行政院國家科學委員會專題研究計畫 成果報告

HPV16 E6/E7 在肺癌 p16 基因甲基化作用機轉之角色
研究成果報告(精簡版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 95-2320-B-040-038-
執 行 期 間 ：95 年 08 月 01 日至 96 年 07 月 31 日
執 行 單 位 ：中山醫學大學醫學研究所

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處 理 方 式 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 96 年 10 月 30 日

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

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成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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行政院國家科學委員會專題計畫成果報告

HPV16 E6/E7 在肺癌p16 基因甲基化作用機轉之角色

The role of HPV 16 E6/E7 in p16 promoter hypermethylation of lung cancer

壹、 中文摘要

已知 HPV 16/18 E6 致癌蛋白始 p53 基因去活化在子宮頸癌的腫瘤化過程扮演了重要的角色。為了了解 HPV16/18 E6 與 p53 表現在肺腫瘤化過程中所扮演的角色，本計畫共收集 122 個肺癌患者的腫瘤組織以免疫組織化學染色分析兩者間的相關性，結果發現 HPV16/18 E6 與 p53 蛋白表現呈負相關，且在連續切片中亦可得到驗證。此外並以即時偵測反轉錄聚合酶連鎖反應偵測 p53 下游基因 $p21^{WAF1/CIP1}$ 及 *mdm-2* mRNA 的表現量，結果發現 $p21^{WAF1/CIP1}$ 及 *mdm-2* mRNA 的表現量在可測得 HPV16/18 E6 表現者低於 HPV16/18 E6 不表現者。為了進一步證明 HPV16/18 E6 可造成 p53 蛋白得去活化，本實驗室由肺癌患者胸水中成功的建立具有 HPV16 感染及不感染的肺腺癌細胞株，在肺癌細胞株的研究中發現 HPV HPV16/18 E6 表現的肺癌細胞株其 p53 蛋白的表現量低於不表現者且其下游基因 $p21^{WAF1/CIP1}$ 及 *mdm-2* mRNA 的表現量也相對較低。免疫沉澱法的分析結果亦發現 HPV16/18 E6 確實與 p53 蛋白結合而造成其去活化，而當把細胞中的 HPV16/18 E6 以 siRNA 方式抑制後 p53 蛋白、$p21^{WAF1/CIP1}$ 及 *mdm-2* mRNA 的表現量均有回復的現象，根據組織及細胞的研究結果證實 HPV 確實存在於肺腫瘤組織中，並可透過始 p53 蛋白去活化而參與肺腫瘤化。此外為了證明 HPV16/18 E6 使 p53 蛋白去活化對 MGMT 基因甲基化的影響，本研究亦利用 siRNA 的方式及轉染突變形式的 p53 基因到肺癌細胞株中，並分析當 p53 蛋白去活化時對 MGMT 基因轉錄起始區甲基化的影響，結果亦發現當 p53 蛋白去活化確實會造成 MGMT 基因轉錄起始區甲基化。因此本研究認為 HPV16/18 E6 除透過使 p53 蛋白去活化而使細胞腫瘤化外亦可造成抑癌基因的甲基化而使細胞癌化。

關鍵字：肺癌，人類乳突瘤病毒

ABSTRACT

Inactivrucial role in cervical tumorigenesis. To investigate the involvement of HPV 16/18 in lung tumorigenesis, the association between HPV 16 or 18 E6 and p53 protein expression in 122 lung tumors was evaluated by immunohistochemistry and data showing that HPV 16/18 E6 expression correlated inversely with p53 expression, which was further confirmed by tissue *in situ* immunostaining. Real-time RT-PCR analysis indicated that E6-positive tumors had lower $p21^{WAF1/CIP1}$ and *mdm-2* mRNA levels than E6-negative tumors. To elucidate the role of E6 on p53 inactivation, we successfully established lung adenocarcinoma cell lines with or without HPV 16 infection from patients' pleural effusions. Western blotting showed that E6 protein was indeed expressed in HPV16-infected cells and a lower level of p53 protein was observed in E6-postive cells compared to E6-negative cells. Moreover, the levels of $p21^{WAF1/CIP1}$ and *mdm-2* mRNA in E6-positive cells were lower than in E6-negative cells. The interaction of E6 with p53 protein was revealed by immunoprecipitation assay showing that p53 could be inactivated by E6 protein. Conversely, p53 proteins, $p21^{WAF1/CIP1}$ and *mdm-2* mRNA expressions were restored in E6 knockdown cells by RNA interference compared with vector control cells. These results reveal that HPV 16/18 E6 may be partially involved in p53 inactivation to downregulate $p21^{WAF1/CIP1}$ and *mdm-2* transcription. In conclusion, HPV 16/18 E6 is indeed expressed in HPV DNA–positive lung tumors and is involved in p53 inactivation to contribute to HPV-mediated lung tumorigenesis. To elucidate whether p53 participates in promoter methylation, we engineered three cell lines: A549 cells with RNA interference (RNAi)-mediated knockdown of p53, and p53 null H1299 cells transfected with either wild-type p53 (WT-p53) or mutant-p53 (L194R-p53). Knockdown of endogenous p53 increased MGMT promoter methylation in A549 cells, and transient expression of WT-p53 in p53 null H1299 cells diminished promoter methylation, whereas the MGMT promoter methylation status was unchanged by expression of L194R. Therefore, we concluded that HPV 16/18 E6 could inactivate p53 protein to induced MGMT gene promoter hypermethylation to promote lung tumorgenesis.

Key words: lung cancer, Human papillomavirus

Introduction

Lung cancer is the leading cause of cancer death for Taiwanese women since 1982. Although cigarette smoking is the major cause of lung cancer worldwide, more than 90% of lung cancer in Taiwanese females is not related to cigarette smoking (1). Therefore, most Taiwanese women may have a unique etiology for lung cancer development. Our previous study indicated that human papillomavirus (HPV) oncogenic subtypes 16/18, which are involved in cervical cancer, also may be involved in the pathogenesis of lung cancer among Taiwanese, because 55% of lung cancer patients had HPV 16/18 DNA compared with 11% of non-cancer control subjects. Also the odds ratio for lung cancer in nonsmoking females with HPV 16/18 infection (~10) was much higher than that for nonsmoking males (~2) (2). Additionally, HPV 16/18 DNA was uniformly detected in lung tumor cells but not in the adjacent non-involved lung tissue. These results strongly suggest that HPV infection with virus subtypes

known to be oncogenic for cervical cancer is associated with lung cancer development in nonsmoking Taiwanese women. In addition, our recent case-control study also clearly revealed that an individual with HPV 16 and 18 DNA in their blood was at a 76-fold risk for lung cancer compared with subjects without HPV 16/18 DNA (3), further implicating HPV in lung tumorigenesis.

Although studies of viral-related lung cancer have been reported (4-6), the molecular pathogenesis of this disease type remains unclear. For example, the impact of the oncogenic DNA virus simian virus 40 (SV40) on the development of malignant mesotheliomas and the high risk of HPV 16/18 in lung cancer were until recently controversial. The integration of high-risk HPV16/18 DNA into host chromosome to express E6 protein plays a crucial role in HPV-induced cervical carcinogenesis (7-9). E6 has many functions that may contribute to its oncogenic potential. The classical function of E6, which is relevant to cellular immortalization, is binding to the tumor suppressor p53, thereby inducing p53 degradation (10). The role of p53 is to safeguard the integrity of the genome by inducing cell cycle arrest or apoptosis upon DNA damage (11). As a transcription factor, p53 upregulates target genes involved in coordinating these responses. For example, $p21^{WAF1/CIP1}$, a cyclin-dependent kinase (CDK) inhibitor that acts on cyclin E/cdk2 complexes and *mdm-2* (12,13). Therefore, p53 inactivation by E6 leads to chromosomal instability and increases the probability of an HPV-infected cell evolving towards malignancy (10). Animal model experiment further demonstrated that HPV16 E6 gene alone is sufficient to induce carcinomas in transgenic mice (14).

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 (15) while Epstein-Barr virus (EBV) causes infectious mononucleosis and is closely associated with Burkit's lymphoma, nasopharyngeal carcinomas, and Hodgkin's disease (16, 17). Furthermore, HPV is now believed to be associated with cervical and oral cancers (18-22) and EBV may involve in breast and gastric cancers (23,24). 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 (25,26), and the association of Simian virus 40 with RASSF1A promoter methylation in malignant mesothelioma have been reported (27,28). Our previous report showed that HPV 16/18 infection was associated with lung cancer development in Taiwanese women nonsmokers (29), 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 (30). 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.

In this study, to understand whether p53 could be inactivated by E6 in HPV-infected lung cancer, the following experiments would be performed: (i) to examine whether E6 could express in lung tumors, (ii) to understand whether E6 protein expression in lung tumor was associated with the inactivation of p53 pathway, and (iii) to elucidate the role of E6 on p53 inactivation in HPV-infected lung cancer cell lines which have been successfully established from patients' pleural effusions.

Result

E6 protein was indeed expressed in lung tumors and adjacent normal tissues: Relationships between E6 and clinical parameters

Our preliminary restriction-specific PCR (RS-PCR) data showed that HPV 16/18 DNA integration occurred in HPV DNA–positive lung tumors. We thus attempted to determine whether HPV 16/18 E6 is expressed in lung tumors by western blotting and immunohistochemistry to verify the association between p53 expression and HPV16/18 E6 expression. Western blotting was first used to detect the presence/absence of HPV 16/18 E6 in 10 randomly selected HPV DNA–positive lung tumors and corresponding adjacent normal lung tissues. The data clearly showed that E6 was predominately expressed in lung tumors, although some of paired adjacent normal tissues had low-level E6 expression (Fig. 1A). Consequently, 122 lung tumors containing or lacking HPV 16/18 DNA were tested for E6 expression using immunohistochemistry. Our present and previous data indicated that HPV 16/18 E6 is only expressed in lung tumors that were previously shown to be positive for HPV 16/18 DNA by nested-PCR (Table 1). HPV 16 or 18 E6 was indeed expressed in tumor cells as well as in adjacent normal cells in tumor tissues such as type II pneumocytes, bronchiole epithelia, blood vessel endothelia, lymphocytes, and alveolar macrophages (Fig. 1B).

The relationships between E6 expression and clinical parameters of lung tumors are shown in Table 1. The expression of HPV 16 or 18 E6 in lung tumors of females, adenocarcinomas, and nonsmokers was significantly higher than in males, squamous cell carcinomas, and smokers, respectively ($P = 0.001$ for gender and tumor type, $P = 0.002$ for smoking status). E6 expression was not associated with other clinical parameters including age, tumor stage, T and N factor, although HPV 18 E6 expression was more

common in advanced tumors and associated with T factor (Table 1).

E6 protein was negatively associated with p53 protein expression in lung tumors

To elucidate whether E6 affects p53 expression, p53 expression in lung tumor tissues was also determined by immunohistochemistry. p53 expression correlated inversely with HPV 16 E6 (P = 0.011) and HPV 16 or 18 E6 expression (P = 0.004), but was marginally associated with HPV 18 E6 alone (P = 0.085) (Table 2). To confirm the reciprocal relationship between HPV 16 or 18 E6 and p53, serial paraffin sections of lung tumors were used to assess protein expression *in vivo*. Protein of p53 was not detected in tumors positive for HPV 16 or 18 E6; conversely, HPV 16– or 18 E6–negative tumors had positive p53 protein expression (Fig. 2). The reverse correlation between HPV 16/18 E6 and p53 expression *in vivo* clearly revealed the possibility that HPV 16/18 E6 may, at least in part, promote the degradation of p53 in HPV-positive lung tumors.

The levels of p21 and mdm2 mRNA in E6 positive tumors were lower than in E6-negative tumors

To elucidate whether p53 was inactivated by E6, mRNA levels of *p21* $^{WAF1/CIP1}$ and *mdm-2*, which function downstream of p53, in lung tumors were measured by real-time RT-PCR. The mRNA levels of *p21* $^{WAF1/CIP1}$ and *mdm-2* in HPV 16 E6–, HPV 18 E6–, and HPV 16 or 18 E6–positive tumors were significantly lower than those of negative tumors (Table 3). However, the expression of these genes did not correlate with p53 mutations and p53 expression, although a negative trend was apparent (Table 3). These results suggest that p53 inactivation caused by HPV 16/18 E6 may play a more important role than p53 mutations or other mechanism(s) in causing p53 accumulation in HPV-positive lung tumors.

The involvement of E6 on p53 inactivation in HPV16-infected lung cancer cells

To elucidate the role of E6 in p53 inactivation in lung cancer, we established three HPV16-infected and one HPV16-noninfected lung adenocarcinoma cell lines from patients' plural effusions. The HPV16 DNA copy numbers of these three cell lines were evaluated by FISH showing that 2-3, 1 and 4-5 of HPV16 DNA copies were revealed in TL-1, TL-2, and TL-3 cells, respectively. Thus, these results clearly showed that HPV16 DNA integrated into chromosomes of these cells (Supplementary Fig. 1). The tumorgenicity of these established lung cancer cell were demonstrated by soft agar assay. HPV-16 infected cells had ability to form larger colonies in soft agar assay compared with the non-infected cells after 14 days culture (data not shown). To our knowledge, this is the first time to establish HPV-infected lung cancer cell lines from pleural effusions of lung cancer patients.

It is well known that E6 was de-repressed by E2 splicing when HPV16 DNA integrated into host chromosomes (19). E6 protein was then evaluated by Western blotting showing that different levels of HPV 16 E6 proteins expressed in HPV 16-infected TL1, TL2, and TL3 cells. As expected, E6 was not detected in HPV16-non-infected TL4 cells. Our data also revealed that p53 protein levels in E6-positive TL1, TL2, and TL3 cells were significantly lower than in E6-negative TL4 cells (Fig. 3A). Immunoprecipitation assay clearly showed that E6 protein was interacted with p53 protein in E6-positive cells, not in E6- negative cells (Fig. 3B). To further verify the interaction between E6 and p53 could be responsible for p53 inactivation, TL1 E6 was knocked down by two RNA interferences (RNAi), western blot showing that E6 protein in siE6-1 (the first RNAi) and siE6-2 (the second RNAi) transient cells was reduced compared with vector control cells, however, the E6 reduction in siE6-2 cells was more efficiency than in siE6-1 cells (Fig. 3C). Conversely, p53 protein levels were markedly increased in both siE6 cells (Fig. 3C). The levels of *p21* $^{WAF1/CIP1}$ and *mdm-2* mRNA evaluated by real-time RT-PCR were significantly restored in siE6-2 cells but relatively renovated in siE6-1 cells compared with vector control cells (Fig. 3D). To explore the growth effects of E6 knockdown by RNAi, TL-1 cells with and without siE6-1 or siE6-2 transfection were evaluated by the doubling time, plating efficiency, and cloning efficiency assay, respectively (Supplementary Fig. 2A). As shown in Fig 5, the doubling time of TL-1 cells with siE6-1 and siE6-2 was extended to 28~32 hr and 36~38 hr compared with 24~26 hr double time of parental TL-1 cells, respectively. The plating efficiency of TL-1 cells was decreased from 42±4% to 22±3% (siE6-1) and 15±1% (si-E6-2). The cloning efficiency was also reduced from 95±7% to 46±4% (siE6-1) and 25±3% (siE6-2). In addition, flow cytometry showed that S-phase cell proportion was significantly decreased in E6 knockdown cells (22.98% for siE6-1, 21.17% for siE6-2) as compared with TL-1 parental cells (38.94%) (Supplementary Fig. 2B). These results clearly indicated that p53 inactivation by E6 may increase the cell proliferation and colony formation.

MGMT promoter methylation is more common in lung cancer cells with p53 negative expression and mutated p53

To elucidate the molecular mechanisms behind this association, we examined the MGMT promoter methylation status of 11 randomly selected lung cancer cell lines with or without p53 mutations. In general, MGMT mRNA expression was higher in wild-type p53 lung cancer cells than in p53-mutated cells. Moreover, the occurrence of MGMT promoter methylation was more common in p53-mutated cells (Fig. 1). Thus, we nvestigated whether mutation of p53 contributes to MGMT promoter methylation in lung cancer patients..

DISCUSSION

One of the key events of HPV-induced carcinogenesis is the integration of the viral genome into a host chromosome (31). HPV genome integration often occurs near common fragile sites of the human genome, such as FRA 3B—a site of frequent integration by HPV 16, which causes loss of heterozygosity of fragile histidine triad (FHIT) in cervical cancer (32, 33). Interestingly, frequent FHIT loss of heterozygosity in HPV DNA–positive lung tumors also has been reported (34). In the present study, 28% (34 cases), 25% (31 cases), and 45% (55 cases) of 122 lung tumors were positive for HPV 16 E6, HPV 18 E6, and HPV 16 or 18 E6, respectively (Table 1). Being stratified tumors with the presence of HPV DNA, the detection frequency of HPV 16 E6, HPV 18 E6, and HPV 16 or 18 E6 was increased to 61, 77, and 67%, respectively. HPV 16/18 E6 expression in cervical cancers is necessary for the maintenance of the transformed phenotype (35). Therefore, the high prevalence of HPV 16/18 E6 expression in HPV DNA–positive lung tumors strongly suggests that HPV 16/18 may play a crucial role in lung tumorigenesis in Taiwanese women.

Our data show that p53 expression was not associated with mutant p53 in lung tumors (Table 2), which is inconsistent with previous study (36) showing that positive p53 expression was due to the increased protein stability by p53 missense mutations. Among eight tumors negative for HPV 16/18 E6 and p53 protein expression, four had p53 deletion mutations. The other four tumors showed HPV 16 E6 mRNA expression by *in situ* RT-PCR, suggesting that E6 expression levels in these four tumors may have been too low to be detected by immunohistochemistry. Among six tumors positive for HPV 16/18 E6 and p53 expression, four were detected with HPV 16 or 18 E6 variants as shown by direct sequencing (data not shown). Nevertheless, these results might partly support the observation that E6-positive tumors had positive p53 expression.

Malanchi et al. (12, 37) reported that HPV 16 E6 could induce cellular proliferation, pRb phosphorylation, and accumulation of gene products that are negatively regulated by pRb, such as p16, CDC2, E2F-1, and cyclin A. Consistent with the hyperphosphorylated state of pRb, cyclin A/CDK2 activity is highly elevated in cells expressing E6 from either HPV 16 or 18. Recently, microarray analysis indicated that a distinct and large subset of cell cycle and cell proliferation genes were up-regulated in HPV-positive head/neck cancer as well as cervical cancer compared with that observed in HPV-negative head/neck cancer, such as cyclin E2, cyclin B1, proliferating cell nuclear antigen (PCNA), E2Fs, and cdc2 (38). Our studies showed higher cell proliferation and S-phase cell proportion in E6-positive lung cancer cells than in E6-knockdown cells. These results support the findings of microarray data that E6 could up-regulate cell cycle- and cell proliferation-regulated gene expressions. (Supplementary Fig. 2B). Malanchi et al. (12, 38) also showed that E6 may strongly downregulate $p21^{WAF1/CIP1}$. Overexpression of $p21^{WAF1/CIP1}$ decreases E6-induced proliferation, indicating that the observed downregulation of endogenous $p21^{WAF1/CIP1}$ in E6-expressing cells is a key mechanism for cell cycle dysregulation. Interestingly, all these events appear to be independent of p53 inactivation. This finding may support the present study showing that the decrease in $p21^{WAF1/CIP1}$ mRNA levels by HPV 16/18 E6 through the p53-independent pathway was more pronounced than the decrease through the p53-dependent pathway upon p53 mutation. The inactivation of p53 by a high-risk HPV E6 oncoprotein is a crucial event during cervical carcinogenesis (10, 35). In our present study, tissue *in situ* immunohistochemistry data clearly showed that E6-positive lung tumors were most often negative for p53 expression. In addition, real-time RT-PCR data revealed that $p21^{WAF1/CIP1}$ and *mdm-2* mRNA expression was significantly reduced in HPV 16/18 E6–positive lung tumors as compared with E6-negative tumors. Collectively, our data show that most lung tumors expressed HPV 16/18 E6 were p53 negative immunostainings. Moreover, E6 appears to downregulate $p21^{WAF1/CIP1}$ and *mdm-2* mRNA expression, which strongly suggests that HPV 16/18 E6 expression in lung tumors could be involved in p53 inactivation. It was well established that the prominent function of E6 stems from its interaction with p53 (followed by p53 degradation) and the pro-apoptotic protein BAK, which results in resistance to apoptosis and increased chromosomal instability (31, 39). Apart from resistance to apoptosis, many other functions for HPV 16/18 E6 in human carcinogenesis have been reported (11). For example, the activation of telomerase and postulated inhibition of the degradation of SRC-family kinases appear to fulfill important functions in stimulating tumor growth (39,40). Nevertheless, these results provide crucial evidence in support of our previous reports showing that HPV 16/18 infection may be associated with lung tumorigenesis, especially for Taiwanese female nonsmokers.

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Table 1. Relationships between HPV 16 E6, HPV 18 E6, and HPV 16 or 18 E6 immunostaining and clinico-p athological parameters in lung tumors.

Parameters	HPV 16 E6			HPV 18 E6			HPV16/18 E6		
	–	+	P	–	+	P	–	+	P
Age									
?65	40	14		38	16		28	26	
>65	48	20	0.690	53	15	0.404	39	29	0.592
Gender									
Female	34	23		38	19		22	35	
Male	54	11	0.005	53	12	0.065	45	20	0.001
Tumor type									
AD	52	31		58	25		37	46	
SQ	36	1	<0.0001	33	6	0.118	30	9	0.001
Smoking									
–	56	31		64	25		41	48	
+	32	3	<0.0001	27	6	0.351	26	7	0.002
Tumor stage									
Early	30	14		38	6		27	17	
Late	58	20	0.530	53	25	0.030	40	38	0.345
T factor									
1+2	72	31		81	22		59	44	
3+4	16	3	0.270	10	9	0.023	8	11	0.316
N factor									
0	32	16		40	8		28	20	
1+2+3	56	18	0.306	51	23	0.090	39	35	0.580
HPV DNA	HPV 16			HPV18			HPV 16/18		
-	66	0		72	0		40	0	
+	22	34	<0.0001	19	31	<0.0001	27	55	<0.0001

Table 2. Correlation of p53 immunostaining with HPV 16 E6, HPV 18 E6, and HPV 16 or 18 E6 immunostaining in lung tumor tissues.

Parameter	p53 immunostaining		P value
	Negative (n=78)	Positive (n=44)	
E6 immunostaining			
HPV 16			
Negative (n=88)	50	38	
Positive (n=34)	28	6	0.011
HPV18			
Negative (n=91)	54	37	
Positive (n=31)	24	7	0.085
HPV 16 or 18			
Negative (n=67)	35	32	
Positive (n=55)	43	12	0.004
p53 mutation			
Negative (n=94)	62	32	
Positive (n=28)	16	12	0.502

Table 3. Correlation between HPV 16/18 E6 and p53 immunostaining and p21$^{WAF1/CIP1}$ and mdm-2 mRNA expression in lung tumor tissues.

	mRNA level (Ct/10^4)			
	p21$^{WAF1/CIP1}$	P value	mdm-2	P value
E6 protein				
HPV 16				
Negative (n=56)	151.73±881.50		189.03±625.74	
Positive (n=34)	16.81±51.68	0.004	43.59±200.31	0.003
HPV 18				
Negative (n=64)	133.35±824.04		123.27±452.53	
Positive (n=26)	20.54±88.33	0.009	16.07±64.43	0.062
HPV 16/18				
Negative (n=40)	199.16±104.06		161.79±541.64	
Positive (n=50)	22.05±75.52	0.003	11.91±49.09	0.003
p53 protein				
Negative (n=63)	34.47±115.81		167.27±603.46	
Positive (n=27)	255.44±1262.73	0.489	566.57±1383.17	0.287
p53 mutation				
Negative (n=69)	122.08±794.09		151.23±574.39	
Positive (n=21)	30.71±99.36	0.625	77.73±198.14	0.147

Fig. 1

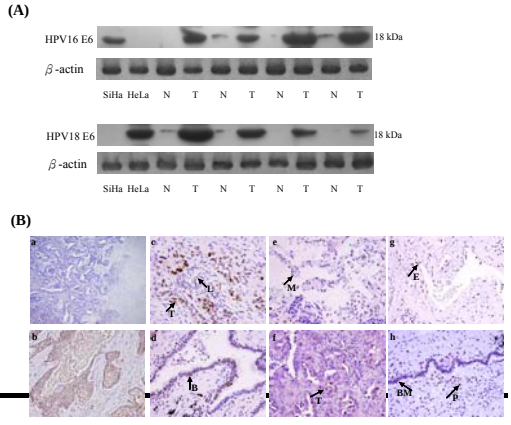


Fig. 1. (A) Western blot analysis for E6 protein expression of HPV 16 and 18. The results of HPV 16 or 18 E6 protein in lung tumor and adjacent normal tissues were shown in T and N. SiHa and HeLa cervical cancer cell lines were used as

positive control for HPV 16 and HPV 18, respectively. (B) Immunohistochemical analysis of HPV 16 or 18 E6 protein in lung tumors and adjacent normal tissues. (a) a negative result of immunostaining in tumor cells (100X), (b) HPV16/18 E6 protein expressed in cervical tumors as positive controls (200X), (c) HPV16 E6 protein expressed in tumor cells (T) and lymphocytes (L) (400X), (d) HPV16 E6 protein expressed in bronchiole epithelial cells (e) (400X), (e) HPV16 E6 protein expressed in alveolar macrophage (M) (400X), (f)HPV18 E6 protein expressed in adenocarcinoma cells (T) (400X), (g) HPV18 E6 protein expressed in endothelial cell of blood vessel (e) (400X), and (h) HPV18 E6 protein in bronchiole metaplasia (BM) and type II pneumocyte (P) (400X).

Fig. 2

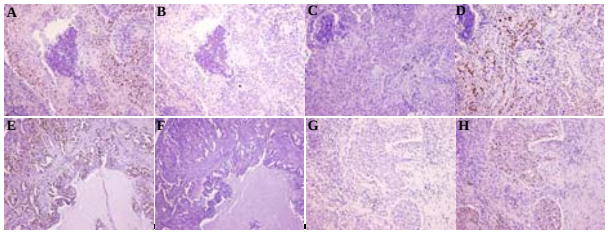


Fig. 2. The representative reciprocal relationships between HPV 16 or 18 E6 and p53 immunostainings in serial paraffin sections of lung tumors. (A) and (B), (C) and (D), (E) and (F), and (G) and (H) were two serial sections from the same lung tumors, respectively. (A) p53 immunostaining positive and (B) HPV 16 E6 immunostaining negative; (C) p53 immunostaining negative and (D) HPV 16 E6 immunostaining positive; (E) p53 immunostaining positive and (F) HPV 18 E6 immunostaining negative, (G) p53 immunostaining negative and (H) HPV 18 E6 immunostaining positive.

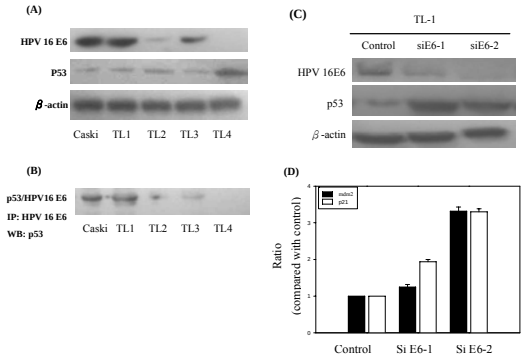


Fig. 3. (A) Detection of HPV 16 E6 and p53 protein expression in lung adenocarcinoma cell lines established from pleural effusion. Cervical cancer cell line Casaki cells was used as positive control and β-actin was used as internal control. (B) A correlation between HPV E6 status and p53 protein expression in established lung adenocarcinoma cell lines. The IP results with E6 antibodies followed by immunoblotting of p53 protein of HPV 16 was showed in p53/HPV16 E6, respectively. Caski cells were used as positive controls. (C) HPV 16 E6 and p53 protein expression in HPV16 E6 knock-down TL-1 cells. SiHa cell was used as positive control and β–actin was used as a internal control. (D) mdm2 and p21 mRNA expression in HPV 16 E6 SiRNA transfected cervical cancer cell line SiHa and lung cancer cell line TL-1 compared with parental control.

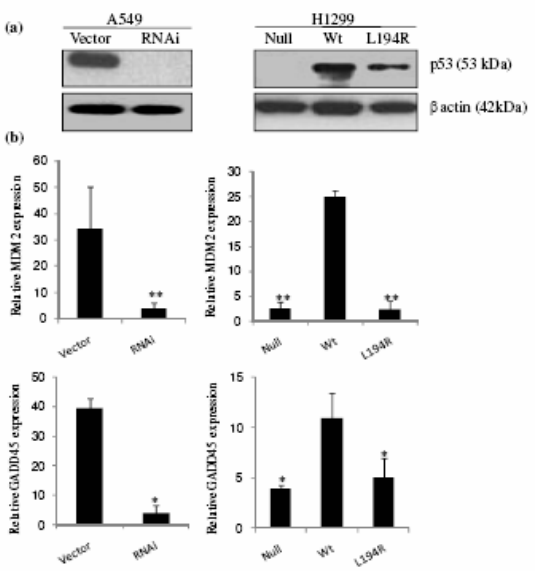
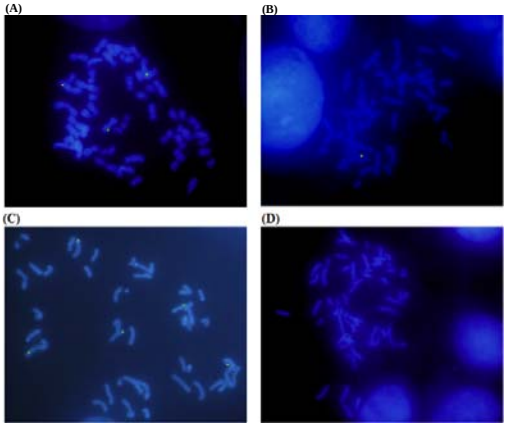
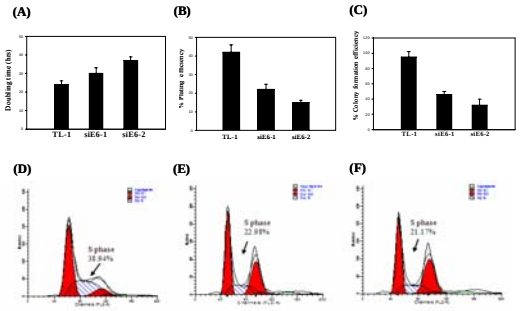


Fig. 4. Analysis of the methylation status of the MGMT promoter in lung cancer cell lines. (a) MSP amplification of DNA fragments representing the unmethylated and methylated MGMT promoter. (b) Expression of MGMT mRNA in lung cancer cell lines was quantified by real-time PCR. All experiments were performed in duplicate. The copy number of MGMT cDNA was normalized to the copy number of 18S in each sample (MGMT cDNA / 18S rRNA × 105) to indicate relative MGMT mRNA expression.



Supplemental Fig. 1. FISH analysis of TL-1 (A), TL-2 (B), TL-3 (C), and TL-4 (D) lung adenocarcinoma cell lines with or without HPV16 infection. The green signal in chromosomes of TL-1, TL-2 and TL-3 cells was presented to be HPV16 DNA copy number. Photos were amplified by 1000X.



Supplemental Fig. 2. Cell growth effects of TL1 cells with or without siE6-1 or siE6 transfection (A) doubling time, (B) plating efficiency, and (C) colony formation efficiency, (D) S-phase cell proportion of TL-1 cells, (E) S-phase cell proportion of TL-1 cells with siE6-1 transfection, (F) S-phase proportion of TL-1 cells with siE6-2 transfection.