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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

人類乳突瘤病毒 16/18 之E6/E7 在肺上皮細胞基因甲基化之角色研究

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中文摘要:

過去本研究室的研究結果發現人類乳突瘤病毒可能參與女性肺癌患者肺癌的形成，但 HPV 感染在肺腫瘤化過程所扮演的角色不清楚。已知高危險型 HPV 16/ 18 E6 蛋白經由將 p53 抑癌蛋白去活化而參與子宮頸癌的腫瘤化過程。因此本計畫共收集 122 個肺癌患者之肺腫瘤組織分析腫瘤組織中 HPV 16/18 致癌蛋白表現與 p53 抑癌蛋白去活化之間的相關性，並利用同步定量反轉錄聚合酶連鎖反應偵測 p53 下游基因 p21 及 mdm2 mRNA 的表現情形，已進一步確認 p53 蛋白是否被 HPV 16/18 蛋白去活化而影響其下游基因的轉錄調控。本研究之研究結果發現確實可利用西方墨點法及免疫組織化學染色在腫瘤組織中測得 HPV 16/18 E6 蛋白的存在，且在女性不抽菸之肺腺癌患者有較高的 HPV 16/18 E6 蛋白表現。而在免疫組織化學染色的結果也發現 p53 蛋白與 HPV 16/18 E6 蛋白表現具有負相關性，且在 HPV 16/18 E6 蛋白表現之腫瘤組織中，p53 下游基因 p21 及 mdm2 mRNA 的表現量也低於 HPV 16/18 E6 蛋白不表現者，以上結果顯示確實可在 HPV 感染之肺腫瘤組織後測得 HPV 16/18 E6 致癌蛋白的存在，而此致癌蛋白確實可經由將 p53 蛋白去活化而參與肺腫瘤的形成。此外，亦利用 siRNA 技術發現當 p53 蛋白不表現時會增加 DNA 的去乙醯化及 DNA 甲基轉移酶結合到 DNA 上的能力，進而促使抑癌基因 MGMT 及 p16 的甲基化，而使其不表現。綜何以上結果，推測 HPV 感染除經由 HPV E6 致癌蛋白的表現使 p53 抑癌基因失去活性外，亦可活化 DNA 甲基化路逕而造成 MGMT 及 p16 抑癌基因的甲基化而使 MGMT 及 p16 抑癌基因不表現。

ABSTRACT

Our previous report indicated that high-risk Human Papillomavirus (HPV) type 16/18 may be associated with lung cancer development among Taiwanese female nonsmokers (Cancer Res., 61, 2799-2803, 2001), however, the role of HPV infection in lung carcinogenesis remains unclear. It is well known that p53 inactivation by HPV 16/18 E6 plays a crucial role in cervical tumorigenesis. To elucidate the involvement of HPV 16/18 in lung tumorigenesis, the association between HPV 16 or 18 E6 and p53 protein expressions in 122 lung tumors was evaluated by immunohistochemistry. Furthermore, $p21^{WAF1/CIP1}$ and mdm-2 mRNA expression levels were determined by real-time RT-PCR to verify whether p53 protein was inactivated by E6. Western blot data showed that E6 protein were detected in some lung tumors with HPV 16 or 18 DNA, but not detected in HPV-negative tumors. Immunohistochemistry data showed that HPV 16 or 18 E6 protein was more frequently detected in female, adenocarcinoma, and nonsmoking lung tumors, compared with that of male, squamous cell carcinoma, and smoking lung tumors. Interestingly, HPV 16/18 E6 expressions were reversely correlated with p53 expression, which was further confirmed by tissue *in situ* immunostaining. Additionally, real-time RT-PCR analysis showed that E6 positive tumors had lower $p21^{WAF1/CIP1}$ and mdm-2 mRNA expression levels than E6 negative tumors. These results revealed that HPV 16/18 E6 may be partially involved in p53 inactivation to down-regulate $p21^{WAF1/CIP1}$ and mdm-2 transcription. In addition, histone modification and DNA methyltransferase binding affinity of promoter of MGMT and p16 in A549 RNAi with p53 deficiency were induced and the promoter hypermethylation of MGMT and p16 were indeed induced in this cell. In conclusion, HPV 16/18 E6 proteins were indeed expressed in HPV DNA-positive lung tumors and involvement in p53 inactivation to contribute to HPV-infected lung tumorigenesis.

目　　　錄

INTRODUCTION

Lung cancer is the leading cause of cancer death in Taiwanese women since 1982. It is well known that cigarette smoking is the major cause of lung cancer, however, more than 90% of female lung cancer in Taiwan could not be related to cigarette smoking (1). Therefore, different etiological factor might be involved in lung cancer development in Taiwanese women. Our previous study indicated that human papillomavirus (HPV) oncogenic subtypes 16/18 may be involved in the pathogenesis of lung cancer among Taiwanese women, since 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 of nonsmoking females with HPV 16/18 infection (~10-fold) was much higher compared with nonsmoking males (an odds ratio of ~2) (2). Additionally, HPV 16/18 DNA was uniformly detected in lung tumor cells, but not in the adjacent non-involved lung. These results strongly suggested that HPV infection with virus subtypes known to be oncogenic for cervical cancer is associated with lung cancer development of nonsmoking Taiwanese women. It is known that oncogenic HPV encode E6 and E7 viral oncoproteins, which inactivate p53 and Rb protein, respectively (3-5). 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 75.7-fold risk for lung cancer compared with those without HPV 16/18 DNA (6), which further authenticated the involvement of HPV in lung tumorigenesis.

Some of viral-related lung cancer studies were reported previously, however, the molecular pathogenesis of viral-associated lung cancer remains unclear (7-9). For example, the impact of the well-known oncogenic DNA virus simian virus 40 (SV40) to the development of malignant mesotheliomas and high risk of HPV 16/18 to lung cancer were until recently controversial.

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Our preliminary restriction specific-PCR (RS-PCR) data showed that HPV 16/18 DNA integration occurred in HPV DNA-positive (HPV-positive) lung tumors. We thus attempted to detect whether HPV 16/18 E6 was expressed in lung tumors by western blot and immunohistochemistry to verify the association between p53 protein expression and HPV 16/18 E6 expression. Furthermore, we also investigated if mRNA levels of p21$^{WAF1/CIP1}$ and mdm2, which are down-stream genes of p53, were reduced in E6 positive lung tumors, as compared with those of E6 negative tumors.

MATERIALS and METHODS

Study subjects

Lung tumor specimens from 122 patients with primary lung cancer were collected. All of these patients, including 57 females and 65 males who admitted into the Department of Thoracic Surgery, Taichung Veteran's General Hospital (TVGH), Taichung, Taiwan between 1998 and 2002, were asked to submit a written informed consent according to a biology study approved by the TVGH Institutional Review Board. None of these subjects had received radiation therapy or chemotherapy prior to surgery. Collected lung tumors have been previously analyzed for the presence of HPV 16 and/or 18 DNA (2, 6), and tumor types and stages were histologically determined according to the WHO classification (World Health Organization, 1981). 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 HPV 16 E6, HPV 18E6 and 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℃ or with polyclonal anti-HPV 16 or 18 E6 antibody (Santa Cruz, California, USA and CHEMICON International, Inc., California, USA) for 90 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

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obtained by leaving out the primary antibody. The intensities of signals were evaluated independently by three observers. Negative immunostaining was defined to be with 0-10% positive nuclei and cases with higher than 10% positive nuclei were decided to be positive immunostaining. Positive control slides for p53 protein detection were purchased from DAKO (Denmark) and the cervical cancer tumor tissues with HPV 16/18 was used as positive control for HPV 16/18 E6. The antibody dilution buffer was used to replace antibodies to serve as a negative control.

Protein extraction and Western blot

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 and β-actin were conducted by incubating the membrane with anti-HPV16 E6, anti-HPV18 E6 (Santa Cruz, California, USA and CHEMICON International, Inc., California, USA), 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).

Preparation of RNA and real-time quantitative RT-PCR

Total RNA of lung tumors were extracted by homogenized in 1 ml TRIzol reagent, followed by chloroform re-extraction and isopropanol precipitation. Three

micrograms of total RNA from lung tumor tissues were reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, California, USA) and oligo d(T)$_{15}$ primer. RTQ-PCR was performed in a final volume of 25 μl containing 1μl of each cDNA template, 10 pmoles of each primer and 12.5μl of a SYBR- Green master mix. The primers were designed using the ABI Prism 7000 SDS Software. Quantification was carried out using the comparative *CT* method and water was used as the negative control. An arbitrary threshold was chosen on the basis of the variability of the baseline. Threshold cycle (*CT*) values were calculated by determining the point at which the fluorescence exceeded the threshold limit. *CT* was reported as the cycle number at this point. The average of target gene was normalized to *18S rRNA* as endogenous housekeeping gene.

Transfection of p53-targeting siRNA into A549 lung cancer cells

The siRNA targeting vectors were kindly provided by Dr. J. T. Chang (Chang et al., 2005). In brief, purified fragments of p53 were cloned with siRNA vectors, and the resultant recombinants were then transfected into human lung cancer A549 cells. On the day prior to transfection, A549 cells were seeded at 1×10^5 cells per well and, after an overnight incubation, the cells were transfected with 1 μg of the siRNA vector using DOTAP transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. After 16 hr, the medium was aspirated, and the cells were washed twice with phosphate-buffered saline (PBS). Stable transfectants were selected by culture in RPMI 1640 medium (Hyclone) containing 400 μg/ml G418 (Promega, Madison, WI). DDH1 mRNA and protein expressions of all clones were evaluated by RT-PCR and Western blot to select high-efficiency knock-down clones for further experiments.

Chromatin immunoprecipitation (ChIP) assay

ChIP analysis was performed using a published procedure with the following modifications. The immunoprecipitated DNA was ethanol precipitated and re-suspended in 25 µl H_2O. Total input samples were re-suspended in 100 µl H_2O and diluted 1:100 before PCR analysis. PCR amplification of immunoprecipitated DNA was carried out with diluted aliquots, using the oligonucleotides 5'-GCCCCTAGAACGCTT-TGC-3' and 5'-CAACACCTGGGAGGCACTT-3' as primers, which encompass the237 bp promoter region of MGMT, PCR products were run on 2% agarose gel andanalyzed by ethidium bromide staining. All ChIP assays were performed at least twicewith similar results.

Statistical analysis

Statistical analysis was performed using the SPSS statistical software program (Version 11.0 SPSS Inc., Chicago, IL, USA). The χ^2 test, Fisher's exact test (two tailed) and Mann-Whitney test were applied for statistical analysis.

RESULTS AND DISCUSSION

HPV 16/18 E6 protein expressed in HPV-positive lung tumors

Western blotting assay was first used to detect HPV 16/18 E6 proteins in 10 randomly selected HPV DNA-positive lung tumors and corresponding adjacent normal lung tissues. Data clearly showed that HPV 16 or 18 E6 proteins were predominately expressed in lung tumors while some of paired adjacent normal tissues were detected with a residual E6 protein expression (Fig. 1). Consequently, 122 lung tumors with the presence or absence of HPV 16/18 DNA were enrolled to detect E6 protein by immunohistochemistry. As shown in Fig. 2, HPV 16 or 18 E6 protein was indeed expressed in tumor cells, additionally expressed in adjacent normal cells in lung tumor tissues such as in type II pneomocyte, bronchiole epithelial cell, endothelial cell of blood vessel, lymphocytes, and alveolar macrophages. The positive E6 immunostaining in surrounding normal cells was further confirmed by western blot. This result was also supported our previous case-control study showing that HPV 16/18 DNA was detected in peripheral blood to be as a risk marker of lung cancer (6). More recently, HPV DNA was detected in peripheral blood from healthy subjects (10) and patients with cervical cancer (11) or neoplastic cervical lesions (12) suggesting that peripheral blood may be the transmission vehicle of HPV and HPV might spread through blood.

The relationships between E6 immunostaining and clinical parameters of lung tumors were shown in Table 1, positive immunostaining of HPV 16 or 18 E6 in lung tumors of females, adenocarcinomas, and nonsmokers were significantly higher than those of males, squamous cell carcinomas, and smokers, respectively (P = 0.001 for gender and tumor type, P = 0.002 for smoking status). The HPV 16 or 18 E6 expression was

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not associated with other clinical parameters including age, tumor stage, T and N factor, although HPV 18 E6 positive immunostaining was more common in advanced tumor stage and T factor (Table 1). HPV 18 has been shown to be more tumorigenic than HPV 16 in cervical carcinogenesis (13, 14) suggesting that HPV 18 E6 might play a more important role than HPV 16 E6 in lung tumor progression.

Our present study clearly indicated that HPV 16/18 E6 protein was indeed expressed in lung tumors which have been evaluated to be positive for HPV 16/18 DNA by nested-PCR and *in situ* hybridization, previously (2). This finding was consistent with our preliminary data from RS-PCR showing that HPV 16/18 DNA was randomly integrated into host chromosomes (data not shown). One of the key events of HPV-induced carcinogenesis is the integration of the HPV genome into a host chromosome (15). HPV genome integration often occurs near common fragile sites of the human genome, such as FRA 3B which was frequently integrated by HPV 16 to cause loss of heterozygosity (LOH) of FHIT in cervical cancer (16, 17). Interestingly, frequent FHIT LOH in HPV-positive lung tumors was also reported previously (18). In this study, 28% (34 cases), 25% (31 cases), and 45% (55 cases) of 122 lung tumors were detected with HPV 16 E6, HPV 18 E6, and HPV 16 or 18 E6, respectively. Among HPV-positive lung tumors, 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 (19). Therefore, the high prevalence of HPV 16/18 E6 expression in HPV-positive lung tumors strongly suggested that HPV 16/18 may play a crucial role in Taiwanese lung tumorigenesis.

Reverse correlation between HPV 16/18 E6 and p53 protein in lung tumors

The E6 protein has many functions that may contribute to its oncogenic potential. Specific relevant to immortalization, the classical function of E6 is binding to inducing the degradation of the p53 tumor suppressor protein (20). The role of p53 is to safeguard the integrity of the genome by inducing cell cycle arrest or apoptosis in the presence of DNA damage (21). As a transcription factor, p53 up-regulates target genes involved in coordinating these responses, such as p21$^{WAF1/CIP1}$, a CDK inhibitor acting on cyclin E/ddk2 complexes and mdm-2 (22, 23). Therefore, its inactivation by the E6 proteins leads to chromosomal instability and increases the probability of an HPV-infected cell evolving towards malignancy (20). To elucidate the correlation between HPV E6 and p53 protein, p53 expression was also determined by immunohistochemistry. As shown in Table 2, p53 immunostaining was reversely correlated with HPV 16 E6 (P = 0.011) and HPV 16 or 18 E6 immunostaining (P = 0.004), but marginally associated with HPV 18 E6 (P = 0.085). To further verify the reciprocal relationship between HPV 16 or 18 E6 and p53, serial paraffin sections of lung tumors were used to detect both protein expressions *in vivo*. As shown in Fig. 3, tumors positive for HPV 16 or 18 E6 were not detected with p53 immunostaining. Inversely, HPV 16 or 18 E6 negative tumors had a positive p53 immunostaining. The tissue *in vivo* reverse correlation between HPV 16/18 E6 and p53 clearly revealed the possibility that HPV 16/18 E6 may, at least in part, promote the degradation of p53 protein in HPV-positive lung tumors.

In this study, p53 immunotaining was not associated with p53 mutation in lung tumors (Table 2). The observation was not consistent with previous studies showing that a positive p53 immunostaining was due to the increased protein stability by p53 missense mutation. Among 8 tumors negative for HPV 16/18 E6 and p53 immunostaining, 4 cases were due to p53 deletion mutation. The other 4 tumors was

detected with HPV 16 mRNA expressions by *in situ* RT-PCR showing the possibility that E6 protein expression of these four tumors was too low to be detectable by immunohistochemistry. Among 6 tumors positive for HPV 16/18 E6 and p53 immunostaining, 4 cases have been 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 a positive p53 immunostaining.

Decreased p21$^{WAF1/CIP1}$ *and mdm-2 mRNA expressions in HPV 16 or 18 E6 positive-lung tumors*

To elucidate whether p53 was inactivated by E6, two p53 down-stream genes, p21$^{WAF1/CIP1}$ and mdm-2 mRNA expression levels in lung tumors were evaluated by real-time RT-PCR. As shown in Table 3, mRNA expression 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. However, both gene expressions were not correlated with p53 mutations and p53 immunostainings although a trend could be observed (Table 3). These results clearly suggested that p53 inactivated by HPV 16/18 E6 may play more important role than that by p53 mutation or other mechanism(s) to cause p53 protein accumulation in HPV-positive lung tumors.

Malanchi et al. (22, 24) have indicated that HPV 16 E6 proteins induce cellular proliferation, pRb phosphorylation, and accumulation of products of genes 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 either of the E6 protein. Malanchi et al. (22, 24) also showed that the E6 protein induce strong down-regulation of the p21$^{WAF1/CIP1}$ gene. Overexpression of p21$^{WAF1/CIP1}$ decreases the E6-proliferation, indicating that

down-regulation of the endogenous p21$^{\text{WAF1/CIP1}}$ gene observed in E6-expressing cells is a key mechanism for cell cycle deregulation. Interestingly, all these events appear to be independent of p53 inactivation. This finding may support the present study showing that p21$^{\text{WAF1/CIP1}}$ mRNA expression decreased by HPV 16/18 E6 through p53 independent pathway was more pronounced than that of p53 dependent pathway by p53 mutation or p53 protein alteration.

The inactivation of p53 by high-risk HPV E6 oncoprotein plays a crucial role during cervical carcinogenesis (19, 20). In this study, tissue *in situ* immunohistochemistry data clearly showed that E6 positive lung tumors were mostly observed to have p53 negative immunostaining. In addition, real-time RT-PCR data revealed that p21$^{\text{WAF1/CIP1}}$ and mdm-2 mRNA expressions were significantly reduced in HPV 16/18 E6 positive compared with those of E6 negative lung tumors. Collectively, HPV 16/18 E6 protein expression was indeed expressed in lung tumors. Moreover, the E6 expression appears to down-regulate p21 and mdm-2 mRNA expressions strongly suggesting that HPV 16/18 E6 protein expressions in lung tumors could involve in p53 inactivation. It was well known that the prominent function of the E6 protein originate from its interaction with, followed by degradation of, p53 and the pro-apoptotic protein BAK, which results in resistance to apoptosis and increase in chromosomal instability (15, 25). Apart from resistance to apoptosis, many functions of HPV 16/18 E6 protein in human carcinogenesis have been reported. For example, the activation of telomerase and postulated inhibition of degradation of SRC-family kinases seem to fulfill important functions in stimulating tumor growth (25, 26). Nevertheless, these results provide the crucial evidence to support our previous reports indicating that HPV 16/18 infection may be associated with lung tumorigenesis, especially for Taiwanese women nonsmokers.

The gene transcription was upregulated with the acetylation of H3 and H4 of

chromatin,

and HDAC, DNMT1, and DNMT3b were dissociated with the acetylated chromatin

(27-32). To verify whether the acetylation of H3 and H4 and the binding activity of

HDAC, DNMT1 and DNMT3b on MGMT promoter were modulated in p53 negative

cells, CHIP assay was performed to elucidate the possibility. As shown in Fig. 4B,

acetylated H3 and H4 chromatin of MGMT and p16 were decreased in p53 deficiency

A549 lung cancer cells. On the contrast, the binding activity of HDAC, DNMT1, and

DNMT3b was significantly increased in this cells (Fig. 4B).These results indicated

that MGMT promoter hypermethylation in p53 deficiency cells was partially

mediated through increase of H3 and H4 acetylation and decrease of the binding

activity of HDAC, DNMT1, and DNMT3b on MGMT promoter.

ACKNOWLEDGMENTS

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Table 1. Relationships between HPV 16 E6, HPV 18 E6, and HPV 16 or 18 E6 immunostainings and clinico-pathological 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

"-" Negative immunostaining; "+" Positive immunostaining.

$\chi 2$ test was used for the statistical analysis.

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

P53 mutations in lung tumors were determined by direct sequencing.

Table 3. Correlation between HPV 16/18 E6 and p53 immunostainings and p21 $^{WAF1/CIP1}$ and

mdm-2 mRNA gene expressions 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

E6 and p53 protein expressions in lung tumors were determined by immunohistochemistry.
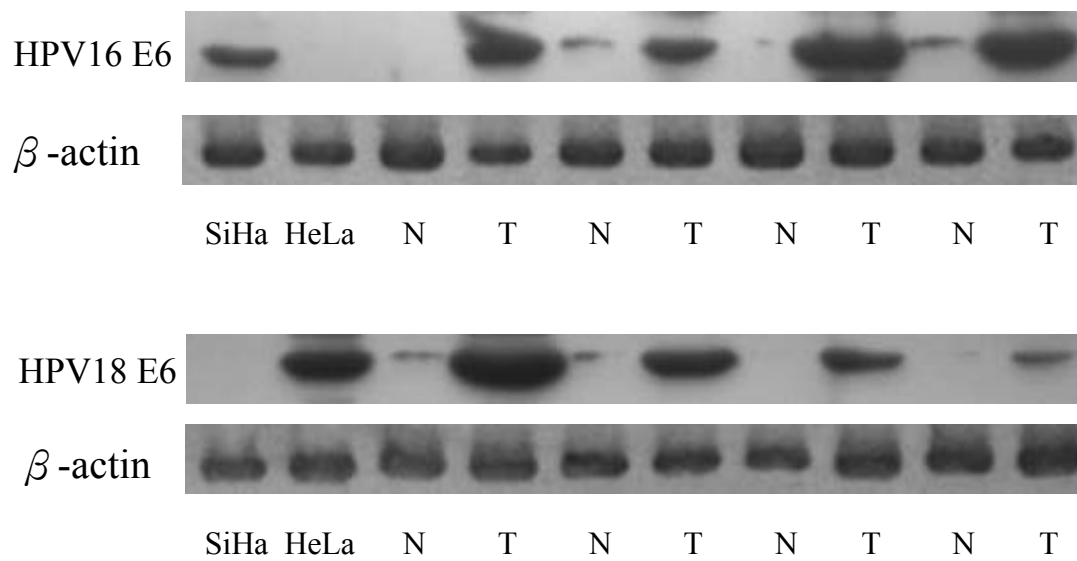
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Fig. 1. Western blot analysis for E6 protein expression of HPV 16 (A) and 18 (B). The results of HPV 16 or 18 E6 in lung tumor and adjacent normal tissues were shown in T and N. SiHa and HeLa cervical cancer cell lines were used as positive control for HPV 16 and HPV 18, respectively.

Fig. 2. 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).
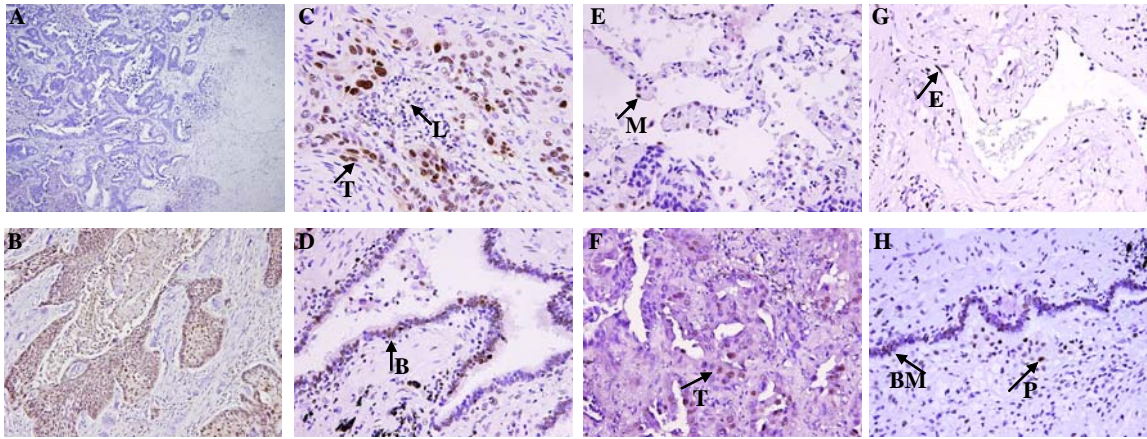
Fig. 3. The representative reciprocal relationships between HPV 16 or 18 E6 and p53 immunostainings in serial paraffin sections of lung tumors. (A) and (B), (C) and (D), (E) and (F), and (G) and (H) were two serial sections from the same lung tumors, respectively. (A) p53 immunostaining positive and (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. 4. Representative analyses of the methylation status of the MGMT promoter using methylation-specific PCR and histone modification of promoter of MGMT and p16 in A549 RNAi with p53 deficiency systems. (A) The presence of a PCR product showing specific amplification of DNA fragments representing unmethylated (U) and methylated (M) of MGMT or p16 promoter. (B) Histone modification of promoter of MGMT and p16 in A549 RNAi with
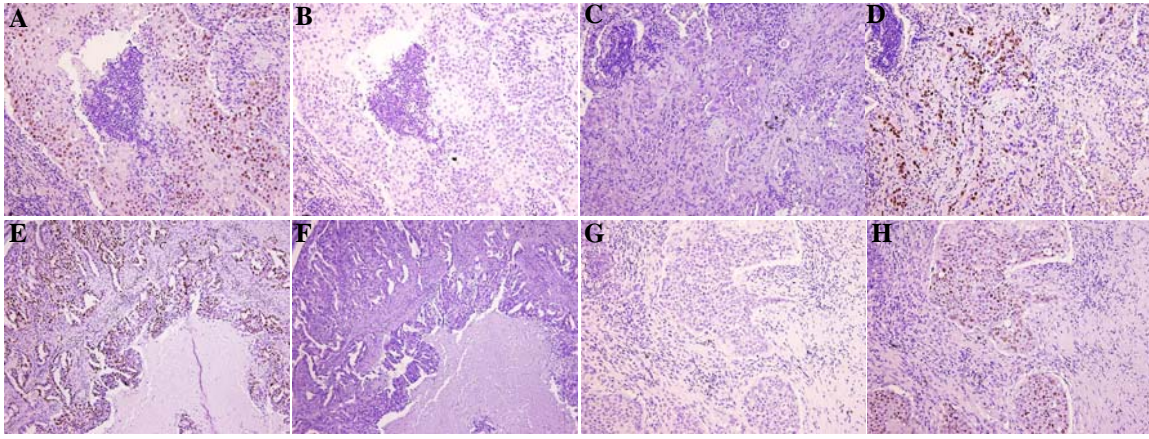
p53 deficiency. Chromatin was isolated and immunoprecipitated with antibody specific for acetylated H3, H4, HDAC and DNMT1.
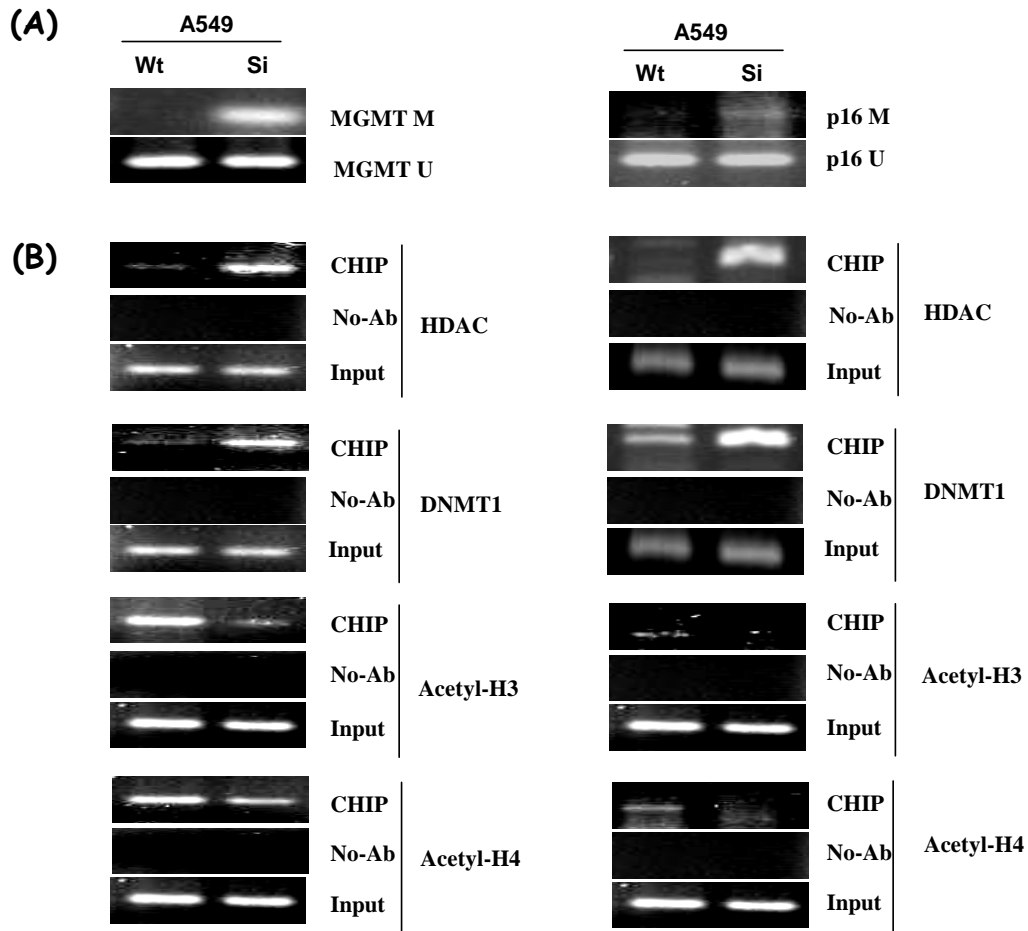
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