCER IN TAIWAN

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Key words: p16INK4a promoter hypermethylation, human papillomavirus, lung cancer.
Running Title: p16INK4a promoter hypermethylation and HPV infection in lung cancer

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ABSTRACT

Inactivation of p16INK4a gene through promoter hypermethylation has been frequently observed in non-small cell lung cancer, however, various studies have shown a controversial correlation between p16INK4a hypermethylation and cigarette smoking. Our recent report showed that human papillomarvirus (HPV) 16/18 infections were associated with the development of nonsmoking female lung cancer in Taiwan and we further speculated that HPV infection may be linked with p16INK4a hypermethylation. To verify the influence of environmental exposure including cigarette smoking, environmental carcinogen exposure, and HPV infections on p16INK4a hypermethylation, tumors from 162 lung patients including 67 smoking males, 41 nonsmoking males and 58 nonsmoking females, were subjected to p16INK4a hypermethylation analysis by methylation-specific PCR (MSP). As the results shown, p16INK4a hypermethylation was detected in 40 (59.7%) of 67 smoking male, 15 (36.6%) of 41 nonsmoking male, and 35 (60.3%) of 58 nonsmoking female lung tumors. This result seemed to reveal that gender and cigarette smoking both possesses an equal influence on p16INK4a hypermethylation. This result also led to a speculation that HPV infection may promote p16INK4a hypermethylation in nonsmoking female lung cancer patients. From our data, p16INK4a hypermethylation frequency in nonsmoking female lung tumors with HPV infection was as high as 70% (30 of 43) compared to those without HPV infection (33%, 5 of 15). In fact, the correlation between HPV infection and p16INK4a hypermethylation was only observed in nonsmoking female lung tumors ($P = 0.017$), but not in smoking male or nonsmoking male lung tumors. Moreover, the reverse correlation between p16INK4a immunostaining and p16INK4a promoter hypermethylation was also only observed in nonsmoking female lung tumors. These results strongly suggested that the involvement of HPV infection in lung tumorigenesis of nonsmoking female cancer patients in Taiwan may be mediated, at least in part, through the increase of hypermethylation to cause p16INK4a

inactivation.

INTRODUCTION

Being mapped to a critical region at chromosome 9p21, which frequently undergoes allele loss, p16INK4a exerts its function by binding to cyclin-dependent protein kinase 4 (CDK4) and inhibits the ability of CDK4 to interact with cyclin D1[1,2]. Several reports indicated that hypermethylation of p16INK4a occurs frequently in non-small cell lung cancer (NSCLC)[3-5]. Belinsky et al.[6] even linked p16INK4a hypermethylation to an early stage in the pathogenesis of lung cancer and suggested that p16INK4a hypermethylation in serum DNA may serve as an early diagnostic marker of lung cancer.

Environment-epigenetic interactions may play a role in the geographic variation of human cancer incidence. Aberrant hypermethylation in the CpG-rich promoter regions of many tumor suppressor genes was associated with the lack of gene transcription and thus, contributed to the formation and progression of lung cancer, such as p16INK4a, DAPK, GSTP1, MGMT, etc[6-9]. Cigarette smoking has been suggested to be the most important causative factor in lung cancer, however, less than 10% of Taiwanese female lung cancer patients were smokers[10]. Thus, environmental exposure other than active cigarette smoking may contribute to the development of nonsmoking female lung cancer in Taiwan. It is conceivable that the role of environment-epigenetic interactions may play a role in lung tumorigenesis of nonsmoking female lung cancer. Our previous reports have revealed that p53 mutation was detected in less than 10% of nonsmoking female lung tumors[10]. This finding led to a hypothesis that an inactivation of p16INK4a tumor suppressor gene through promoter hypermethylation may be involved in lung tumorigenesis of these patients. Several reports showed that p16INK4a hypermethylation was correlated with tobacco carcinogen exposure[11-14], but conflicting results were also reported by other groups[15,22]. Viral exposure is another possible factor to cause epigenetic alterations in human cancer, such as the association of HBV and HCV infections

with promoter hypermethylation of p16INK4a and estrogen receptor in HCC[15-16] and the association of Simian virus 40 with RASSF1A promoter hypermethylation in malignant mesothelima[17-18]. Furthermore, our recent report showed that HPV 16/18 infection was associated with lung cancer in Taiwanese women nonsmokers[19]. In this study, p16INK4a hypermethylation status were compared among smoking male, nonsmoking male and female lung cancer patients to verify which factors including cigarette smoking, exposure to environmental carcinogens, and HPV infection, were more important in inducing epigenetic alteration for p16INK4a gene inactivation.

PATIENTS AND METHODS

Study subjects

Studied cancer tissues, obtained by surgical resections, were from 166 NSCLC patients, including 67 smoking males, 41 nonsmoking males and 58 nonsmoking females, admitted to Taichung Veterans General Hospital, Taichung, Taiwan between 1993 and 2001. No-smoking female lung tumors were available for this study since less than 10% of female lung cancer patients were smokers in Taiwan. The histology, types and stages of tumors were determined according to the WHO classification method (WHO, 1982). Information on smoking history of lung cancer patients was obtained from hospital records and used to categorize patients into smoking and non-smoking groups. Tissues were stored at −80°C right after resections until used.

DNA extraction

Homogenized tissues were digested with proteinase K (100 μg/ml) in 10 mM Tris, 0.1 M NaCl, 25 mM EDTA (pH 8.0), and 0.5% SDS at 56℃ for least 16-18 hr. Genomic DNA was isolated by conventional phenol-chloroform extraction and ethanol precipitation and finally dissolved in 20 μl of sterile distilled water.

Methylation-specific PCR (MSP) and direct sequencing

MSP was conducted according to procedures described in a previous study[20]. Briefly, approximate 6 μg of extracted DNA was treated with sodium bisulfate (Sigma, USA) and purified using Wizard DNA Clean-Up System (Promega Corporation, USA) according to the manufacturer's instruction. The purified DNA was mixed with 0.6 N NaOH to a final concentration of 0.3 N and incubated for 10 min at room temperature followed by an ethanol precipitation. Resultant DNA preparations were re-suspended in 10-15 μl ddH_2O and stored at

-20°C until the subsequent PCR, using primers specific for the methylated p16INK4a sequence. Sense and antisense primers for the methylated sequence were 5'-TTATTAGAGGGTGGGGCGGATCC-3'and 5'-GACCCCGAA -CCGCGACCGTA-A-3', respectively. All bisulfate-treated DNA was also amplified by using primers specific for the unmethylated p16INK4a sequence. Sense and antisense primers for the unmethylated sequence were 5'-TTATTAGAGGGTGGGGTGGATTGT-3' and 5'-CAACCC -CAAACCACAACCATAA-3, respectively. Both PCR reactions were initialized with a denaturation at 95°C for 5 min followed by 40 cycles of 94°C for 60 s, 65°C (for methylated p16INK4a)/60°C (for unmethylated p16INK4a) for 50 s, and 72°C for 50 s, and a final extension at 72°C for 10 min. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. The p16INK4a CpG islands were amplified from bisulfate-treated genomic DNAs by PCR with the same primer pairs as described above. Amplified products were directly sequenced using the ABI sequencing system (Applied Biosystems) to confirm MSP results.

Immunohistochemistry assay

All specimens were formalin fixed and paraffin embedded. Briefly, 3 μm sections were cut, mounted on glass, and dried overnight at 37℃. All sections were then deparaffinized in xylene, rehydrated through alcohol, and washed in phosphate-buffered saline. Sections for p16INK4a detection were heated in a microwave oven twice for 5 min in citrate buffer (pH 6.0). A mouse anti-p16INK4a monoclonal antibody (at a dilution of 1:200) (Santa Cruz) was used as the primary antibody and the incubation time was 60 min at room temperature followed by a conventional streptavidin peroxidase method (DAKO, LSAB Kit K675, Denmark). Signals were developed with 3, 3'-diaminobenzidine for 5 min and counter-stained with hematoxylin. Negative controls were performed by leaving out the primary antibody. The results were

evaluated independently by three observers and scored for the percentage of positive nuclei: score 0, no positive staining; score +, from 1% to 10%; score ++, from 11% - 50%; score +++, more than 50% positive cells. In this study, scores +, ++, and +++ were considered to be a positive immunostaining, and score 0 was seen as a negative immunostaining.

Statistical analysis

Statistical analysis of frequency distributions was done by x^2 test, and the correlations between p16INK4a protein expression, promoter hypermethylation, HPV infection and clinico-pathological parameters were analyzed by statistical software SPSS 10.0.

RESULTS

The relationships between p16INK4a hypermethylation and clinico-pathological parameters of lung cancer patients

Dissected tumor specimens from 166 cancer patients were collected and subjected to MSP analysis for p16INK4a hypermethylation. Representative data from MSP analysis was shown in Fig. 1. The relationships between p16INK4a hypermethylation and clinico-pathological parameters of these tumors were statistically analyzed with no significant correlation being found for parameters including age, gender, tumor type, grade, tumor stage, smoking status, and T and N values (Table 1). After being categorized by gender and smoking status, p16INK4a hypermethylation frequencies of 3 categories including nonsmoking female, nonsmoking male, and smoking male tumors were 60.3% (35 of 58), 36.6% (15 of 41), and 59.7% (40 of 67), respectively (Table 2). The p16INK4a hypermethylation frequency of nonsmoking male tumors was significantly lower than those of nonsmoking female and smoking male tumors ($P = 0.033$). Surprisingly, nonsmoking female tumors had a similar high frequency of p16INK4a hypermethylation compared with that of smoking male tumors.

Correlation of p16INK4a hypermethylation with p16INK4a protein expression

To understand whether the promoter hypermethylation was indeed responsible for the inactivation of p16INK4a gene, *in situ* p16INK4a protein expression in lung tumors was examined by immunohistochemistry. Our data showed that p16INK4a protein expression was significantly correlated with p16INK4a promoter hypermethylation in overall cases ($P < 0.001$, Table 3). Among the three categories, a significant correlation was only observed in nonsmoking female tumors, but not in smoking or nonsmoking male tumors. This result

indicated that p16INK4a gene inactivation, which led to a negative immunostaining for p16INK4a in female lung cancer, was predominately mediated though promoter hypermethylation.

Correlation between p16INK4a hypermethylation and HPV infection

Based on the results described above, we hypothesized that the high frequency of p16INK4a hypermethylation in nonsmoking female lung cancer which caused p16INK4a negative immunostaining may be correlated to HPV 16 or 18 infections. Therefore, the correlation between HPV infection and p16INK4a hypermethylation in the three categories of lung tumors was examined to elucidate the association between HPV infection and the high frequency of p16INK4a hypermethylation in nonsmoking female lung tumors. As shown in Table 4, HPV 16 or 18 infection was indeed correlated with p16INK4a hypermethylation only in nonsmoking female lung tumors ($P = 0.017$), but not in nonsmoking male and smoking male lung tumors. Furthermore, we surprisingly found a reverse correlation between p16INK4a hypermethylation and HPV infection in smoking male lung cancer although such reverse correlation did not reach a statistical significance ($P = 0.05$).

Correlation of p16INK4a protein expression with HPV infection

To elucidate whether p16INK4a protein expression was indeed decreased by HPV infection via an alteration in promoter methylation, the correlation of p16INK4a protein expression with HPV infection among the three categories was statistically analyzed. The results showed that a significantly reverse correlation between p16INK4a protein expression and HPV 16 or 18 infection was observed only in nonsmoking female lung tumors ($P = 0.034$, Table 5). In contrast, a positive correlation was found between p16INK4a protein expression and HPV

infection in nonsmoking and smoking male tumors. This result strongly suggested that HPV infection was involved only in female lung tumorigenesis by promoting a hypermethylation of p16INK4a promoter to cause p16INK4a gene inactivation.

DISCUSSION

In this study, we have revealed that p16INK4a gene inactivation through a promoter hypermethylation may be linked with HPV infection in nonsmoking female cancer. Therefore, a higher prevalence of HPV infection in nonsmoking female lung cancer compared to that of nonsmoking male lung cancer may explain why a high frequency of p16INK4a hypermethylation was also observed in nonsmoking female lung cancer, rather than in nonsmoking male lung cancer. In this study, a total of 166 lung tumors, including 90 from a previous study, were analyzed for HPV 16/18 infection by nested-PCR and the result showed a HPV prevalence similar to that from our previous study[19]. Thus, this result supported our previous report showing that HPV infection was associated with nonsmoking female lung cancer in Taiwan. Additionally, it suggested that the involvement of HPV infection in lung tumorigenesis may be partly through p16INK4a inactivation by promoter hypermethylation.

Previous studies have indicated that p16INK4a hypermethylation was more frequently observed in men than in women, and p16INK4a hypermethylation was more frequent in squamous carcinomas than in adenocarcinomas[13]. Another study has shown a reverse observation in p16INK4a hypermethylation in Chinese lung cancer with an extremely high frequency of p16INK4a hypermethylation in female lung patients (12 of 13, 92%) compared with that of male patients (36 of 62, 58%)[21]. In this study, the difference of p16INK4a promoter hypermethylation between various genders and tumor types was not observed in overall cases (Table 1). However, in the further analysis for the correlation of HPV infection with p16INK4a hypermethylation among different tumor types and gender, we found that p16INK4a hypermethylation was significantly increased by HPV infection in female adenocarcinoma, but decreased by HPV infection in male adenocarcinoma and squamous cell carcinoma. No correlation was observed between HPV infection and p16INK4a

hypermethylation in female squamous cell carcinoma. These results suggested that in various genders and various tumor types, HPV infection may contribute differently in p16INK4a hypermethylation.

A previous study in an animal model had shown associations between specific carcinogen exposures and hypermethylation of estrogen receptor gene[22-23]. In addition, a recent study[13] has demonstrated an association between cigarette smoking and p16INK4a hypermethylation in human lung cancers, and Kim et al. (2001) have described a higher frequency of p16INK4a hypermethylation in smoking lung cancer patients. However, others have suggested that p16INK4a inactivation by promoter hypermethylation was frequently observed in lung tumors arising both in even and never smokers[15]. In our study, a higher frequency of p16INK4a hypermethylation in smoking lung cancer supported some previous reports indicating that cigarette smoking may be related with p16INK4a hypermethylation. Interestingly, a significant difference in p16INK4a hypermethylation frequency between nonsmoking male and female lung cancer reflected that different aetiological factors other than cigarette smoking may be linked with p16INK4a hypermethylation, especially in nonsmoking female lung cancer in Taiwan.

Hepatocellular carcinoma (HCC) tumors from patients with hepatitis (caused by hepatitis B virus, hepatitis C virus), from patients with cirrhosis, or from patients living in high-risk geographic areas had much higher levels of hypermethylation of p16INK4a and estrogen receptor (ER) than do tumors arising spontaneously[24]. Moreover, recent reports further indicated that intensive promoter hypermethylation of O6-methylguanine-DNA methyltransferase (MGMT), hMLH1, and Ras association domain family 1A (RASSF1A) was associated with hepatitis viral infection[24-26]. For the association between promoter hypermethylation and HPV infection, some reports indicated a reverse correlation between

RASSF1A hypermethylation and the presence of HPV infections in squamous cervical carcinomas[27]. In the other hand, RASSF1A hypermethylation was more common in cervical adenocarcinomas and not correlated with HPV infection[28]. These results suggested that these two events, RASSF1A hypermethylation and HPV infection, may independently play a role in cervical tumorigenesis. However, in our study, frequent p16INK4a hypermethylation was correlated with HPV 16 or 18 infections in nonsmoking female lung cancer patients. It is well known that E7 of HPV 16 or 18 was involved in Rb inactivation and released histone deacetylase (HDAC) from the complex of E2F-Rb-HDAC to enhance p16INK4a hypermethylation through chromatin remodeling by HDAC[29]. Our preliminary data showed that p16INK4a immunostainings marginally positively correlated with Rb immunostainings seemed to support the above speculation. The detail molecular mechanism for the association between p16INK4a promoter hypermethylation and HPV infection should be further investigated.

In summary, p16INK4a hypermethylation was frequently observed in HPV-infected female lung cancer as compared with that in male lung cancer. Moreover, the contribution of p16INK4a hypermethylation to p16INK4a gene inactivation was only observed in female lung cancer, but not in male case. Therefore, we believed that HPV infection may be partly involved in p16INK4a gene inactivation through promoter hypermethylation in HPV infected-female lung cancer in Taiwan. Our results may provide an example to evidence that environment-epigenetic interactions may play a role in the geographic variation of human cancer development.

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Table 1. Relationships between p16INK4a hypermethylation and clinicopathological parameters.

Clinicopathological parameters	P16INK4a hypermethylation		
	Absent n (%)	Present n (%)	P
Age (yr)			
<65	40 (49)	42 (51)	0.444
≧65	36 (43)	48 (57)	
Gender			
Female	23 (40)	35 (60)	0.246
Male	53 (49)	55 (51)	
Tumor type			
Adenocarcinoma	44 (46)	51 (54)	0.873
Squamous cell carcinoma	32 (45)	39 (55)	
Differentiation grade			
Well	4 (67)	2 (33)	0.565
Moderate	47 (46)	56 (54)	
Poor	25 (44)	32 (56)	
Stage			
I	35 (49)	37 (51)	0.403
II	10 (34)	19 (66)	
III	31 (48)	34 (52)	
T factor			
T1	3 (50)	3 (50)	0.979
T2	57 (45)	70 (55)	
T3	12 (48)	13 (52)	
T4	4 (50)	4 (50)	
N factor			
N0	40 (48)	44 (52)	0.202
N1	7 (29)	17 (71)	
N2	29 (50)	29 (50)	
Smoking status			
Nonsmoking	49 (49)	50 (51)	0.243
Smoking	27 (40)	40 (60)	

Table 2. The difference in p16INK4a hypermethylation between groups with various genders and smoking status.

	Nonsmoking		Smoking	
	Female	Male	Male	
p16INK4a hypermethylation	n (%)	n (%)	n (%)	P
Negative	23 (40)	26 (63)	27 (40)	0.033
Positive	35 (60)	15 (37)	40 (60)	
P		0.02	0.02	

Table 3. Correlation between p16INK4a hypermethylation and p16INK4a protein immunostainings in all studied cases and three categories.

P16INK4a protein Immunostaining	p16INK4a hypermethylation							
	All studied cases (n=166)		Nonsmoking				Smoking	
			Female (n=58)		Male (n=41)		Male (n=67)	
	Absent n (%)	Present n (%)	Absent n (%)	Present n (%)	Absent n (%)	Present n (%)	Absent n (%)	Present n(%)
Negative	46 (37)	78 (63)	8 (22)	29 (78)	19 (58)	14 (42)	19 (35)	35 (65)
Positive	30 (71)	12 (29)	15 (71)	6 (29)	7 (88)	1 (12)	8 (62)	5 (38)
P	<0.001		<0.001		0.22		0.117	

The three categories of lung tumors in this study were nonsmoking female, nonsmoking male, and smoking male lung tumors which were categorized based on gender and cigarette smoking status.

Table 4. The correlation between p16INK4a hypermethylation and HPV 16 or 18 infections in all studied cases and three categories.

	p16INK4a hypermethylation							
	All studied cases (n=166)		Nonsmoking				Smoking	
			Female (n=58)		Male (n=41)		Male (n=67)	
HPV 16 or 18 Infections	Absent n (%)	Present n (%)	Absent n (%)	Present n (%)	Absent n (%)	Present n (%)	Absent n (%)	Present n (%)
Negative	33 (44)	42 (56)	10 (67)	5 (33)	9 (56)	7 (44)	14 (32)	30 (68)
Positive	43 (47)	48 (53)	13 (30)	30 (70)	17 (68)	8 (32)	13 (57)	10 (43)
P	0.675		0.017		0.446		0.05	

The three categories of lung tumors in this study were nonsmoking female, nonsmoking male, and smoking male lung tumors which were categorized based on gender and cigarette smoking status.

Table 5. Correlation between p16INK4a protein immunostaings and HPV 16 or 18 infections in all studied cases and three categories.

	p16INK4a protein immunostaining							
	All studied cases		Nonsmoking				Smoking	
	(n=166)		Female (n=58)		Male (n=41)		Male (n=67)	
HPV 16 or 18 Infections	Absent n (%)	Present n (%)	Absent n (%)	Present n (%)	Absent n (%)	Present n (%)	Absent n (%)	Present n (%)
Negative	61 (81)	14 (19)	6 (40)	9 (60)	16 (100)	0 (0)	39 (89)	5 (11)
Positive	63 (69)	28 (31)	31 (72)	12 (28)	17 (68)	8 (32)	15 (65)	8 (35)
P	0.074		0.034		0.014		0.047	

The three categories of lung tumors in this study were nonsmoking female, nonsmoking male, and smoking male lung tumors which were categorized based on gender and cigarette smoking.

LEGENDS

Fig. 1. Representative results of MSP and direct DNA sequencing analysis of p16INK4a CpG islands from four lung tumors. Lane M was PCR product with primers specific for methylated DNA and lane U was that with primers specific for unmethylated DNA. The DNA templates of T1, T2, T3, T4, and T5 were prepared from tumors of various patients. The templates of P1 and P2, serving as positive controls respectively for methylated and unmethylated reactions, were prepared from Calu-1 cells and lymphocytes of healthy subject, respectively, while distilled water was used as templates for N, serving as a negative control (A). CpG-methylated cytosines remained as cytosines, whereas 5-methylcytosines remain unaltered in example (★) (B). p16INK4a Unmethylated cytosines changed to thymidines in the PCR products (C).

Fig. 2. Representative of p16INK4a positive and negative protein immunostaining in paraffin sections of lung tumors. A representative negative immunostaining was shown in (A) while positive immunostaining in lung adenocarcinoma and squamous cell lung carcinoma were shown in (B) and (C), respectively.

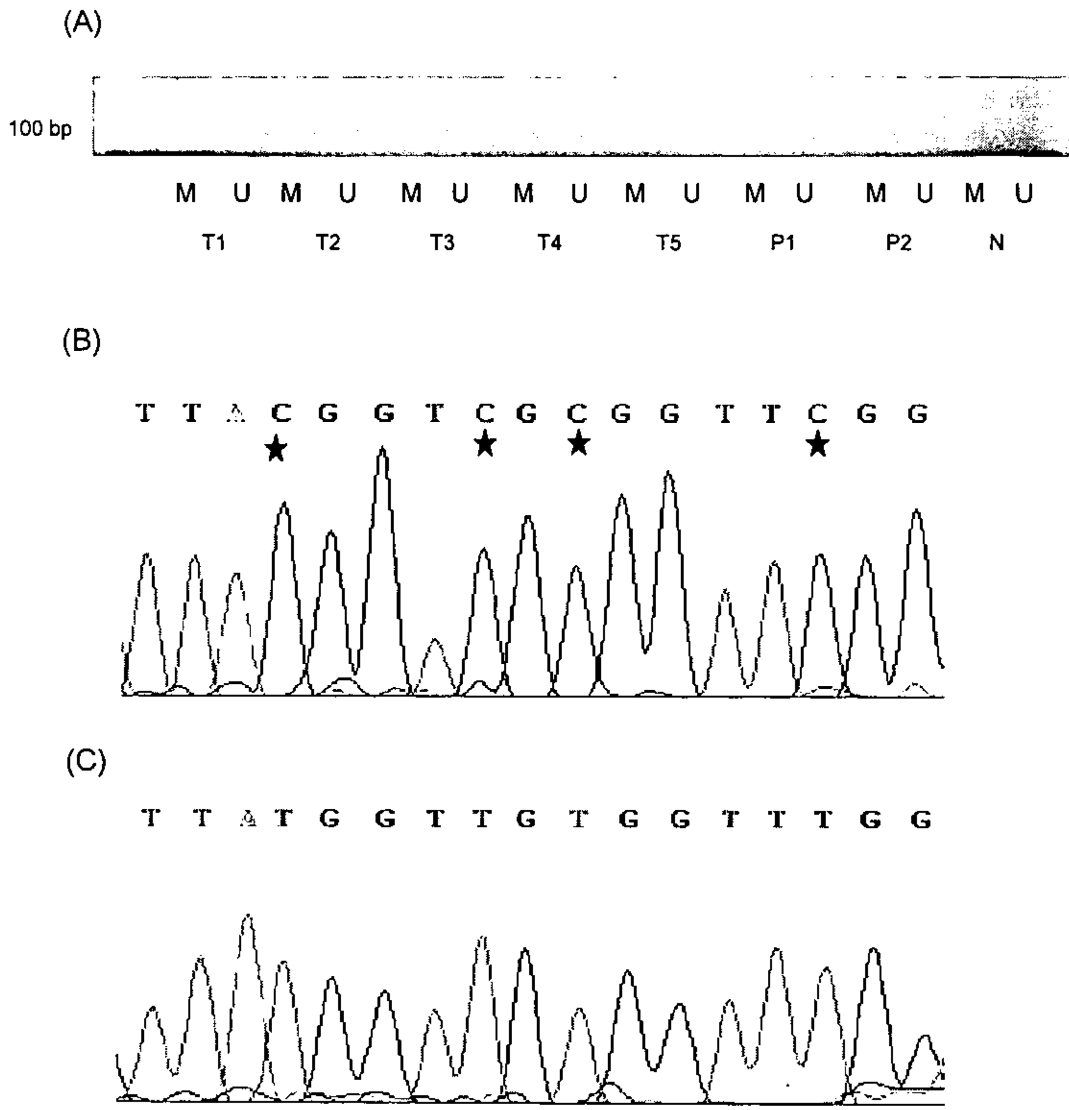

Fig. 1. Representative results of MSP and direct DNA sequencing analysis of p16INK4a CpG islands from four lung tumors. Lane M was PCR product with primers specific for methylated DNA and lane U was that with primers specific for unmethylated DNA. The DNA templates of T1, T2, T3, T4, and T5 were prepared from tumors of various patients. The templates of P1 and P2, serving as positive controls respectively for methylated and unmethylated reactions, were prepared from Calu-1 cells and lymphocytes of healthy subject, respectively, while distilled water was used as templates for N, serving as a negative control (A). CpG-methylated cytosines remained as cytosines, whereas 5-methylcytosines remain unaltered in example (★) (B). p16INK4a Unmethylated cytosines changed to thymidines in the PCR products (C).

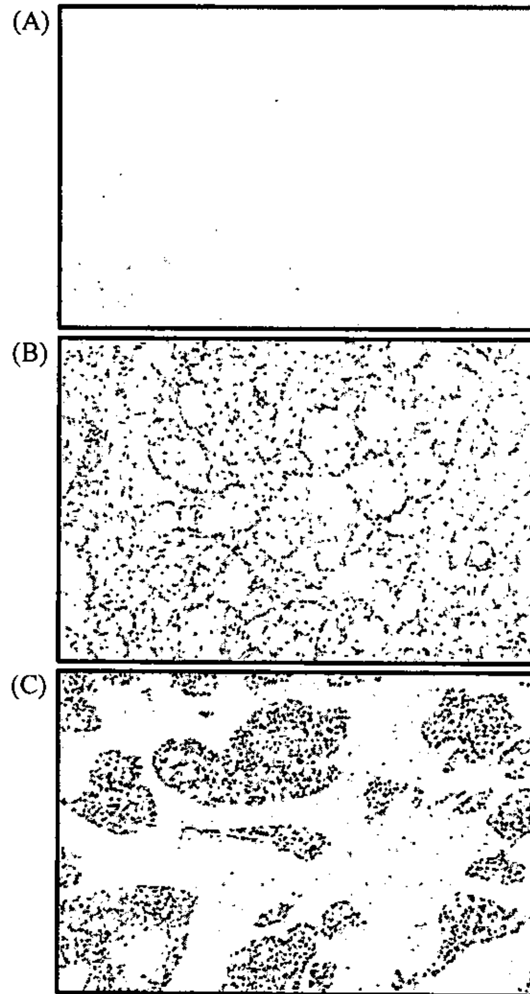

Fig. 2. Representative of p16INK4a positive and negative protein immunostaining in paraffin sections of lung tumors. A representative negative immunostaining was shown in (A) while positive immunostaining in lung adenocarcinoma and squamous cell lung carcinoma were shown in (B) and (C), respectively.

AN ASSOCIATION OF DNMT3b PROTEIN EXPRESSION WITH P16INK4a PROMOTER HYPERMETHYLATION IN NON-SMOKING FEMALE LUNG CANCER WITH HUMAN PAPILLOMAVIRUS INFECTION

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Key words: DNA methyltransferase, human papillomavirus, lung cancer.
Running title: Increased DNMT3b protein expression and HPV infection

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ABSTRACT

Our recent report indicated that HPV infection may be associated with the increased frequency of p16INK4a promoter hypermethylation to cause p16 inactivation. In this study, we further speculated that the HPV infection may be linked with DNA methyltransferase (DNMT) protein expression in lung cancer patients. Our data indicated that an association of p16INK4a promoter hypermethylation with HPV infection was observed, but only in female cases ($P < 0.0001$). Interestingly, DNMT3b protein expression was significantly correlated with p16INK4a promoter hypermethylation ($P=0.023$) and HPV 16/18 infections ($P< 0.001$), respectively. Moreover, the correlation between p16INK4a promoter hypermethylation DNMT3b protein expression was exclusively seen in female cases ($P = 0.035$). These results strongly suggested that the involvement of HPV infection in nonsmoking female lung tumorigenesis may be mediated, at least in part, through the increase of DNMT3b protein expression to cause p16INK4a promoter hypermethylation.

INTRODUCTION

The inactivation of tumor suppressor genes is one of the main events leading to the development and progression of various human cancers (1). This inactivation occurs through intragenic mutations, genomic deletions and also very often by epigenetic silencing associated with the hypermethylation of the CpG islands located at the promoter regions of these putative tumor suppressor genes (TSGs) (2, 3). Examples of widely recognized TSGs undergoing CpG island promoter hypermethylation in NSCLC, include p16INK4a, DAPK, GSTP1, MGMT, etc (4-6). However, the mechanisms involved in hypermethylated DNA loci remain unclear. Global cytosine methylation patterns in mammals appear to be established by a complex interplay of at least three independently encoded DNA methyltransferases (DNMTs) including DNMT1, DNMT3a and DNMT3b (7, 8). DNMTs are commonly classified as *de novo* (DNMT3a and DNMT3b) and maintenance (DNMT1) enzymes (7, 8). Most interesting, overexpression of DNMT1 and DNMT3b is a common event in human tumors, such as sporadic breast carcinomas, myelogenous leukemia (7, 8). However, the role in the epigenetic silencing of TSGs is still not well characterized.

Approximately, 15% of all cancers worldwide appeared to be associated with viral infections, and several human DNA viruses are now accepted as causative factors of specific malignancies. Human papillomavirus (HPV) has been well known to cause cervical and anogenital cancer (9) while Epstein-Barr virus (EBV) causes infectious mononucleosis and is closely associated with Burkit's lymphoma, nasopharyngeal carcinomas, and Hodgkin's disease (10, 11). Furthermore, HPV is now believed to be associated with cervical and oral cancers (12-16) and EBV may involve in breast and gastric cancers (17, 18). Viral exposure is a possible factor to cause epigenetic variation in human cancer, for example, the associations of HBV and HCV infections

with promoter hypermethylation of p16INK4a and estrogen receptor in hepatocullular carcinomas (19, 20), and the association of Simian virus 40 with RASSF1A promoter methylation in malignant mesothelioma have been reported (21, 22). Our previous report showed that HPV 16/18 infection was associated with lung cancer development in Taiwanese women nonsmokers (23), and indicated that a high frequency of p16INK4a promoter hypermethylation was frequently observed in nonsmoking female lung cancer with HPV infection as compared with those without HPV infection (24). In this study, we hypothesized that certain DNMTs, such as DNMT1 and DNMT3b, might be involved in p16INK4a promoter hypermethylation and these protein expressions may be linked with HPV 16/18 infections. These results revealed a possibility that the involvement of HPV16/18 infections in non-smoking lung tumorigenesis may be through the increase of DNMTs protein expression to cause p16 hypermethylation.

MATERIALS AND METHODS

Study subjects

Fifty-seven primary non-smoking lung cancer patients including 32 females and 25 males, who had undergone thoracic surgery at Changhua Christian Hospital, were enrolled. None of the subjects had received radiation therapy or chemotherapy prior to surgery. The histology of tumor types and stages were determined according to the WHO classification method. Information on smoking history of the lung cancer patients was obtained from hospital records and then patients were classified into smoking and non-smoking groups.

Immunohistochemical analyses

All specimens were formalin fixed and paraffin embedded. Sections were cut at a thickness of 3 μm, mounted on glass, and dried overnight at 37℃. All sections were then deparaffinized in xylene, rehydrated through a series of alcohol, and washed in phosphate-buffered saline. This buffer was used for all subsequent washes. Sections for DNMT1 and DNMT3b detection were heated in a microwave oven twice for 5 min in citrate buffer (pH 6.0). Mouse anti-DNMT1 (IMGENEX, USA) and anti-DNMT3 monoclonal antibody (Gene Therapy Systems, USA) was used as the primary antibody and the incubation time was 60 min at room temperature followed by a conventional streptavidin peroxidase method (DAKO, LSAB Kit K675, Denmark). Signals were developed with 3, 3'-diaminobenzidine for 5 min and counter-stained with hematoxylin. Negative controls were obtained in the absence of the primary antibody. Negative controls were obtained by leaving out primary antibody. The results were evaluated independently by three observers and scored for the percentage of positive nuclei: score 0, no positive staining; score +, from 1% to 10%; score ++, from 11% - 50%; score +++, more than 50% positive cells. In this

study, scores +, ++, and +++ were considered to be positive immunostaining, and score 0 was seen as a negative immunostaining.

DNA Extraction and nested polymerase chain reaction (Nested-PCR).

Genomic DNA from tumor tissues was isolated by a conventional phenol-chloroform extraction, ethanol precipitation and finally dissolved in sterile distilled water. HPV DNA was first amplified with type consensus primers MY09 and MY11 (23, 25) followed by a second round of amplification with type specific primers flanking the L1 region to identify the subtype. Ten microliters of the final PCR product was loaded onto a 2% agarose gel, stained with ethidium bromide and visualized under UV illumination. Appropriate negative and positive controls were included in each PCR reaction. A part of the β-actin gene in all samples was amplified to exclude false-negative results while DNA preparations from SiHa cell (containing HPV 16) and HeLa cell (containing HPV 18) were used as positive controls.

Methylation-specific PCR (MSP) and direct sequencing

MSP was conducted according to procedures described in a previous study (20, 24). Briefly, approximate 6 μg of extracted DNA was treated with sodium bisulfate (Sigma, USA) and purified using Wizard DNA Clean-Up System (Promega Corporation, USA) according to the manufacturer's instruction. The purified DNA was mixed with 0.6 N NaOH to a final concentration of 0.3 N and incubated for 10 min at room temperature followed by an ethanol precipitation. Resultant DNA preparations were re-suspended in 10-15 μl ddH_2O and stored at -20°C until the subsequent PCR, using primers specific for the methylated p16INK4a sequence. Sense and antisense primers for the methylated sequence were 5'-TTATTAGAGGGTGGGGCGGATCC-3'and 5'-GACCCCGAACCGCGACCGT

AA-3', respectively. All bisulfate-treated DNA was also amplified by using primers specific for the unmethylated p16INK4a sequence. Sense and antisense primers for the unmethylated sequence were 5'-TTATTAGAGGGTGGGGTGGATTGT-3' and 5'-CAACCC -CAAACCACAACCATAA-3, respectively. Both PCR reactions were initialized with a denaturation at 95°C for 5 min followed by 40 cycles of 94°C for 60 s, 65°C (for methylated p16INK4a)/60°C (for unmethylated p16INK4a) for 50 s, and 72°C for 50 s, and a final extension at 72°C for 10 min. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. The p16INK4a CpG islands were amplified from bisulfate-treated genomic DNAs by PCR with the same primer pairs as described above. Amplified products were directly sequenced using the ABI sequencing system (Applied Biosystems) to confirm MSP results.

Statistical Analysis

Statistical analysis was performed using the SPSS statistical software program (SPSS Inc., Chicago, IL). The Fisher's exact or Chi-square was applied for statistical analysis. A $P < 0.05$ was considered to be statistically significant.

RESULTS

The relationships between protein expressions of DNMTs and clinicopathological parameters of lung cancer patients

Dissected tumor specimens from 57 non-smoking lung cancer patients including 29 adenocarcinoma and 28 squamous cell carcinomas were collected and subjected to immunohistochemical analysis for DNMT1 and DNMT3b protein expressions. Representative results were shown in Fig. 1. As shown in Table 1, DNMT3b protein was more frequently observed in adenocarcinomas and female patients than in squamous cell carcinoma and males, respectively (P=0.033 for tumor type and P= 0.015 for gender). However, no correlation was observed between DNMT1 and any analyzed clinico-pathological parameter, including age, gender, tumor type, tumor stage, T and N value (Table 1).

The association of DNMTs with p16INK4a promoter hypermethylation and HPV infection

As shown in Table 2, p16INK4a promoter hypermethylation was significantly correlated with HPV infection in all study cases (P < 0.0001), and the correlation was only observed in female cases (P < 0.0001), but not in male cases. These results were consistent with our previous report with different study population (24). To verify whether the increased p16INK4a promoter hypermethylation was associated with DNMT3b protein expression, our data showed that DNMT3b protein expression was positively associated with p16INK4a promoter hypermethylation (P = 0.023), but such association was not observed for DNMT1 (Table 2). Meanwhile, such association was also only found in female cases (P = 0.035), but not in male cases (P = 0.428). Additionally, we also found that DNMT3b protein expression, but not DNMT1, was significantly correlated with HPV infection (P = 0.001) (Table 3).

The correlation between HPV infection, DNMT3b protein expression and p16INK4a promoter hypermethylation

Our recent report indicated that a higher frequency of p16INK4a promoter hypermethylation was observed in nonsmoking female lung cancer with HPV infection as compared with those without HPV infection (24). The significant correlation between p16INK4a promoter hypermethylation and HPV infection was also revealed in this study, therefore we suspect that HPV infection may alter the DNMT3b protein expression to promote p16INK4a promoter hypermethylation. As show in Table 4, the frequency of p16INK4a promoter hypermethylation in HPV+/DNMT3b+ (94.7%, 18 of 19) was significantly higher than that of other three groups including HPV-/DNMT3b- (40.9%, 9 of 22), HPV-/DNMT3b+ (33.3%, 2 of 6) and HPV+/DNMT3b- (70.0%, 7 of 10). Thus, this result suggested that HPV infection might induce the protein expression of DNMT3b to promote p16INK4a promoter hypermethylation in lung tumorigenesis.

DISCUSSION

Our recent report indicated that p16 gene inactivation through promoter hypermethylation was frequently observed in nonsmoking female lung cancer patients with HPV infection (30 of 43, 70%) as compared with those without HPV infection (5 of 10, 33%) (24). Furthermore, the prevalence of HPV infection in this study population was quite consistent to that reported previously (24). Based on these results, we strongly suggested that DNMT3b protein expression might be increased by HPV infection to cause p16INK4a promoter hypermethylation.

Although no somatic defects have been reported in these enzymes in human tumors, DNMT1 and DNMT3b seemed to be up-regulated in the majority of human neoplasms. A germline genetic defect in DNMT3b has been described, causing the syndrome ICF (immunodeficiency, centromeric region instability, and facial anomalies) (26), but its effect in cancer risk has not been reported. To date, methylation of CpG dinucleotides has been known to be mediated by at least three DNMTs, including DNMT1, DNMT3a, and DNMT3b (7). Two of these enzymes, DNMT3a and DNMT3b, are thought to be responsible for an initial setup of methylation patterns for developing genomes (27). DNMT1 is constitutively expressed in proliferating cells and functions as maintenance enzyme to ensure that the methylation patterns are faithfully copied to daughter cells during DNA replication (7, 27). Antisense DNMT1 treatment of tumor cell lines with methylated p16 resulted in demethylation of the locus, re-expression of p16, and cessation of cell growth (28). On the other hand, homogygous deletion of DNMT1 in HCT116m a diploid human colon cancer cell line, had no effect on the status of methylated and silenced wild type p16 allele (29). These results implied either that another DNMT is necessary for maintaining p16INK4a promoter hypermethylation or that prolonged

inactivation of DNMT1 by genetic deletion induces compensatory activity. To evidence the possibility, antisense suppression of DNMT3b in tumor cell lines could restore TSGs expression (30), as did deletion of DNMT3b in DNMT1-deficient HCT116 (31). Two siRNAs of DNMT1 and DNMT3b engineered specifically to suppress, these gene had differential effects on DNA methylation and gene reactivation in the ovarian cancer cell line CP70 (32). The epigenetic alteration appeared less effective in cells transfected with DNMT3b siRNA and a synergistic relationship between DNMT3b and DNMT1 in this cell line. These results suggested that whereas DNMT1 plays a key role in methylation maintenance, DNMT3b may act as an accessory to support the function in CP70 cells (32). A recent report showed that normal human bronchial epithelial (NHBE) cells expressing telomerase, SV40 large antigen, and activated Ras were immortal, formed colonies in soft agar, and expressed DNMT3b (33), while antisense suppression of DNMT3b prevented soft agar growth. Furthermore, mouse embryo fibroblasts expressing T antigen and Ras formed soft agar colonies and large tumors, but fibroblasts from *dnmt3b-/-* mice did not grow in soft agar and were much less tumorigenic *in vivo*. The TSGs, FHIT, TSLC1, and RASSF1A were downregulated in transformed NHBE cells, and antisense DNMT3b treatment resulted in re-expression of FHIT and TSLC1, suggesting that DNMT3b may silence genes by several mechanisms including direct DNA methylation or recruitment of protein that modify chromatin. More importantly, the abovementions indicated that DNMT3b plays an important role in transformation (33). In this study, the frequency of p16INK4a promoter hypermethylation in lung tumors with DNMT3b+/DNMT1+ (75%, 9 of 12) was exactly the same as the frequency of p16INK4a promoter hypermethylation in lung tumors with DNMT3b+/DNMT1- (75%, 18 of 24). These results may support our present data showing that a high prevalence of p16INK4a promoter hypermethylation in lung tumors with HPV

infection was more favorably associated with DNMT3b than with DNMT1.

To our knowledge, this is the first study to link DNMT3b protein expression and p16INK4a promoter hypermethylation in lung cancer, and results further demonstrated our recent report (24) suggesting that DNMT3b protein expression might be promoted by HPV infection to increase p16INK4a promoter hypermethylation involvement in HPV associated-lung tumorigenesis among Taiwanese women nonsmokers.

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Table 1. The association between DNMT1, DNMT3b protein expression and clinical characteristics in non-smoking lung cancer patients.

Parameters	DNMT1		DNMT3b	
	Negative (n=43)	Positive (n=14)	Negative (n=32)	Positive (n=25)
Age				
≦64 (n=27)	19	8	16	11
>64 (n=30)	24	6	16	14
P value	0.540		0.790	
Gender				
Female (n=32)	22	10	13	19
Male (n=25)	21	4	19	6
P value	0.227		0.015	
Tumor type				
AD (n=29)	22	7	12	17
SQ (n=28)	21	7	20	8
P value	1.000		0.033	
Tumor stage				
I (n=32)	25	7	16	16
II (n=4)	2	2	3	1
III (n=21)	16	5	13	8
P value	0.514		0.500	
T factor				
1 (n=6)	2	4	3	3
2 (n=39)	30	9	20	19
3 (n=10)	9	1	7	3
4 (n=2)	2	0	2	0
P value	0.064		0.310	
N factor				
0 (n=34)	27	7	17	17
1 (n=8)	6	2	6	2
2 (n=15)	10	5	9	6
P value	0.643		0.399	

χ^2 test was used for statistical analysis.
AD: adenocarcinoma, SQ: squamous cell carcinoma.

Table 2. An association between p16INK4a promoter hypermethylation and HPV 16/18 infection, p16INK4a promoter hypermethylation and DNMT3b protein expression in tumor tissues of lung cancer patients.

	p16INK4a promoter hypermethylation					
	All subjects		Female		Male	
Parameters	Negative (n=21)	Positive (n=26)	Negative (n=11)	Positive (n=21)	Negative (n=10)	Positive (n=15)
HPV 16/18 infection						
Negative	17	11	10	2	7	9
Positive	4	25	1	19	3	6
P value	<0.0001		<0.0001		0.691	
DNMT1 protein						
Negative	16	27	8	14	8	13
Positive	5	9	3	7	2	2
P value	1.000		1.000		1.000	
DNMT3b protein						
Negative	12	9	6	3	6	6
Positive	9	27	5	18	4	9
P value	0.023		0.035		0.428	

Table 3. An association between DNMT3b protein expression and HPV 16/18 infection in tumor tissues of lung cancer patients.

DNMT	HPV16/18 infection		
protein expression	Negative (n= 28)	Positive (n=29)	p value
DNMT1			
Negative	23	20	
Positive	5	9	0.358
DNMT3b			
Negative	22	10	
Positive	6	19	0.001

Table 4. An association between DNMT3b protein expression, p16INK4a promoter hypermethylation and HPV 16/18 infection in tumor tissues of lung cancer patients.

	p16INK4a promoter hypermethylation		
HPV 16/18 infection / DNMT3b protein	Negative (n=21)	Positive (n=36)	P value
Negative/ Negative	13	9	
Negative/ Positive	4	2	
Positive/ Negative	3	7	
Positive/ Positive	1	18	0.001

LEGENDS

Fig. 1. Representative positive and negative immunostainings for DNMT1and DNMT3b proteins in paraffin sections of lung tumors. A representative negative immunostaining was shown in (A) while DNMT1 and DNMT3b positive immunostaining in lung carcinoma was shown in (B) and (C), respectively.

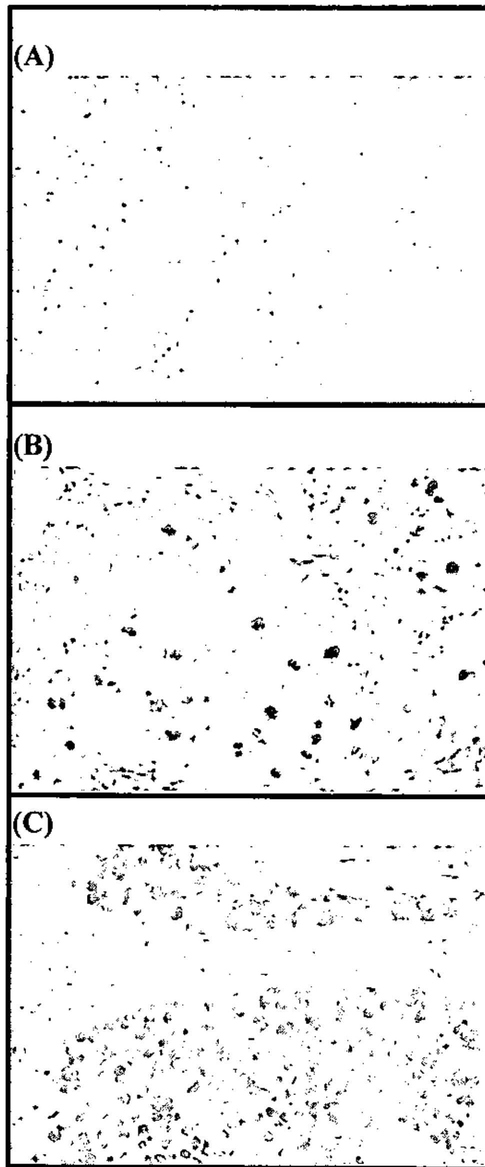

Fig. 1. Representative positive and negative immunostainings for DNMT1and DNMT3b proteins in paraffin sections of lung tumors. A representative negative immunostaining was shown in (A) while DNMT1 and DNMT3b positive immunostaining in lung carcinoma was shown in (B) and (C), respectively.

INVOLVEMENT OF HPV 16/18 E6 STATUS IN TAIWANESE LUNG CANCER WITH P53 PROTEIN EXPRESSION

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Keywords: HPV 16/18 E6 mRNA, lung cancer, p53 inactivation

Running title: Involvement of E6 with p53 in lung cancer

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ABSTRACT

Our recent report have showed that high-risk HPV 16/18 infections were associated with female lung cancer incidences in Taiwan. To elucidate the involvement of HPV 16/18 infections in lung tumorigenesis, the interaction between the status of the HPV 16/18 E6 and p53 protein in lung tumors were evaluated to verify whether E6 was linked with p53 inactivation. We first examined HPV 16/18 E6 mRNA and p53 protein expressions in serial sections of lung tumors by *in situ* RT-PCR and immunohistochemistry, respectively. With half of HPV DNA positive tumors being detected with HPV 16 and/or 18 E6 mRNA, a reciprocal relationship between E6 and p53 was most frequently observed in tumors positive for both of HPV 16 and 18 E6 mRNA (HPV 16+/18+) among four categories including HPV 16/18-, HPV 16+, HPV 18+, and HPV 16/18+ (P = 0.009). To understand whether p53 immunostaining positive was correlated to the occurrence of E6 mRNA variants, direct autosequencing was performed to reveal E6 variants in 10 of 12 p53-positive tumors (83.3%). Results of western blot and immunoprecipitation (IP) showed that the interaction between E6 and p53 proteins was detected in 32 lung tumors with HPV 16+ and/or 18+, but not in those 5 HPV 16/18-. A relatively higher p53 protein levels were detected in tumors with different HPV 16/18 E6 variants than those with wild type E6 protein. Moreover, p53 protein was stabilized in lung fibroblast cells WI-38 being transfected with different E6 variants. These results strongly suggested that wild type or variant E6 proteins may interact with p53 protein and therefore involve in HPV-infected lung tumorigenesis.

INTRODUCTION

It has been known that one-half of the overall lung cancer incidences in Taiwan cannot be attributed to cigarette smoking and over 90% of Taiwanese female lung cancer patients were nonsmokers. Furthermore, the gender discrepancy in lung cancer mortality in Taiwan (with a male to female ratio of 2) was lower than that of other areas worldwide, where the ratio ranged from 2.3 to 8.6 [1, 2]. Our previous report showed that infection of high-risk HPV, type 16/18, was associated with lung cancer in Taiwanese female nonsmokers[3]. In addition, our recent case-control study also clearly revealed that a person with HPV 16 and 18 DNA in their blood had 75.7-fold risk of lung cancer compared with those without HPV 16/18 DNA [4], which further authenticated the involvement of HPV in lung tumorigenesis.

Tumorigenic progression of high-risk HPV-associated lesions is always accompanied by the physical integration of the viral genome into a host chromosome, which interrupts the E2 open reading frame, eliminates the suppression of E2 on E6 and E7 expressions, and eventually leads to the over-expression of E6 and E7 oncoproteins [5-7]. The hallmark of HPV being involved in cervical carcinogenesis is the immortalization of primary human cervical, epidermal, mammary, and bronchial epithelial cells by the E6 protein of high-risk of HPVs [8,9]. It is believed that the E6 protein of high-risk HPV binds to the proteins encoded by the host p53 tumor-suppressor genes to cause cell cycle progression [9-12]. A most significant observation related to the function of E6 protein was made initially by revealing the binding of cellular p53 protein to E6 followed by experiments demonstrating that this binding promotes the degradation of p53 mediated by the cellular ubiquitin-proteasomal pathway[9]. Recent experiments[13] have also shown that in HPV-positive cancer cells the Mdm2 pathway is completely inactivated, while p53 degradation depends entirely on E6. It is clear that the E6-p53

interaction represents one of the key events in E6 induced malignancy. In this study, to verify the involvement of HPV infection-related mechanism in lung tumorigenesis, the presence of E6 mRNA and p53 protein expressions in lung tumor tissues were determined by *in situ* RT-PCR and immunohistochemistry. Additionally, western blot and immunoprecipitation (IP) assay were performed to prove the interaction between E6 protein and p53 protein to cause p53 inactivation.

MATERIALS and METHODS

Study Subjects

Seventy-three primary lung cancer patients including 38 females and 35 males, who had undergone thoracic surgery from 1998 to 2000 at Veterans General Hospital-Taichung and analyzed for the presence of HPV 16 and/or 18 DNA previously, were enrolled. None of these subjects had received radiation therapy or chemotherapy prior to surgery. Tumor types and stages were determined according to the WHO classification (World Health Organization, 1981) and pathological material was processed for conventional histological procedures.

Immunohistochemistry

Formalin fixed and paraffin embedded specimens were sectioned at a thickness of 3 μm. All sections were then deparaffinized in xylene, rehydrated through serial dilutions of alcohol, and washed in PBS (pH 7.2), the buffer which was used for all subsequent washes. For p53 detection, sections were heated in a microwave oven twice for 5 min in citrate buffer (pH 6.0), and then incubated with a monoclonal anti-human p53 antibody (DAKO, DO7, Denmark; at a dilution of 1:250) for 60 min at 25℃. The conventional streptavidin peroxidase method (DAKO, LSAB Kit K675, Copenhagen, Denmark) was performed to develop signals and the cells were counter-stained with hematoxylin. Negative controls were obtained by leaving out the primary antibody. The intensities of signals were evaluated independently by three observers and scored by the percentage of positive nuclei: score 0-10%, no positive staining; score >10%, positive staining.

In Situ RT-PCR

In situ RT-PCR was used to detect the location of HPV 16/18 E6 gene expression. The

deparaffinized and rehydrated sections of 5 μm were first digested with proteinase K followed by RNase-free DNase treatment to remove DNA. After 3 times of washes, reverse transcription was performed in a reaction containing RT reaction buffer (Life Technologies, Grand Island, NY, USA), 10 mM DTT, 100 pmole of random primer, 0.5 mM each of dNTPs, and 200 units of M-MLV reverse transcriptase (Life Technologies, Grand Island, NY, USA). The reactions were carried out at 42°C for 50 min and then terminated by heating at 75°C for 10 min followed by quickly chilling on ice. After 3 times of washes, target sequences were amplified in a reaction containing primers specific for E6 of HPV 16 or HPV 18, 1 nmol digoxigenin-11-dUTP (Roche Applied Science, Mannheim, Germany), 2.5 units of Taq polymerase (TaKaRa, Shiga, Japan), 0.5 mM dNTPs, and 5 μl PCR reaction buffer. An initial cycle was performed for 5 min at 94°C, followed by 15 cycles of 40 sec at 94°C, 40 sec at 45°C, and 1 min at 72°C. The sequences of primers used for PCR amplification were as follow: HPV16 E6: 5'-atgcaccaaaagagaactgca-3' (sense) and 5'- ttactgctgggtttctctacgtg-3' (antisense); HPV18 E6: 5'- atggcgcgctttgaggatccaac -3' (sense) and 5'- ttatacttgtgtttctctgcgtcg -3' (antisense). Thereafter, the detection reagent (anti-DIG antibody conjugated with alkaline phosphatase; Roche Applied Science, Mannheim, Germany) was applied onto sections followed by an incubation with NBT/BCIP solution to allow the signals to develop. After the signal development, the sections were counterstained with methylgreen, rinsed briefly in absolute ethanol, mounted, and then observed for signals under a microscope (200×). Cervical cancer tissues known to be positive for-HPV 16/18 infections served as positive controls.

Detection of HPV 16/18 E6 variants

RT-PCR and autosequencing were employed to analyze for E6 variants with mRNA preparations being studied materials. Total RNA was extracted by homogenizing

tissues in 1 ml TRIzol reagent (Life Technologies, Grand Island, NY, USA), followed by chloroform re-extraction and isopropanol precipitation. The preparation of cDNA was performed in a reaction with a total volume of 20 μl, containing 5 μg of total RNA in RT reaction buffer (Life Technologies, Grand Island, NY, USA), 10 mM DTT, 100 pmole of oligo d(T)18, 0.5 mM each of dNTPs, and 200 units of M-MLV reverse transcriptase (Life Technologies, Grand Island, NY, USA). The reactions were incubated at 42°C for 50 min and then terminated by heating at 75°C for 10 min followed by a quick chilling on ice. Target sequences were amplified in a 50 μl reaction mixture containing 20 pmole primers specific for full-length E6 of HPV 16 or HPV 18, 2.5 units of Taq polymerase (TaKaRa, Shiga, Japan), 0.5 mM dNTPs, 5 μl PCR reaction buffer, and 1 μl cDNA preparation. An initial cycle was performed for 5 min at 94°C, followed by 35 cycles of 40 sec at 94°C, 40 sec at 54°C, and 1 min at 72°C. The PCR products were analyzed using 2% agarose gel electrophoresis and those with HPV 16/18 E6 mRNA splicing and/or variant were then further analyzed by an autosequence machine (3100-Avant Genetic Analyzer) according to manufacture's protocol (Applied Biosystems, California, USA).

Expression of HPV 16 or 18 E6 in human fibroblast cell line WI-38

Full length E6 of HPV 16 or 18 was amplified by PCR from CasKi and HeLa cell lines, containing the HPV 16 and HPV18 virus genome, respectively. Different variant forms of HPV 16 or 18 were directly amplified from lung tumor tissues and the resulted PCR products were purified with GENECLEAN III kit and then cloned into an eukaryotic expression vector, pcDNA3.1/V5-His TOPO TA Expression Kit (Invitorogen, California, USA) and recombinant plasmid were transfected into WI-38. On the prior day of transfection, WI-38 cells was seeded at 1×10^5 cells per well and after an overnight incubation, cells, at 30-50% confluent, were washed twice with

phenol red-free DMEM medium without FBS. After cells being incubated with 1 ml phenol red-free DMEM medium with 10% FBS for 3 hours, calcium chloride-Hepes-buffer saline, and recombinant DNA solution was added dropwise to the medium in each well of plate and then the cells were put back to the incubator. After 4 hours, the medium was aspirated, the cells were shocked with glycerol solution for 30s and washed twice with phosphate-buffered saline. Stable transfectants were selected by culturing those transfected cells in the medium containing antibiotic G418.

Protein Extraction, Western Blot, and IP

Total proteins were extracted from fresh lung tumor tissues with a lysis buffer (100 mM Tris, pH 8.0, 1% SDS) and recovered protein concentrations were determined using the Bio-Rad protein assay kit followed by a separation with SDS-PAGE (12.5% gel, 1.5 mm thick). After an electrophoretic transfer to a PVDF membrane, nonspecific binding sites were blocked with 5% nonfat milk in TBS-Tween 20. The detection of HPV 16 or 18 E6, p53 and β-actin were conducted by incubating the membrane with anti-V5, (Invitorogen, California, USA) anti-HPV16 E6, anti-HPV18 E6 (Santa Cruz, California, USA and CHEMICON International, Inc., California, USA), monoclonal anti-human p53 and β-actin antibodies (DAKO, Copenhagen, Denmark) for 60 min at room temperature, followed by subsequent incubation with a peroxidase-conjugated secondary antibody (1:5000 dilution). Extensive washings with TBS-Tween 20 were performed between incubations to remove non-specific binding. The protein bands were visualized using enhanced chemiluminescence (NEN Life Science Products Inc., Boston MA, USA). For immunoprecipitation, protein extracts were precleared with prewashed Pierce protein A /G agarose beads. The precleared protein extracts were incubated with an anti-p53 (DAKO, Copenhagen, Denmark)

antibody for 8 hrs at 4℃ on a rotating rocker, and then with the prewashed Pierce protein A/G agarose beads for an additional 2 hrs. The immunocomplex was washed four times with Nonidet P-40 buffer, dissolved in SDS loading buffer, and fractionated on SDS / 12.5% polylacrylamide gels. The separated proteins were then transferred to a PVDF membrane and incubated with an anti-V5, (Invitorogen, California, USA) anti-HPV16 E6, anti-HPV18 E6 (Santa Cruz, California, USA and CHEMICON International, Inc., California, USA), monoclonal anti-human p53 and β-actin antibodies and an appropriate horseradish peroxidase (HRP)-coupled secondary antibody (Santa Cruz, California, USA). The membrane was washed in TBS-Tween 20 and the resulting signals were visualized by enhanced chemiluminescence (NEN Life Science Products, Inc. Boston MA, USA).

Statistical Analysis

Statistical analysis was performed using the SPSS statistical software program (Version 11.0 SPSS Inc., Chicago, IL, USA). The χ^2 test was applied for statistical analysis.

RESULTS

The concordant existence of E6 mRNA in HPV 16/18 DNA positive tumors

To elucidate whether HPV infection is implicated in lung tumorigenesis, HPV 16/18 E6 mRNA on paraffin sections of 73 lung tumors, which have been analyzed for the presence of HPV 16 and/or 18 DNA in our previous study[3], were analyzed by *in situ* RT-PCR. Results showed that HPV 16 E6 mRNA was detected in 27 (58.7%) of 46 HPV 16 DNA-positive tumors while HPV 18 E6 mRNA was detected in 25 (46.3%) of 54 HPV18 DNA-positive tumors. However, E6 mRNA was detected in none of HPV DNA-negative tumors (Table 1). This result has proved, to a certain extent, the accuracy of HPV DNA detection in our previous study.

Tissue *in vivo* reciprocal relationships between E6 mRNA and p53 protein

Based on the results of E6 mRNA detection, all 73 study subjects were grouped into 4 categories, including HPV 16/18+ (positive for HPV 16 and 18 E6 mRNAs), HPV 16+ (positive for HPV 16 mRNA only), HPV 18+ (positive for HPV 18 mRNA only) and HPV 16/18- (negative for both HPV 16 and 18 mRNAs). The serial paraffin sections of each tumor specimen were evaluated for the presence of HPV 16/18 E6 mRNA and p53 protein expressions by *in situ* RT-PCR and immunohistochemistry, respectively. As indicated by the representative data in Fig. 1, HPV 16 and/or 18 E6 mRNA were detected in lung tumor concurrently with p53 negative immunostaining (Fig. 1 A and B) and HPV 16 E6 mRNA was not detected in lung tumors with p53 positive immunostaining (Fig. 1 C and D). A reverse correlation between the presence of E6 mRNA and p53 protein was commonly observed in all four categories while most frequently in HPV 16/18+ group ($P = 0.009$, Table 2), followed sequentially by groups of HPV 16+, HPV 18+, and HPV 16/18-. It was notable that all eleven patients in HPV 16/18+ group were nonsmoking female. These results revealed a possibility

that HPV 16/18 E6 mRNA may play a role in the inactivation of p53 protein in lung tumors.

A correlation between HPV 16/18 E6 variants and p53 positive immunostainings

Previous studies have shown that E6 variants with mutations in the N-terminal region lost the ability to bind with E6AP and failed to cause p53 protein degradation[9]. To verify whether E6 variants were present in tumors with E6 mRNA expression and p53 positive immunostainings, HPV 16/18 E6 mRNA was analyzed by RT-PCR and autosequencing (Fig. 2 and 3). HPV 16 or 18 E6 variants, including G188C, T200C and splicing 227-408 of HPV 16 E6 variants, and A192T, A194C, and A400C of HPV 18 E6 variants, were detected in 13 (31.7%) of 41 tumors positive for HPV 16 and/or 18 mRNA (Table 3). Among those six types of E6 variants detected, five were base-substitution mutations that cause amino acid changes of E29Q, K33R, I30L, L102F, and one was transcriptional splicing to result in E6*I and E6*II splicing forms which coexisted with wild type of E6 mRNA. However, such coexistence did not occur for wild type E6 and E6 base-substitution variants. One E6 variant with a silent mutation, A194C (I30I), was found in a tumor, which was also detected with an 8 bp deletion in p53 gene, therefore, this silent mutation may be responsible for the resulting p53 negative immunostaining of this particular tumor. Among those E6 variants with base-substitution, mutation sites of 3 variants were within the N-terminal sequence (E29Q, K33R, and I30L) and another was found in the C-terminal sequence (L102F). Meanwhile, positive immunostaining of p53 protein was observed in tumors with E29Q, K33R or I30L E6 variants, but not in those 2 tumors with L102F variant. Interestingly, no E6 variant was found in HPV 16/18+ tumors that were all from female patients. In addition, after being stratified by gender, the occurrence of E6 variants in male tumors was significantly higher than that in

female (55.6% vs. 13.0%). Among all tumors positive for HPV 16 and/or 18 E6 mRNA in this study, HPV 16 or 18 E6 variants were detected in 10 (83.3%) of 12 tumors positive for p53 immunostaining. These results suggested that the presence of HPV 16 or 18 E6 variants were correlated with p53 protein stability in lung tumors.

A correlation between E6 variants and p53 protein stability in lung cells

To verify whether E6 variants may to lead to a degradation of p53 protein, different E6 variants were transfected into lung fibroblast WI-38 cells and western blot data indicated that E6 protein was detected in WI-38 cells transfected with HPV 16 E29Q, HPV 16 K33R, HPV 18 I30L, and wild type of HPV 16 and 18 E6, but not observed in HPV 16 E6*I- and HPV 16 E6*II-transfected cells (Fig. 4). Meanwhile, p53 protein was detected in WI-38 cells transfected with all E6 variants, not detected in cells transfected with wild type E6 of HPV 16 and 18 (Fig. 4). This experiment on cell lines indicated that the E6 variants found in lung tumor cases had lost the capability of p53 degradation and subsequently caused positive p53 immunostaining in lung tumors with E6 mRNA signals.

The involvement of HPV E6 in p53 protein degradation in lung tumor tissues

To elucidate the involvement of E6 protein in p53 inactivation, western blot and IP were used to examine the interaction between E6 and p53 proteins. The p53 protein level evaluated by western blot was similar to that detected by immunohistochemistry. With E6 expressions in SiHa (for HPV 16) and HeLa (for HPV 18) cells being adequately used as positive controls (Fig. 5), IP data showed that the binding between HPV 16 and/or HPV 18 E6 with p53 protein were detected in all tested 32 lung tumors with HPV 16 and/or 18 E6 mRNA, but not in 5 lung tumors without E6 mRNA, which acted as negative controls in this analysis. Interestingly, p53 protein

levels in tumors with I30L and E29Q E6 variants were relatively higher than those with wild type E6 protein. These results strongly suggested that E6 protein might be involved in p53 inactivation in lung tumors.

DISCUSSION

Most molecular investigations of the involvement of HPV 16/18 infections in human cancer were performed in cell lines, rarely on tumor tissues[5]. In this study, we collected data from tissue *in vivo* to address such issue and the data supported the possible role of HPV 16/18 E6 in p53 inactivation in lung tumors. Previously, the high-risk HPV 16/18 E6 gene expressions have been evaluated by RT-PCR, RNR-RNA *in situ* hybridization, and immunohistochemistry[14-18]. Although, RT-PCR is more sensitive in detection of HPV 16/18 E6 mRNA than *in situ* hybridization and immunohistochemistry, results from RT-PCR detection may be misleading due to contamination of non-tumor RNA in tumor tissues. To eliminate the false-positive possibility of HPV E6 mRNA in lung tumors, HPV DNA integration was evaluated by RS-PCR and positive results were detected in all tumors positive for HPV 16 and/or 18 E6 mRNA, but in none of HPV E6 mRNA negative tumors (data not shown). The products amplified from RS-PCR experiments were eluted from agarose gels and then subjected to direct sequencing to confirm the integration of HPV DNA in HPV E6 mRNA positive tumors (data not shown). Thus, the simultaneous occurrence of HPV DNA integration and E6 mRNA expression observed in one-half of HPV 16/18 DNA positive lung tumors supported the possibility that high risk HPV 16/18 DNA may integrate into cellular chromosome of tumor cells and expression of E6 to involve in lung tumorigenesis.

P53 is a tumor suppressor gene, which regulates cell proliferation and inhibits development or survival of cancer cells. Inactivation of p53 tumor suppressor gene has been found in many types of human cancers. Our previous immunostaining studies and DNA sequencing data[19, 20] revealed a significantly higher frequency of p53 negative immunostaining but a lower p53 mutation frequency in Taiwanese lung

cancer patients. To verify whether such p53 inactivation was through the p53 mutation, direct DNA sequencing data indicated that mutations in p53 genes were detected in only 8 of 73 HPV DNA positive tumors (11%). This frequency was relatively lower compared with that of HPV negative tumors reported previously[19, 20]. Coexistence of E6 expression with p53 mutation was found in only 4 HPV 16-/18+ tumors (1-bp deletion, P179L, A276T, and 8-bp deletion), neither in HPV 16+/18- nor in HPV 16+/18+ tumors. In addition, 2 of 4 HPV 18+ tumors with p53 mutation (P179L and an 8-bp deletion) had the same E6 variant, I30L. In this study, no p53 mutation was found in lung tumors with HPV 16 E6 mRNA expression, which was consistent with a previous observation in head and neck cancer[21].

The p53 codon72 polymorphism has recently been linked to the risk of cervical cancer. The arginine allele at codon 72 of p53 was found to be more susceptible to p53 degradation by HPV E6 protein than the proline allele *in vivo* and *in vitro*[22, 23]. Individuals with homozygous arginine-72 alleles were about seven times more susceptible to HPV-associated tumorigenesis than those with heterozygotes and therefore the arginine-encoding allele represented a significant risk factor in the development of HPV-associated cancers[24]. To exclude the effect of p53 codon72 polymorphism in HPV-related p53 protein degradation, the relationship between p53 codon 72 polymorphism and HPV infection in lung cancer patients with negative p53 immunostaining were also analyzed. In our study, no difference between HPV E6- and HPV E6+ lung cancer patients in the distribution of p53 genotypes was found and p53 immunostaining was not associated with p53 polymorphism (data not shown). p53 immunostaining was negative in 16 of 20 (80%) HPV E6+ lung tumors with Arg/Pro genotype, such frequency was relatively higher compared to tumors with Arg/Arg and Pro/Pro, but the occurrence of E6 variants in tumors with Arg/Pro

genotype were relatively lower (4 of 20, 20%). These results revealed that the correlation of HPV E6 status with the positive p53 immunostaining in HPV infected lung tumors was more significant than that with the p53 codon 72 polymorphism.

Previous studies[9, 10, 25] have reported that HPV E6 oncoprotein acts by promoting the p53 protein degradation via the ubiquitin-proteasomal pathway. From the results of this study, it was revealed that not all HPV E6 expression resulted in p53 negative immunostaining. As shown in Table 2, in our study group, positive p53 immunostaining was shown in 4 and 8 lung tumors with E6 mRNA of HPV 16 and 18, respectively. Interestingly, E6 variants, which were incapable of causing p53 degradation, were present in 10 of 12 tumors (83.3%) with positive p53 immunostaining. E6 reactivates the degradation of p53 by recruiting E6AP, a uibiquitin ligase[9]. High risk HPV E6 binds to E6AP via its N-terminal substrate recognition domain[26] and formation of a stable E6-E6AP complex precedes an association with p53 degradation[27]. Furthermore, mutated HPV E6 proteins generated by site-directed mutagenesis failed to form E6-p53-E6AP complex to interfere p53 in vivo stability[26]. Several previous[28, 29] studies have shown that alternatively spliced HPV 16 and 18 E6 mRNA inhibited the E6-directed, ubiquitin-mediated degradation of p53 *in vitro*. In our study, p53 immunostaining was positive in 8 of 8 (100%) and 2 of 4 (50%) lung tumors with HPV 16 and 18 E6 variants, respectively. Moreover, all of them were detected to have a variation in N-terminal of the E6 protein that may hinder the binding of E6 with E6AP and then lead to p53 protein accumulation. However, HPV 18 E6 variant L102F, detected in a lung tumor negative for p53 immunostaining, has a mutation in C-terminal of E6 protein and such mutation may not affect the formation of E6-p53-E6AP complex to cause p53 degradation by ubiquitin-proteasomal pathway. Our E6 variants transfection experiment strongly

supported the interaction between E6 variants and p53 protein observed in lung tumor tissues. Thus, the conflicting results of E6 and p53 both positive observed in some lung tumors were explained by that the capability for p53 degradation of E6 variants was decreased compared with wild type E6.

Several studies[9, 30] have reported that high-risk HPV E6 proteins could result in dysfunction of p53 not only by promoting the p53 protein degradation via the ubiquitin-proteasomal pathway but also by binding with the C-terminal of p53 protein to inhibit the transcription activity that causing cell cycle progression. Clearly, the cell cycle control would be lost if p53 was bound with high-risk HPV E6[9, 31]. We have detected E6 proteins bound with p53 in all tested 32 lung tumors positive for HPV E6 mRNA in this study. From that about 46% of Taiwanese lung cancers were detected with HPV 16/18 DNA in our previous study, together with that about 50% of HPV DNA-positive lung tumors HPV DNA were detected with HPV E6 expressions in the present study. In summary, the *in vivo* reciprocal relationships between high-risk HPV 16/18 E6 mRNAs and p53 immunostainings, as well as the binding of wild-type and variants of E6 protein with p53 protein in lung tumor tissues, have strongly supported our previous unexpected finding that high-risk HPV 16/18 infection may be associated with Taiwanese lung tumorigenesis. Our present results further suggested that the binding of p53 protein with HPV 16/18 E6 oncoprotein may play a role in lung tumorigenesis in Taiwanese lung cancer patients.

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Table 1. Correlations between HPV 16/18 DNA and E6 mRNA expressions in lung tumors.

HPV16/18 E6 mRNA	HPV 16/18 DNA		
	Negative	Positive	P
HPV16	(n=91)	(n=46)	
Negative (n=110)	91	19	
Positive (n=27)	0	27	<0.0001
HPV18	(n=83)	(n=54)	
Negative (n=112)	83	29	
Positive (n=25)	0	25	<0.0001

Data of HPV 16/18 DNA were obtained from a previous study (Cheng et al., 2001).

χ^2 test was used for statistical analysis.

Table 2. Correlations between HPV 16/18 E6 mRNA expression and p53 protein immunostaining in lung tumor tissues.

p53 protein immunostaining	HPV 16/18- (n=32)	HPV 18+ (n=14)	HPV 16+ (n=16)	HPV16/18+* (n=11)	p
Negative (n=48)	19	10	8	11	
Positive (n=25)	13	4	8	0	0.009

*All 73 study subjects were grouped into 4 categories, including HPV 16/18+ (positive for HPV 16 and 18 E6 mRNAs), HPV 16+ (positive for HPV 16 mRNA only), HPV 18+ (positive for HPV 18 mRNA only) and HPV 16/18- (negative for both HPV 16 and 18 mRNAs) as text described. χ^2 test was used for statistical analysis.

Table 3. The association of p53 protein immunostainings with p53 gene mutation, codon 72 polymorphism, E6 mRNA status in HPV 16/18 E6 mRNA positive patients.

Patient no.	Gender	Age	Smoking habits	Tumor		p53 gene mutation		p53 codon72 genotype	P53 IHC	HPV16/18 E6 mRNA	HPV E6 variant	
				Type	Stage	Location (codon)	Type				Location (nt)	Amino acid change (codon)
8144	F	41	-	AD	IIIa	-	-	Arg/Arg	-	+/-	-	-
9649	F	55	-	AD	IIb	-	-	Arg/Arg	-	+/-	-	-
8367	F	58	-	AD	IIIa	-	-	Pro/Pro	-	+/-	-	-
9538	F	46	-	AD	I	-	-	Arg/Arg	+	+/-	T200C	K33R
3154	F	71	-	SQ	I	-	-	Arg/Pro	-	+/-	-	-
9931	F	72	-	AD	IIb	-	-	Arg/Pro	-	+/-	-	-
9984	F	70	-	SQ	I	-	-	Arg/Pro	-	-/+	-	-
0586	F	37	-	AD	IIIa	243-246	8 bp del	Pro/Pro	-	-/+	A194C	I30I
4391	F	40	-	AD	IIIa	-	-	Arg/Pro	-	-/+	-	-
9141	F	58	-	AD	IIIa	-	-	Arg/Pro	-	-/+	A410C	L102F
0396	F	46	-	AD	I	-	-	Arg/Arg	-	-/+	-	-
8110	F	67	-	AD	IIIB	-	-	Arg/Arg	-	-/+	-	-

Table 3. (Continued)

Patient no.	Gender	Age	Smoking habits	Tumor		p53 gene mutation		p53 codon72 genotype	P53 IHC	HPV16/18 E6 mRNA	HPV E6 variant	
				Type	Stage	Location (codon)	Type				Location (nt)	Amino acid change (codon)
4253	F	73	-	AD	I	-	-	Arg/Pro	-	+/+	-	-
6923	F	52	-	AD	I	-	-	Arg/Pro	-	+/+	-	-
2466	F	76	-	AD	IIIb	-	-	Arg/Arg	-	+/+	-	-
6380	F	58	-	SQ	I	-	-	Arg/Pro	-	+/+	-	-
4924	F	84	-	AD	IIIb	-	-	Arg/Arg	-	+/+	-	-
9291	F	48	-	AD	I	-	-	Arg/Arg	-	+/+	-	-
0158	F	64	-	AD	IIa	-	-	Arg/Pro	-	+/+	-	-
9638	F	74	-	SQ	IIIa	-	-	Arg/Pro	-	+/+	-	-
0641	F	76	-	AD	IIIa	-	-	Arg/Pro	-	+/+	-	-
9047	F	84	-	AD	IIIa	-	-	Arg/Pro	-	+/+	-	-
2265	F	57	-	AD	I	-	-	Arg/Arg	-	+/+	-	-
6167	M	71	+	SQ	IIIa	Codon 261	1-bp del	Pro/Pro	-	-/+	-	-
4000	M	62	-	AD	I	-	-	Arg/Pro	-	-/+	-	-

Table 3. (Continued)

Patient no.	Gender	Age	Smoking habits	Tumor		p53 gene mutation		p53 codon72 polymorphism	P53 IHC	HPV16/18 E6 mRNA	HPV E6 variant	
				Type	Stage	Location (codon)	Type				Location (nt)	Amino acid change (codon)
8577	M	72	+	AD	IIIa	-	-	Pro/Pro	-	-/+	A410C	L102F
7979	M	42	+	AD	I	-	-	Arg/Pro	-	+/-	-	-
9548	M	67	-	AD	I	-	-	Arg/Pro	-	+/-	-	-
6076	M	38	-	SQ	IIIa	-	-	Arg/Arg	-	+/-	-	-
4014	M	72	+	SQ	I	-	-	Arg/Pro	-	-/+	-	-
5868	M	68	+	SQ	I	Codon 179	P179L	Arg/Pro	+	-/+	A192T	I30L
3780	M	64	+	SQ	IIb	-	-	Pro/Pro	+	-/+	-	-
3402	M	43	+	SQ	IIb	-	-	Arg/Pro	+	-/+	-	-
3896	M	66	-	AD	IIb	276	A276T	Arg/Arg	+	-/+	A192T	I30L
9362	M	56	+	SQ	IIb	-	-	Arg/Arg	+	+/-	G188C	E29Q
4853	M	66	+	AD	IIb	-	-	Arg/Arg	+	+/-	G188C	E29Q
9440	M	49	-	AD	IIa	-	-	Arg/Pro	+	+/-	T200C	K33R
8164	M	66	+	SQ	IIIa	-	-	Arg/Pro	+	+/-	G188C	E29Q
9904	M	66	+	SQ	IIb	-	-	Arg/Arg	+	+/-	Splicing	E6*I & E6*II
6920	M	63	+	SQ	IIIa	-	-	Arg/Arg	+	+/-	G188C	E29Q
1604	M	62	-	AD	I	-	-	Pro/Pro	+	+/-	T200C	K33R

LEGENDS

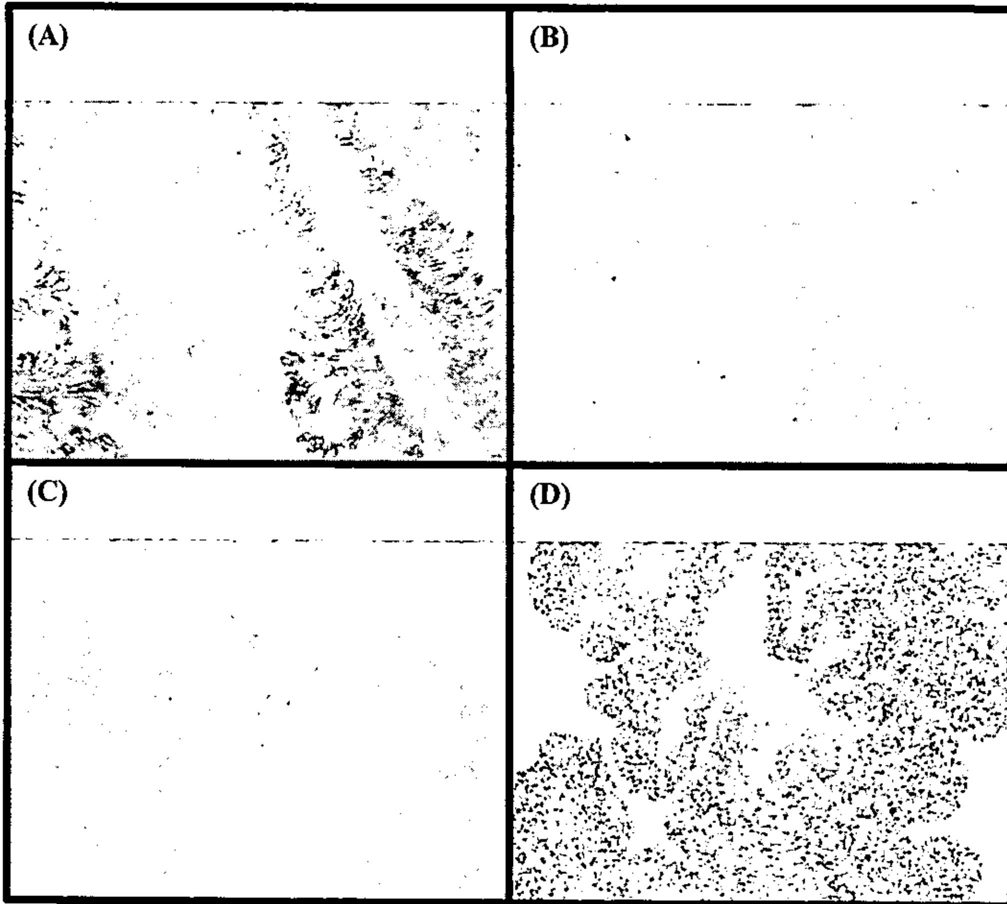
Fig. 1. The representative reciprocal relationships between HPV 16 E6 mRNA expression and p53 immunostaining in two serial paraffin sections of lung tumors. (A) the presence of HPV 16 E6 mRNA on a lung tumor section detected by *in situ* RT-PCR, (B) the lack of p53 signal on the serial section of that in (A) detected by immunohistochemistry, (C) the absence of HPV E6 mRNA signal on a lung tumor section, and (D) the presence of p53 immunostaining signals on the serial section of that in (C).

Fig. 2. Autosequencing analysis of HPV16 E6 variants. Sequences of wild type cDNA of HPV 16 were shown in (A), (B) and (C), respectively while those of HPV 16 E6 variants, G188C, T200C, and HPV16 E6*I splicing, were shown in (D), (E), and (F), respectively.

Fig. 3. Autosequencing analysis of HPV18 E6 variants. Sequences of wild type E6 cDNA of HPV 18 were shown in (A), (B) and (C), respectively while those of HPV 18 E6 variants A194C, A192T, and A400C, were shown in (D), (E), and (F), respectively.

Fig. 4. A correlation between HPV E6 and p53 protein expression in human fibroblast WI-38 cell line with different HPV16 or 18 E6 status. Parental cells without being transfected (1) and cells being transfected with a specified E6 construct: vector control (2), HPV 16 E6*I (3), HPV 16 E6*II (4), HPV16 E29Q (5), HPV 16 K33R (6), HPV18 I30L (7), HPV16 E6 (8), and HPV18 E6 (9), were subjected to western blot analysis for HPV 16/18 E6, p53 and β-actin protein expressions as shown in HPV 16/18 E6, p53 and β-actin lane, respectively.

Fig. 5. A correlation between HPV E6 status and p53 protein expression in lung tumor tissues in two independent experiments of different lung cancer patients. Western blot analysis for p53 and β-actin protein expression were shown in p53 and β-actin lane, respectively. The results of IP by p53 antibodies followed by immunoblotting of E6 protein of HPV 16 or 18 were shown in p53/HPV16 E6 and p53/HPV 18 E6, respectively. HeLa and SiHa cells were used as positive controls. P53 protein levels in two tumors with E6 variants (I30L and E29Q) were relatively higher than those with wild type of E6.



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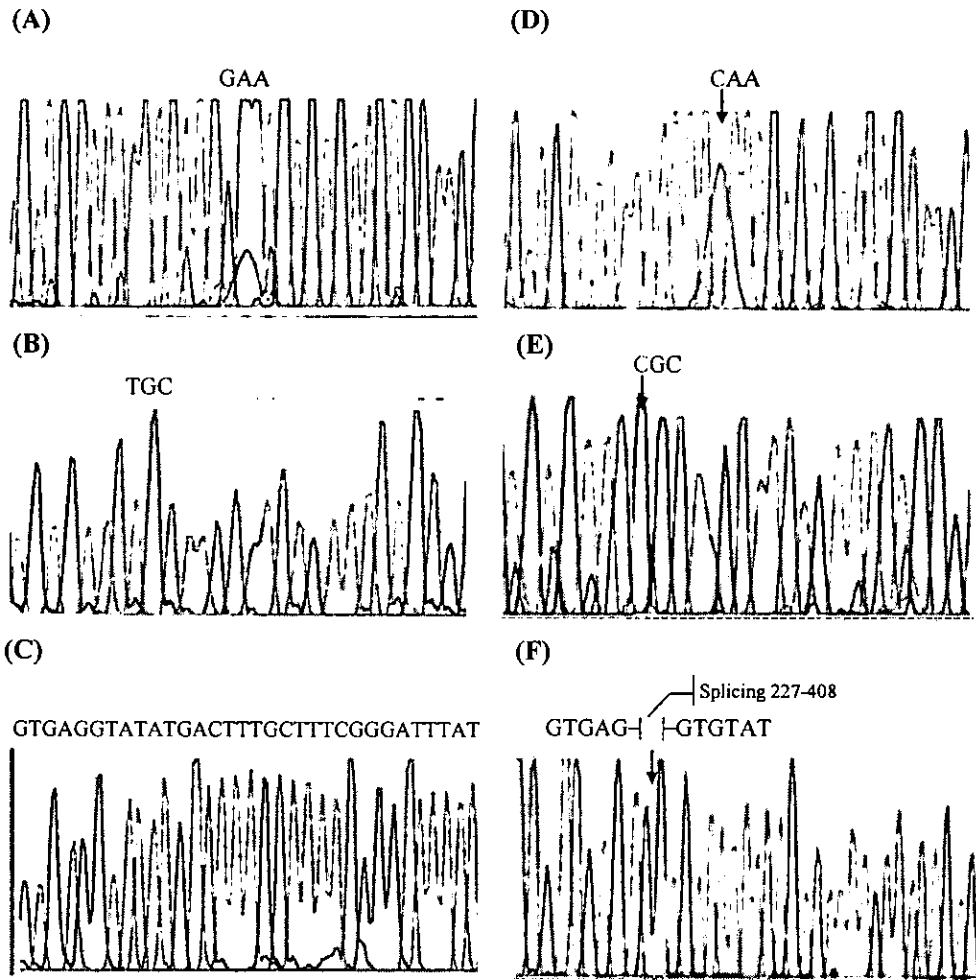

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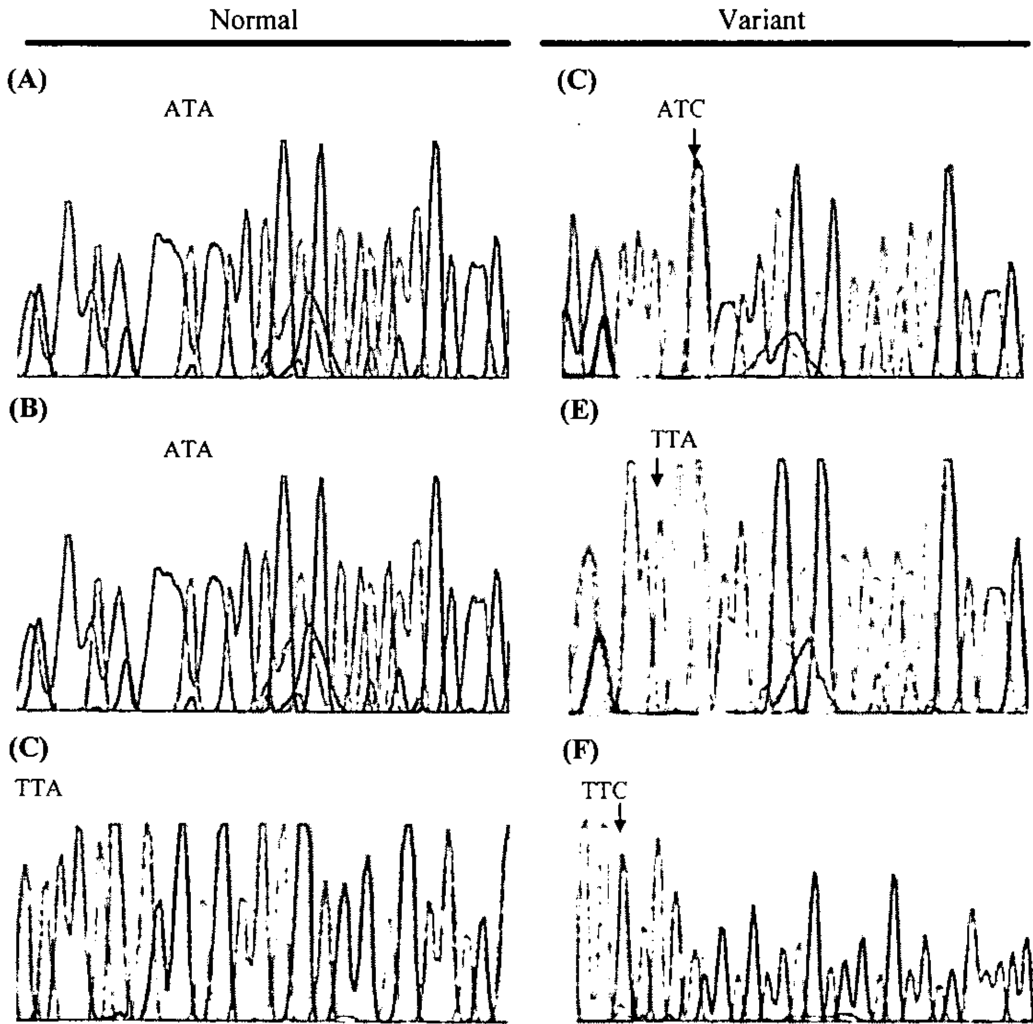
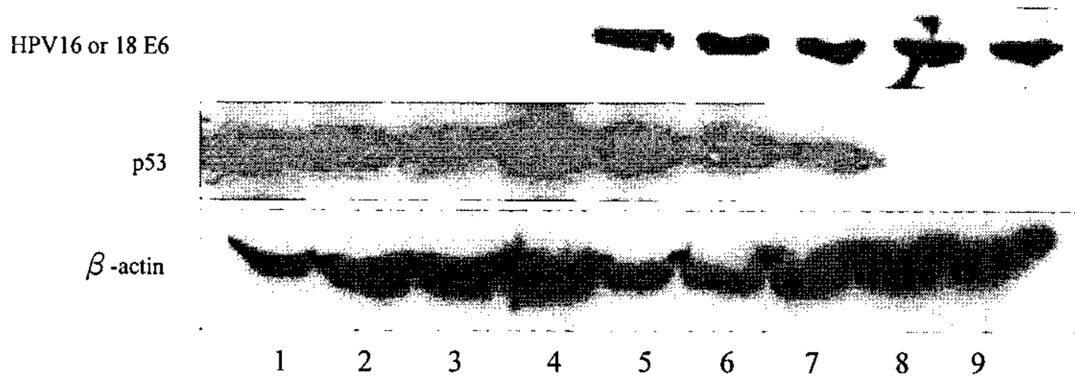


Fig. 3. Autosequencing analysis of HPV18 E6 variants. Sequences of wild type E6 cDNA of HPV 18 were shown in (A), (B) and (C), respectively while those of HPV 18 E6 variants A194C, A192T, and A400C, were shown in (D), (E), and (F), respectively.



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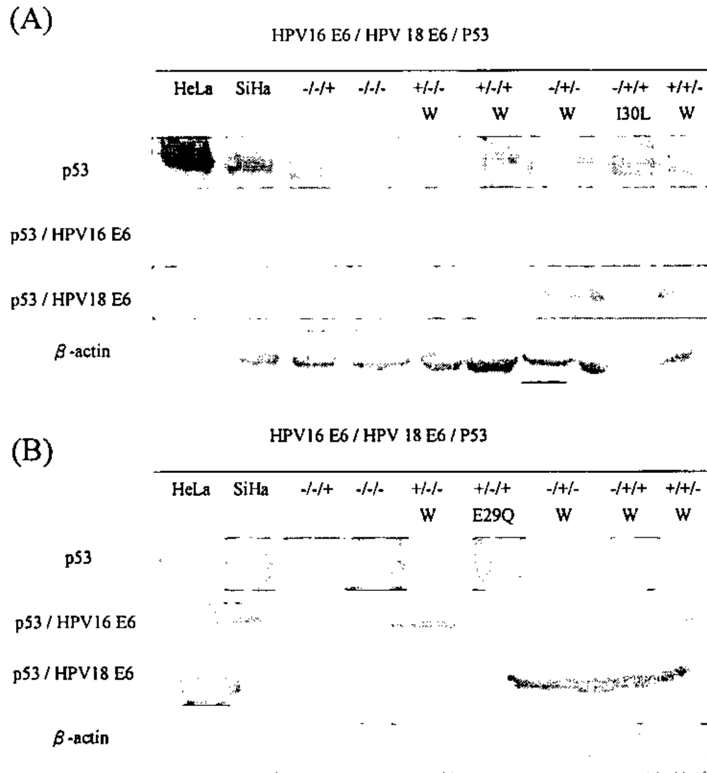

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FREQUENT *FHIT* GENE LOSS OF HETEROZYGOSITY IN HUMAN PAPILLOMARVIRUS-INFECTED NONSMOKING FEMALE LUNG CANCER IN TAIWAN

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Key words: FHIT, LOH, HPV-associated lung cancer

Running Title: FHIT LOH in HPV-infected nonsmoking female lung cancer

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ABSTRACT

The fragile histidine triad (FHIT), located in chromosome region 3p14.2, had been reported to be a frequent allele with loss of heterozygosity (LOH) in smoking lung cancer and HPV-associated cervical cancer and may act as a tumor suppressor gene to involve in smoking- lung tumorigenesis and HPV-related cervical tumorigenesis, respectively. In our previous report, a high prevalence of HPV 16/18 infection has been observed in nonsmoking female lung cancer patients, and thus it was speculated that HPV 16/18 infection may increase the occurrence of FHIT LOH in female cases to implicate in lung tumorigenesis. In this study, 157 lung cancer patients were enrolled and subjected to FHIT LOH analysis with three microsatellite markers. The frequency of FHIT LOH in males, smokers, and squamous cell carcinomas lung cancer patients was significantly higher than that of their corresponding counterpart ($P = 0.020$ for gender, $P < 0.001$ for smoking status, and $P = 0.038$ for tumor type). Additionally, among patient groups of nonsmoking female, nonsmoking male and smoking male, FHIT LOH frequency (58%) was the highest in smoking male lung cancer patients. During an investigation for the effect of HPV infection on the occurrence of FHIT LOH, a correlation between HPV 16 infection and FHIT LOH was only observed in female lung cancer cases. To be more specifically, FHIT LOH frequency was remarkably increased from 18% (6 of 33) in HPV 16 non-infected female cases to 46% (11 of 24) in HPV 16 infected cases. These results suggest that HPV 16 may, at least in part, contribute to the increase of FHIT LOH frequency to involve in HPV associated lung tumorigenesis in Taiwanese non-smoking women.

INTRODUCTION

The fragile histidine triad (FHIT) gene, located in chromosome region 3p14.2, undergoes frequent allele loss of heterozygosity (LOH) and occasional homozygous deletions in various cancer types including lung cancer and cervical cancers[1]. FHIT protein inactivation is a frequent event in bronchial premalignant lesions and in stage I non-small cell lung cancer (NSCLC), suggesting that loss of FHIT expression occurs early in the progression from normal to malignant lesions[2]. Importantly, loss of FHIT expression plays a potential role in smoking-related lung tumorigenesis[2]. On the other hand, human papillomarivus (HPV) infection has an initiating role in cervical cancer[3]. A complex series of molecular events are associated with HPV infection in cervical tumorigenesis and the most important event is HPV DNA integration into the host genome[4, 5]. HPV can insert its genes into the fragile site FRA3B adjacent to FHIT to occur allele loss of the gene[6]. Thus, loss of FHIT gene expression may be an excellent marker of HPV associated-cervical cancer.

Our previous case-control study indicated that Taiwanese women with HPV 16/18 infections in lung tissues had a up to 12-fold of lung cancer risk compared to that of Taiwanese men[7]. Thus, we speculated that HPV may be a possible aetiological factor of lung cancer since over 90% of Taiwanese women with lung cancer were nonsmokers[8, 9] while lung cancer has been the leading cause of cancer mortality since 1982 (Department of Health, ROC, Life Statistics, 1996). It has been shown that the integration of HPV viral genome frequently occurred at the fragile site FRA3B adjacent to FHIT to cause allele loss of the gene[4,6], therefore, to further explore the involvement of HPV infection in lung tumorigenesis, FHIT may be considered to be a suitable candidate gene to verify whether more frequent FHIT LOH was observed in HPV-infected lung cancer compared with that of non-HPV infected lung cancer,

especially in nonsmoking female cases.

MATERIALS AND METHODS

Study subjects

A total of 172 primary lung cancer patients including 60 females and 112 males, who had undergone thoracic surgery at Veterans General Hospital-Taichung, were enrolled. None of the subjects had received radiation therapy or chemotherapy prior to surgery. The histology of tumor types and stages were determined by qualified pathologists according to the WHO classification. Information on smoking history of the lung cancer patients was obtained from hospital records to categorize the patients into smoking or non-smoking groups. Since less than 10% of female lung cancer patients were smokers, and most of female cases were diagnosed with late-stages and therefore not suitable for surgical resection therapy, we were unable to collect any tumor tissues from smoking female patients for this study.

DNA Extraction and Nested polymerase chain reaction (Nested PCR).

Genomic DNA from tumor and non-tumor tissues were isolated by conventional phenol-chloroform extraction, ethanol precipitation and finally dissolved in sterile distilled water. HPV viral DNA was first amplified with type consensus primers MY09 and MY11[10] followed by a second round of amplification with type specific primers flanking the L1 region to identify the subtype. The final PCR product (10 μl) was loaded onto a 2% agarose gel, stained with ethidium bromide and visualized under UV illumination. Appropriate negative and positive controls were included in each PCR reaction with a part of the β-actin gene in all samples being amplified to exclude false-negative results and DNA preparations from the SiHa cell (containing HPV 16) and the HeLa cell (containing HPV 18) being used as positive controls.

PCR and fluorescent DNA analysis of FHIT LOH

The sequences of nucleotide markers for microsatellite analysis, *D3S1300*, *D3S1234*, and *D3S1313*, are available through the Genome Database. PCR amplification was carried out in a final volume of 15μl with 50 ng of genomic DNA template; 100 ng of each labeled primer; 25mM dGTP, dATP, dTTP, and dCTP; 1.5 mM $MgCl_2$;10 × buffer and 0.6 unit of Amplitaq Gold (Applied Biosystems). Samples were processed through 35 cycles, with each cycle consisting of 40 s at 94°C, 40 s at an annealing temperature of 57°C to 60°C, as appropriate for each primer, and 40 s at 72°C. Afterwards, 1 μl of the 1:20 diluted PCR product were mixed with 10 μl of Hi-Di formamide (Applied Biosystems) and 0.5 μl liz500 size standard (Applied Biosystems). The mixture was denatured at 95°C for 5 min, chilled on ice, and analyzed by ABI PRISM 310 automatic sequencer. The image was analyzed using the Genescan and Genotyper software (Applied Biosystems). Q^{LOH} was calculated as the ratio of the allele height ratios in tumor and non-tumor DNA, as in [tumor allele 1/tumor allele 2]/[non-tumor allele 1/ non-tumor allele 2]. When this value was greater than one, Q^{LOH} was set to be the inverse. Tumor: non-tumor ratios were scored as: negative if $Q^{LOH} > 0.49$; LOH if $Q^{LOH} \leqq 0.49$. The cut-off value was established based on the 99.7% reference range of mean allele ratios from normal tissue samples, and the inter-assay variability was then assessed. This figure was calculated from mean allele ratios of normal samples of $0.79 \pm 3SD$ (SD=0.1, n = 40). Thus, the cut off point of allele ratio was 0.49.

Statistical Analysis

Fisher's exact test and χ^2 test were used for statistical analyses. All analyses were performed using the SPSS Version 11.0 statistical package.

RESULTS

LOH of FHIT in lung cancer patients was determined by three markers, D3S1300, D3S1234, and D3S1313, of 3p14.2. A wide range of allele ratios were recorded at the three loci in lesions. The rates of informativity at the three loci were 75, 79, and 84%, respectively. Applying the experimentally established LOH allele ratio cut-off point of 0.49, 30% of cases overall had LOH at the D3S1300 locus, 32% at the D3S1234 locus, and 21% at the D3S1313 locus while 42% (64/153) of cases informative at each one loci had LOH at both microsatellite regions. The representative FHIT LOH examined by each marker was shown in Fig. 1. The relationships of FHIT LOH with the clinico-pathological parameters of lung cancer patients were shown in Table 1. FHIT LOH was associated with tumor type and smoking status, but not related to other studied parameters including age, tumor stage, and T, N values. Namely, the frequency of FHIT LOH in males, smokers, and patients with squamous cell carcinomas was significantly higher compared to that of females, nonsmokers, and patients with adenocarcinomas, respectively ($P = 0.020$ for gender, < 0.001 for smoking status, and $P = 0.038$ for tumor type). To understand the contribution potentials of gender and cigarette smoking for the occurrence of FHIT LOH, the FHIT LOH frequencies in patient groups, including smoking male, nonsmoking male and nonsmoking female, were compared. As shown in Table 2, the highest frequency of FHIT LOH was observed in smoking male cases (58%) while nonsmoking male and female cases have a same FHIT LOH frequency (30%).

To verify the implication of HPV infection in the occurrence of FHIT LOH, the association between FHIT LOH and HPV infection was analyzed for all study cases as well as three groups including nonsmoking female, nonsmoking male, and smoking male lung cancer patient. As shown in Table 3, only a nearly significant association

between HPV 16 or 18 infection and FHIT LOH was observed in nonsmoking female lung cancer patients (P = 0.078), but not in other groups. After further analyses with HPV 16 or HPV 18 infection being individual factor, the abovementioned association was then only observed in nonsmoking female lung cancer patients with HPV 16 infection, but not with HPV 18 infection (P = 0.024 for HPV 16, P = 0.0498 for HPV 18). More interestingly, the frequency of FHIT LOH was markedly increased from 18 % (6 of 33) in nonsmoking female cases without HPV 16 infection to 46 % (11 of 24) in those with HPV 16 infection (P = 0.024).

DISCUSSION

Many human cancers, including those of lung, breast, kidney, head and neck, ovary, and cervix, have been demonstrate to be with 3p LOH involving at least four distinct chromosomal regions[11-14]. Of interest is the 3p14.2 locus, which contains the apidicoline-inducible FRA3B fragile site[15, 16]. This site has also been reported to be an integration site for HPV 16 and demonstrated to be frequent LOH in cervical carcinoma[6]. Within this region and spanning FRA3B is the candidate tumor suppressor gene, FHIT gene[15]. Previous reports showed that active smokers exhibited a significantly higher frequency of fragile site expression, including FRA3B[17], compared to that of nonsmokers, since 45% of healthy former smokers have LOH at 3p14.2 in bronchial epithelium[18], and nearly 100% of small cell lung cancer patients showed LOH on 3p14.2[19-21]. These results suggested that active tobacco exposure may increase the potential for chromosome breakage and/or rearrangement at the fragile sites that, in turn, contribute to carcinogenesis. In this study, we found FHIT LOH frequency in smoking lung cancer patients was significantly higher than in nonsmoking lung cancer patients (Table 1). This data was consistent with previous reports showing that a higher FHIT LOH frequency in smoking lung tumors and our data supported that allele loss of FHIT may play a role in smoking-related lung tumorigenesis.

Our data clearly indicated that FHIT LOH frequency was significantly increased by HPV 16 infection in nonsmoking female lung cancer patients. The frequency of FHIT LOH in female cases was increased to 46% in HPV 16 infected cases, an occurrence similar to that of smoking cases. This result suggested that HPV 16 infection may contribute to LOH of FHIT in lung cancer. The contribution of HPV infection was apparent to be similar to cigarette smoking. In the present study, three markers,

D3S1300, D3S1234, and D3S1313 were used to determine FHIT LOH, and LOH occurred at any one of three markers was considered to be FHIT LOH positive. In the verification to detect which marker for FHIT LOH detection was more associated with HPV 16 infection in female cases, our data indicated that HPV 16 infection was only significantly correlated with FHIT LOH detected by D3S1300 (P = 0.013), which was the most near FRA3B site among those three markers. In cervical cancer studies, HPV 16 has been reported to integrate within intron 4 of FHIT gene, and HPV 16 DNA more preferred to integrate into FRA3B site to involve in FHIT LOH than other types of high risk of HPV had been intensively investigated[4, 6]. This result strongly reflected the fact that HPV 16 DNA may integrate into 3p14.2 at FRA3B to involve in FHIT LOH of HPV infected-lung tumors.

In conclusion, our data provided the evidence to show that the involvement of HPV 16 in Taiwanese nonsmoking female lung tumorigenesis may be, at least in part, mediated through an increase of the frequency of FHIT LOH.

ACKNOWLEDGMENTS

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Table 1. The association between FHIT LOH and clinical characteristics in lung cancer patients.

Parameters	No.	FHIT LOH		P value
		Negative	Positive	
Age (year)				
≦66	79	44	35	0.522
＞66	74	45	29	
Gender				
Female	57	40	17	0.020
Male	96	49	47	
Smoking status				
Smoker	65	27	38	＜0.001
Non-smoker	88	62	26	
Tumor type				
AD	89	58	31	0.038
SQ	64	31	33	
Stage				
I	65	41	24	0.428
II	23	11	12	
III	65	37	28	
T				
1	7	5	2	0.296
2	122	71	51	
3	19	12	7	
4	5	1	4	
N				
0	72	45	27	0.278
1	30	15	15	
2	48	26	22	
3	3	3	0	

χ^2test were used for statistical analysis.

AD: adenocarcinoma, SQ: squamous cell carcinoma.

Table 2. The differences of FHIT LOH between lung cancer patients with various gender and smoking habits.

FHIT LOH	Nonsmoking female n(%)	Nonsmoking male n(%)	Smoking male n(%)	P value
Negative	40(70)	22(70)	27(42)	0.002
Positive	17(30)	9(30)	38(58)	
P value	0.938	0.007		

χ^2 test were used for statistical analysis.

Table 3. A correlation between HPV infection and FHIT LOH in all studied cases and three categories.

HPV Infection	FHIT LOH							
	All studied cases		Nonsmoking				Smoking	
	(n=153)		Female (n=57)		Male (n=31)		Male (n=65)	
	Negative n	Positive n	Negative n	Positive n	Negative n	Positive n	Negative n	Positive n
16 or 18								
Negative	51	33	21	4	13	5	17	24
Positive	38	31	19	13	9	4	10	14
P	0.481		0.078		1.000		0.987	
16								
Negative	63	43	27	6	16	7	20	30
Positive	26	21	13	11	6	2	7	8
P	0.634		0.024		1.000		0.646	
18								
Negative	67	48	27	13	17	6	23	29
Positive	22	16	13	4	5	3	4	9
P	0.968		0.498		0.660		0.532	

Three categories of lung tumors in this study, including nonsmoking female, nonsmoking male, and smoking male, were categorized based on gender and cigarette smoking status.

LEGEND

Fig. 1. Representative results of FHIT LOH determined by marker of (A) D3S1234, (B) D3S1300 and (C) D3S1313 in lung tumor and adjacent normal tissues.

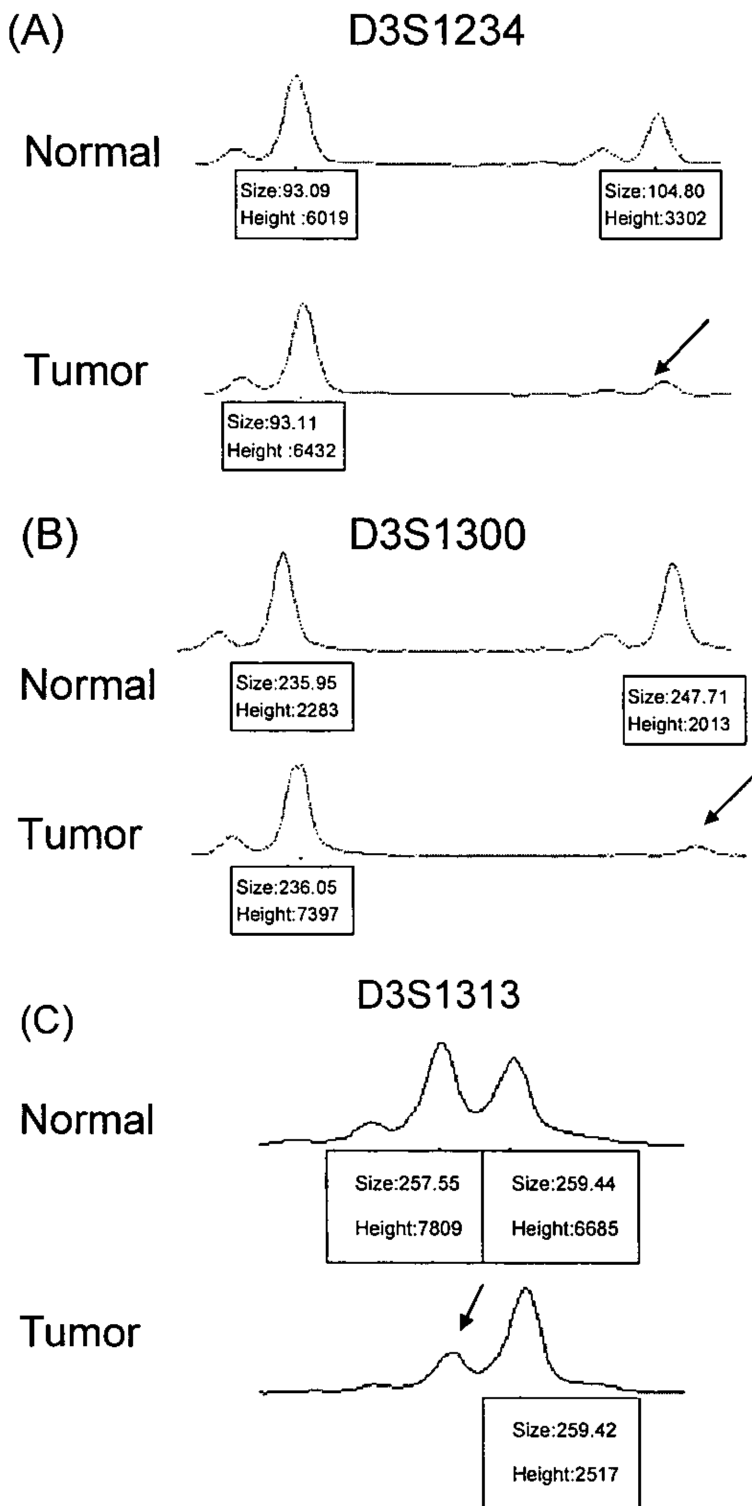

Fig. 1. Representative results of FHIT LOH determined by marker of (A) D3S1234, (B) D3S1300 and (C) D3S1313 in lung tumor and adjacent normal tissues.

The Correlation between Aberrant Connexin 43 mRNA Expression Induced by Promoter Methylation and Nodal Micrometastasis in Non-Small Cell Lung Cancer[1]

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ABSTRACT

Reduced connexin (Cx) 43 gene expression has been shown in most of lung tumors and cancer cell lines. Although aberrant Cx43 gene expression was linked with lung tumorigenesis, our understanding to the mechanism was still limited. We hypothesized that the evidence of aberrant Cx43 gene expression was gradually intensified from adjacent normal lung tissues surrounding tumors toward tumor tissues. In this study, 90 lung tumors and adjacent normal tissues were collected to examine Cx43 mRNA expression by reverse transcription-PCR (RT-PCR). Our data showed that Cx43 mRNA expression in adjacent normal lung tissue was significantly correlated with nodal involvement ($P = 0.03$), but the similar trend was not observed in tumor tissues. To verify whether lack of Cx43 mRNA expression resulted from promoter methylation, PCR-based methylation assay was performed for Cx43 promoter methylation analysis. A higher frequency of promoter methylation was observed in Cx43 mRNA-negative patients (21 of 33, 63.7%) compared with Cx43 mRNA-positive patients (3 of 57, 5.3%, $P < 0.0001$). To elucidate whether aberrant Cx43 gene expression originated from adjacent normal lung tissues, 25 lung tumors and each of five adjacent normal tissues at various distances from tumor tissues were collected to examine Cx43 mRNA and protein expression by RT-PCR and Western blot, respectively. The results show that Cx43 mRNA and protein expressions gradually decreased from adjacent normal lung tissues to tumor tissues with a positive correlation to the distance from the tumor tissues. Gel-shift assay data also revealed that shifted band binding with AP1 was only observed in adjacent normal tissues, which were far from the tumor tissues. These results indicate that promoter methylation may interfere with AP1 binding to the promoter to cause aberrant Cx43 gene expression. Thus, Cx43 mRNA in adjacent normal tissue surrounding lung tumor simply detected by RT-PCR may act as a molecular marker of nodal micrometastasis in non-small cell lung cancer.

INTRODUCTION

Lung cancer is the leading cause of cancer death in many developed countries, including Taiwan, in which 20% of cancer deaths have been caused by lung cancer. Late diagnosis in lung metastasis has resulted in <20% of lung cancer patients having a 5-year survival (1). Thus, suitable molecular markers for early diagnosis of lung cancer metastasis are helpful for reducing lung cancer mortality rates. Currently, the presence of lymph node metastasis along with extent of primary tumor (T) and distant metastasis (M) status represents the most accurate factor available for the prediction of prognosis in patients who undergo complete surgical resection. However, tumor recurrence has occurred in ~30% of patients with pathological stage I NSCLC[4] and ultimately led to death, despite complete surgical resection. This suggests that occult micrometastatic tumor cells, which are not detected by current clinical staging examinations and conventional histopathologic methods, have already spread to the regional lymph node or distant mesenchymal organs at the time of surgery. Therefore, for an accurate prediction of prognosis, it is necessary to assess the lymph node status and take account of nodal micrometastasis.

Many human tumors, including lung cancer, have been reported to be deficient in expression of Cx43 mRNA and protein levels (2–7). Moreover, the decreased connexin 43 mRNA is correlated with its protein levels (8). This finding suggests that reduced Cx43 gene expression in human lung cancer cell lines and lung tumors may be caused by promoter methylation. After the transfection of Cx43 cDNA into a human lung carcinoma cell line deficient in Cx43 gene expression, the Cx43 transfectants show a reduced growth rate and inhibition of tumorigenicity (9). Similar findings were also observed in human glioblastoma cells (10). In contrast, when rat glioma cells

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[4] The abbreviations used are: NSCLC, non-small cell lung cancer; RT-PCR, reverse transcription-PCR.

were transfected by a mutant Cx43 cDNA, the growth capacity of the transfected clones was restored, and the tumorigenicity of these cells was reinforced (11). Furthermore, some evidence supports the hypothesis that Cx43 may be a tumor suppressor gene, which suggests that reduced Cx43 gene expression is involved in tumor progression, including invasion and metastasis (9–13), *e.g.*, a mouse skin carcinogenesis experiment showed that clear reduction of Cx43 was observed in squamous cell carcinomas at invasive sites, and additionally, when squamous cell carcinomas metastasized into lymph nodes, few carcinoma cells expressed Cx43 (14, 15). This suggests that quantitative and qualitative changes in Cx43 expression are associated with tumor progression, including the loss of differentiation and invasion and metastasis, during multistage mouse skin carcinogenesis (15). In this study, we found that Cx43 mRNA expression in adjacent normal lung tissues from lung cancer patients was related with nodal micrometastasis. Moreover, promoter methylation was responsible for the aberrant Cx43 gene expression.

MATERIALS AND METHODS

Study Subjects. Between 1994 and 1997, 90 consecutive patients with operable NSCLC underwent surgery at the Department of Thoracic Surgery, Veterans' General Hospital-Taichung, Taiwan, ROC. Noncancer patients with lung disease, including pneumothorax, tuberculosis, chest wall deformity, and cryptococcal infection, who underwent thoracic surgery at Cheng-Kung University Hospital, Taiwan or Changhua Christian Hospital, Changhua, served as control subjects. None of the subjects received radiation therapy or chemotherapy before surgery. After surgery, pathological material from different areas of the tumor was processed by conventional histological procedures, and the fresh samples were immediately stored at −70°C until further use.

Preparation of RNA and RT-PCR. Total RNA was extracted by homogenizing the tissues in 1 ml of TRIzol reagent (Life Technologies, Inc., Grand Island, NY), followed by chloroform re-extraction and isopropanol precipitation. The preparation of cDNA was performed in a reaction with a total volume of 20 μl, containing 5 μg of total RNA in the reverse transcriptase reaction buffer (Life Technologies, Inc.), 10 mM DTT, 100 pmol of oligo d(T)18, 0.5 mM each of deoxynucleotide triphosphates, and 200 units of M-MLV reverse transcriptase (Life Technologies, Inc.). The reactions were incubated at 42°C for 50 min and then terminated by heating at 75°C for 10 min followed by quick chilling on ice. Target sequences were amplified in a 50-μl reaction mixture containing 20 pmol of each of the following primers: Cx43-S (5′-GCGTGAGGAAAG-TACCAAAC-3′) and Cx43-AS (5′-CCCGTTGGAACTCAA-GAAGG-3′), 2.5 units of Taq polymerase (TaKaRa, Shiga, Japan), 0.5 mM deoxynucleotide triphosphates, 5 μl of PCR reaction buffer, and 1 μl of cDNA preparation. An initial cycle was performed for 5 min at 94°C, followed by 35 cycles of 40 s at 94°C, 40 s at 54°C, and 1 min at 72°C. The PCR products were analyzed using 1% agarose gel electrophoresis.

PCR-based Methylation Assay. The methylation status of genomic DNA was analyzed using the modified PCR methylation assay. Genomic DNA (1 μg) was digested overnight with 10 units of methylation-sensitive (*Msp*I) and methylation-insensitive (*Hind*III) enzyme. For the assessment of the methylation status of the promoter of the Cx43 gene, 100 ng of digested DNA were amplified by PCR using primers flanking the restriction sites. Undigested and digested DNA were used as controls and included for every site examined. To rule out the possibility of incomplete digestion, all samples were digested twice with each of the enzymes in independent experiments. PCR amplifications from each of the duplicate digests were repeated at least twice to ensure reproducibility of the results.

Gel-shift Assay. Nuclear extract was prepared as described previously. DNA binding was measured using a gel retardation assay. A complementary pair of synthetic oligonucleotides containing the sequence 5′-CCCAGTTGAGTC-AGTGGCTTG-3′ of the AP1 binding was synthesized. The oligonucleotides were 32p-labeled at the 5′-end using T4-polynucleotide kinase and [α-32p]dATP. Nuclear extract (15 μg) was incubated with 1 μl of poly[d(I-C)] and 20 ng of 32p-labeled AP1 binding site at room temperature for 20 min. The reaction mixture was loaded onto a 5% polyacrylamide gel and electrophoresed at 120 V for ~3 h in 25 mM Tris to 195 mM glycine. Gel was dried and exposed on X-ray film overnight.

Protein Extraction and Western Blot. Total protein extracts from fresh lung tumor tissues were prepared with a lysis buffer [100 mM Tris (pH 8.0) and 1% SDS]. The protein concentration was determined using the Bio-Rad protein assay kit, and this was followed by separation with SDS-PAGE (12.5% gel, 1.5 mm thick). After electrophoretic transfer to Hybond-C extra nitrocellulose, the nonspecific binding sites were blocked with 5% nonfat milk in TBS-Tween 20. Cx43 protein was detected by incubating the membrane with monoclonal antihuman Cx43 antibody (Zymed Laboratories, Inc., dilution 1:1000) for 60 min at room temperature, followed by extensive washing with TBS-Tween 20 and subsequent incubation with peroxidase-conjugated secondary antibody (1:500 dilution). The Cx43 band was visualized using enhanced chemiluminescence (NEN Life Science Products, Inc., Boston, MA).

Statistical Analysis. Statistical analysis was performed using the SPSS statistical software program version 10.0 (SPSS, Inc., Chicago, IL). Analyses of the associations between Cx43 mRNA expression and prognostic factors for lung cancer (including age, sex, T, N, M, tumor stage, tumor type, and tumor grade) and Cx43 gene methylation status were, respectively, performed using the Pearson χ^2 test, Fisher's exact test, and likelihood ratio test.

RESULTS

Our previous studies have shown that Cx43 protein was not detected in most of lung tumor tissues (151 of 165, 92%; unpublished data). In this study, 90 lung tumors and adjacent normal tissues were collected to examine the correlation of Cx43 mRNA expression with clinical-pathological parameters. Among the parameters, Cx43 mRNA expression in adjacent normal lung tissues was only associated with nodal involvement (Table 1; $P = 0.03$). To verify whether lack of Cx43 mRNA expression resulted from promoter methylation, PCR-based methylation assay for Cx43 promoter methylation was performed. Patients with Cx43 mRNA negative had a higher fre-

Table 1 Relationships between Cx43 mRNA expression in lung tumors and adjacent normal tissues from lung cancer patients and their clinical parameters

	Cx43 mRNA expression			
	Adjacent normal tissue		Tumor tissue	
Parameter	Negative	Positive	Negative	Positive
Tumor type				
Adenocarcinoma	15	40	20	35
Squamous cell carcinoma	14	21	13	22
P	0.250		1.000	
Tumor stage				
I	10	21	12	19
II	3	12	3	12
III	16	28	18	26
P	0.504		0.334	
T factor				
1	2	4	2	4
2	22	41	23	40
3	3	11	4	10
4	2	5	4	3
P	0.801		0.640	
N factor				
0 (n = 42)	12	30	15	27
1 (n = 20)	3	17	4	16
2 (n = 28)	14	14	14	14
P	0.030		0.103	

Table 2 Correlation between Cx43 mRNA expression and its promoter methylation in adjacent normal lung tissues from lung cancer patients

	Cx43 mRNA expression		
Promoter methylation	Negative	Positive	*P*
Negative	12	54	
Positive	21	3	<0.0001

quency of promoter methylation (21 of 33, 63.7%) compared with those with Cx43 mRNA positive (3 of 57, 5.3%; Table 2; Fig. 1). Thus, promoter methylation may play an important role in aberrant Cx43 transcription in NSCLC.

To verify that down-regulated Cx43 gene expression in adjacent normal lung tissues gradually decreased with distance from lung tumors, Cx43 mRNA and protein expressions in 25 surgically resected adjacent normal lung tissues at various distances surrounding lung tumors were evaluated by RT-PCR and Western blot, respectively. Our data show that CX43 mRNA and protein were normally expressed in lung tissues of noncancer controls (Fig. 2*A*) but gradually decreased in adjacent normal tissues with being closer to the lung tissues. Similar decreases in Cx43 protein expression levels, after being adjusted with that of β-actin, were also observed (Fig. 2*B*). To further elucidate the mechanism for aberrant Cx43 mRNA transcription, 5 of 25 lung cancer patients were used for gel-shift assays to examine the binding affinity between AP1 and the Cx43 gene promoter. Our results show that aberrant Cx43 mRNA expression in lung tumors and adjacent normal tissues were correlated

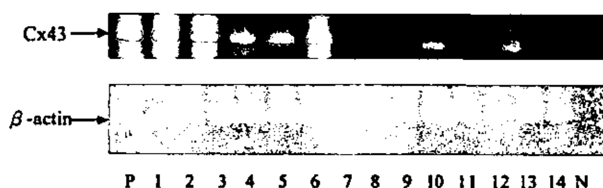

Fig. 1 Cx43 promoter methylation status in adjacent normal lung tissues surrounding tumors. DNA was digested with *Msp*I and analyzed by methylation-specific PCR. *P*, positive control; *N*, negative control. *Lanes 1–14* were taken from different lung cancer patients. β-actin was used as an internal control.

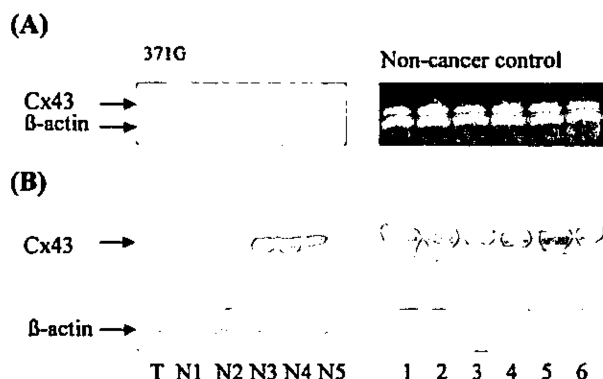

Fig. 2 Representative Cx43 mRNA and protein expressions in lung tumor (*T*) and its adjacent normal tissues with various distances (*N1–N5*) compared with Cx43 mRNA and protein expressions in normal lung tissues from six noncancer control subjects. *A*, Cx43 mRNA expressions in lung cancer patients (*371G*) and noncancer controls; *B*, Cx43 protein in lung cancer patients (*371G*) and noncancer controls. Cx43 mRNA and protein expressions were analyzed by RT-PCR and Western blot, respectively. β-actin was used as an internal control.

to the reduction of promoter binding with AP1 (Fig. 3). Loss of AP1 binding was found in tumors with the absence of Cx43 mRNA expression and gradually decreased in adjacent tissues in direct relation to the decreasing distance from the tumor tissues. This result strongly indicates that the binding affinity of AP1 with the promoter region may be responsible for the gradual decrease in Cx43 gene expression. In conclusion, the gradual decrease in Cx43 gene expression in adjacent normal lung tissues strongly suggests that the absence of Cx43 mRNA may be useful as an early diagnostic molecular marker for nodal micrometastasis in NSCLC patients.

DISCUSSION

There is a growing body of evidence suggesting that connexin gap junction proteins act as tumor suppressors. Their tumor inhibitory effect is usually attributed to their main function of cell coupling through gap junctions (6–12, 16, 17). Aberrant Cx43 gene expression has been found in several types of tumor, including liver, prostate, kidney, skin, breast, and lung cancers (2–6). In our previous report, Cx43 protein expression was not detected in most of the lung tumors (unpublished data), and the findings were similar to previous studies. Ruch *et al.* (2) indicated that Cx43 mRNA and protein levels of various human

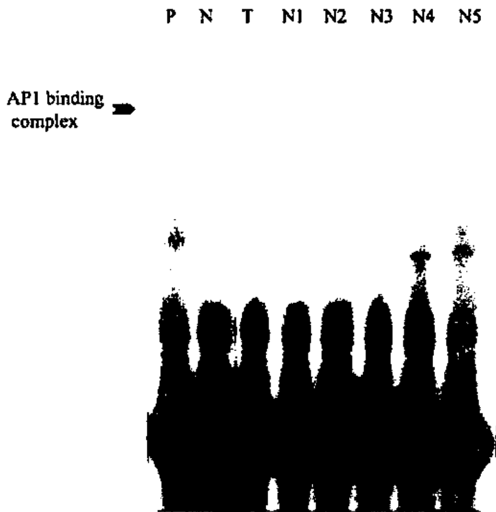

Fig. 3 Representatiave gel retardation assay of nuclear extracts from lung tumor (*T*) and its adjacent normal tissues at various distances (*N1–N5*) from synthetic AP-1 binding sequence on Cx43 promoter region. Nuclear extracts of WI38 human lung fibroblast cells were used as positive controls. WI38 cell nuclear extracts incubated with an excess of unlabeled AP-1 probe to determine the specificity were used as negative controls (*N*).

lung cell lines were all significantly reduced in comparison with nontransformed lung epithelial cells (2). The decrease in Cx43 mRNA levels in these lung carcinoma cells was correlated well with Cx43 protein expression (8). However, no direct evidence is available to demonstrate the involvement of Cx43 in lung tumorigenesis. In the present study, we report for the first time how gradual increases in aberrations of Cx43 gene expression through promoter methylation in adjacent normal lung tissues compare with corresponding tumors. Moreover, aberrant Cx43 mRNA expression in adjacent normal lung tissue is correlated to nodal micrometastasis of NSCLC. This finding may be used to support previous findings showing that aberrant Cx43 gene expression was involved in human lung tumorigenesis.

In a multistage mouse chemical-induced skin carcinogenesis model, the expression of Cx43 in squamous cell carcinomas was significantly decreased compared with surrounding nontumorous epidermis and papillomas (14, 15). Moreover, Cx43 was expressed in only few squamous cell carcinomas that had metastasized into lymph nodes (15). In human lung carcinoma cells, Cx43 mRNA was not detected in a highly metastatic lung carcinoma cell line, PG, by Northern blot (9). Furthermore, a transfection with exogenous Cx43 cDNA into PG cells could result in a marked suppression of growth *in vitro* and *in vivo* (9). This seems to reveal that aberrant Cx43 gene expression may be associated with lung tumor metastasis. A similar observation was made in breast cancer where a correlation was found between metastatic potential and the decrease of Cx43 gene expression (18).

It is well established that promoter methylation can result in decreased expression of tumor suppressor genes, such as *p16*, *p15*, and *hMLH-1*, contributing to increased tumorigenicity in various tumors (19). Promoter methylation has been shown to link with the aberrant transcription of Cx43 in rat liver cells (20). Additionally, treatment of Cx43-negative HeLa cells with 5-aza-2′-deoxycytidine resulted in expression of Cx43, suggesting gene silencing via DNA methylation (21). In the present study, the majority of Cx43 mRNA-negative samples was found to possess promoter methylation. The promoter methylation was not only detected by PCR-based methylation asasy but also confirmed by Southern blot (data not shown). Moreover, the promoter methylation responsible for the decrease of Cx43 gene expression was further confirmed by gel-shift binding assay data. Previous reports have indicated that the AP1 binding site was located at the region of Cx43 promoter methylation (21–24). Thus, we suggest that aberrant AP1 binding with promoter region by meththylation may be responsible for the aberrant Cx43 gene transcription in NSCLC.

In summary, gradually aberrant Cx43 gene expressions in adjacent normal lung tissues surrounding tumors mediated through promoter methylation strongly suggest that Cx43 mRNA in adjacent normal tissue simply detected by RT-PCR may be act as a molecular marker of nodal micrometastasis in NSCLC.

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