Human Papillomavirus 16/18 E6 Oncoprotein Is Expressed in Lung Cancer and Related with p53 Inactivation

Ya-Wen Cheng,1 Ming-Fang Wu,5 John Wang,6 Kun-Tu Yeh,8 Yih-Gang Goan,9,10,11 Hui-Ling Chiou,7 Chih-Yi Chen, and Huei Lee7

Abstract

Inactivation of p53 by human papillomavirus 16/18 E6 plays a crucial role in cervical tumorigenesis. To investigate the involvement of HPV16/18 in lung tumorigenesis, the association between HPV16 or HPV18 E6 and p53 protein expression in 122 lung tumors was evaluated by immunohistochemistry, and data showed that HPV16/18 E6 expression correlated inversely with p53 expression, which was further confirmed by tissue in situ immunostaining. Real-time reverse transcription-PCR analysis indicated that E6-positive tumors had lower p21WAF1/CIP1 and mdm2 mRNA levels than E6-negative tumors. To elucidate the role of E6 in p53 inactivation, we successfully established lung adenocarcinoma cell lines with or without HPV16 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-positive cells compared with E6-negative cells. Moreover, the levels of p21WAF1/CIP1 and mdm2 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 and p21WAF1/CIP1 and mdm2 mRNA expressions were restored in E6-knockdown cells by RNA interference compared with control cells. These results reveal that HPV16/18 E6 may be partially involved in p53 inactivation to down-regulate p21WAF1/CIP1 and mdm2 transcription. In conclusion, HPV16/18 E6 is indeed expressed in HPV DNA-positive lung tumors and is involved in p53 inactivation to contributing to HPV-mediated lung tumorigenesis. [Cancer Res 2007;67(22):10686–93]

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 and 18, which are involved in cervical cancer, may also be involved in the pathogenesis of lung cancer among Taiwanese because 55% of lung cancer patients had HPV16/18 DNA compared with 11% of noncancer control subjects. In addition, the odds ratio for lung cancer in nonsmoking females with HPV16/18 infection (~10) was much higher than that for nonsmoking males (~2; ref. 2). Additionally, HPV16/18 DNA was uniformly detected in lung tumor cells but not in the adjacent noninvolved 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 HPV16 and HPV18 DNA in their blood was at a 76-fold risk for lung cancer compared with subjects without HPV16/18 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 effect of the oncogenic DNA virus SV40 on the development of malignant mesotheliomas and the high risk of HPV16/18 in lung cancer were controversial until recently. 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 classic 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 on DNA damage (11). As a transcription factor, p53 up-regulates target genes involved in coordinating these responses [e.g., p21WAF1/CIP1, a cyclin-dependent kinase (cdk) inhibitor that acts on cyclin E/cdk2 complexes and mdm2; refs. 12, 13]. Therefore, p53 inactivation by E6 leads to chromosomal instability and increases the probability of a HPV-infected cell evolving toward malignancy (10). Animal model experiment further showed that HPV16 E6 gene alone is sufficient to induce carcinomas in transgenic mice (14).

In this study, to understand whether p53 could be inactivated by E6 in HPV-infected lung cancer, the following experiments would be done (a) to examine whether E6 could express in lung tumors; (b) to understand whether E6 protein expression in lung tumor was associated with the inactivation of p53 pathway; and (c) to elucidate the role of E6 in p53 inactivation in HPV-infected lung cancer cell lines that have been successfully established from patients’ pleural effusions.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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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 were admitted into the Department of Thoracic Surgery, Taichung Veteran’s General Hospital, Taichung, Taiwan between 1998 and 2002, were asked to submit a written informed consent approved by the Institutional Review Board. None of these subjects had received radiation therapy or chemotherapy before surgery. Collected lung tumors have previously been analyzed for the presence of HPV16 and/or HPV18 DNA (2, 3), and tumor types and stages were histologically determined according to the WHO (1981) classification. Pathologic material was processed for conventional histologic 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 HPV16 E6, HPV18 E6, 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 antibody to p53 antibody (DAKO, D07; at a dilution of 1:250) for 60 min at 25°C or with polyclonal anti-HPV16 or HPV18 E6 antibody (Santa Cruz Biotechnology and Chemicon International, Inc.) for 90 min at 25°C. The conventional streptavidin-peroxidase method (DAKO, LSAB Kit K675) was used to develop signals and the cells were counterstained with hematoxylin. Negative controls were 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% to 10% positive nuclei, and cases with >10% positive nuclei were decided to be positive for immunostaining. Positive control slides for p53 protein detection were purchased from DAKO and the cervical cancer tumor tissues with HPV16/18 were used as positive control for HPV16/18 E6. The antibody dilution buffer was used to replace antibodies to serve as a negative control.

Direct sequencing. Mutations in exons 5 to 8 of the p53 gene were determined by direct sequencing of PCR products amplified from the DNA of tumor cells isolated by microdissection of the lung tumor tissues. DNA lysis buffer was used to lyse cells and then the solution was subjected to proteinase K digestion and phenol-chloroform extraction. Finally, the DNA was precipitated with ethanol with the addition of linear polyacrylamide to increase the efficiency of DNA precipitation (15). Target sequences were amplified in a 50-μL reaction mixture containing 20 pmol of each primer, 2.5 μL of genomic DNA as the template. PCR products were 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 whereas DNA preparations from SiHa cells (containing HPV16) and HeLa cells (containing HPV18) were used as positive controls.

Establishment of HPV16-infected and noninfected lung cancer cell lines from patients’ pleural effusions. Lung tumor cells were cultured and established from pleural effusions of three patients by the Ficol-Paque method. The pathologic diagnosis of the patients was adenocarcinoma of lung including a 72-year-old female nonsmoker with T2N1M1, a 54-year-old male smoker with T3N2M0, and a 53-year-old male ex-smoker with T1N1M1.

Naked PCR. Genomic DNA was prepared from a tissue section and isolated by conventional phenol-chloroform extraction, ethanol precipitated, and finally dissolved in 20 μL of sterile distilled water. HPV viral DNA was first amplified with type consensus primers MY09 and MY11 (2), followed by second round of amplification with type-specific primers flanking the L1 region to identify the subtype. Ten microliters of the final PCR product were 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 whereas DNA preparations from SiHa cells (containing HPV16) and HeLa cells (containing HPV18) were used as positive controls.

Silencing of endogenous HPV16 E6 expression by RNA interference.

The target sequences for RNA interference (RNAi) for HPV16 E6 were previously verified (16, 17). The sequence of the siE6-1 sense strand–directed small interfering RNA (siRNA) was 5'-GGAGGUAUGUGCUUCUdTdT-3' (16) and that of the siE6-2 sense strand–directed siRNA was 5'-GAAGUUGUGACUGAAGGdTdT-3' (17). To suppress transcription of the endogenous HPV16 E6 gene, SiHa and TL-1 cells were transiently transfected with the synthetic siRNAs against HPV16 E6 using Oligofectamine reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, 24 h before transfection, 1.4 × 10⁵ cells were seeded in each well of a six-well plate. Oligofectamine reagent (3 μL) was added to 12 μL of OPTI-MEM (Invitrogen). After 5 min, 60 pmol of each siRNA in 175 μL of OPTI-MEM were combined with the Oligofectamine mixture. After a 20-min incubation at 25°C, siRNA/Oligofectamine mixtures were added to the cells. After 48 h of incubation at 37°C, the cells were harvested and subjected to real-time quantitative PCR and Western blot analysis.

Fluorescence in situ hybridization. Fluorescence in situ hybridization (FISH) was done by the metaphase presentation of established lung cancer cells. A FITC-DUTP HPV type 16 probe was used for HPV detection (Roche Diagnostics). Briefly, the hybridizing probe was prepared by PCR amplification with a HPV type 16–specific primer (16UP-5'TACTAATCTTAAGGAGTACC-3'; 16DN-5'TGTGATGTGTTTGAACGATT-3'; ref. 2). Slides were treated with RNase for 30 min at 37°C, and FISH was done as previously described (18). The hybridization was done in a humidified chamber at 48°C for 16 h followed by washing with sodium chloride sodium citrate, and then counterstaining with 4',6-diamidino-2-phenylindole was observed for signals under a microscope.

Preparation of RNA and real-time quantitative RT-PCR. Total RNA of lung tumors was extracted by homogenization in 1-mL TRIzol reagent, followed by chloroform reextraction and isopropanol precipitation. Three micrograms of total RNA from lung tumor tissues were reverse transcribed and

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using SuperScript II Reverse Transcriptase (Invitrogen) and oligo d(T)15 primer. Quantitative RT-PCR was done in a final volume of 25 
A containing 1 
A of each cDNA template, 10 pmol of each primer, and 12.5 
A of a SYBR 
Green master mix. The primers were designed using the ABI Prism 7000 
SDS Software. Quantification was carried out using the comparative 
threshold cycle (C_T) method and water was used as the negative control. An 
arbitrary threshold was chosen on the basis of the variability of the baseline. 
C_T values were calculated by determining the point at which fluorescence 
exceeded the threshold limit. C_T was reported as the cycle number at this 
point. The average of target gene was normalized to 18S rRNA 
as endogenous housekeeping gene.

Flow cytometry analysis. Distribution of the cells in the cell cycle was 
determined by propidium iodide staining. In brief, cells (2 \times 10^6/mL) were 
fixed with PBS containing 80% ethanol, then incubated at 4 C in 0.2-mL 
PBS solution containing 0.05 mg/mL propidium iodide, 1 mmol/L EDTA, 
0.01% Triton X-100, and 1 mg/mL RNase A for 1 h. Analysis was done with a 
FACSCalibur cytometer (Becton Dickinson). Cells with subdiploid DNA 
content were considered to be apoptotic cells. Cell cycle distributions were 
analyzed by CellQuest software.

Effects of E6 RNAi on the doubling time, plating efficiency, and 
cloning efficiency of TL-1 lung cancer cells. TL-1 cells (10^3/mL) 
transfected with or without siE6-1 and siE6-2 were seeded in a 35-mm 
dish and cultured for 24, 48, 72, 96, 120, and 144 h, and then the cell number 
at each culture time point was counted for calculation of the doubling time. 
The plating and cloning efficiency assay was done with 10^3 cells in a 35-mm 
dish cultured for 5 to 7 and 14 days, respectively.

Statistical analysis. Statistical analysis was done using the SPSS 
statistical software program (version 11.0, SPSS, Inc.). The \( \chi^2 \) test, Fisher 
extact test (two tailed), and Mann-Whitney test were applied for statistical 
analysis.

Results

E6 protein was indeed expressed in lung tumors and 
adjacent normal tissues: relationships between E6 and clinical 
parameters. Our preliminary restriction-specific PCR data showed 
that HPV16/18 DNA integration occurred in HPV DNA–positive 
lung tumors. We thus attempted to determine whether HPV16/18 
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 or absence of HPV16/18 E6 in 10 
randomly selected HPV DNA–positive lung tumors. We thus attempted to determine whether HPV16/18 
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first used to detect the presence or absence of HPV16/18 E6 in 10 
randomly selected HPV DNA–positive lung tumors and corres-
ponding 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 
HPV16/18 DNA were tested for E6 expression by immunohisto-
chemistry. Our present and previous data indicated that HPV16/18 
E6 is only expressed in lung tumors that were previously shown to
be positive for HPV16/18 DNA by nested PCR (Table 1). HPV16 or HPV18 E6 was indeed expressed in tumor cells as well as in adjacent normal cells in tumor tissues such as type II pneumocytes, bronchiol 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 HPV16 or HPV18 E6 in lung tumors of females, adenocarcinoma patients, and nonsmokers was significantly higher than in lung tumors of males, squamous cell carcinoma patients, 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, and T and N factor, although HPV18 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 HPV16 E6 (P = 0.011) and HPV16/18 E6 expression (P = 0.004), but was marginally associated with HPV18 E6 alone (P = 0.085; Table 2). To confirm the reciprocal relationship between HPV16 or HPV18 E6 and p53, serial paraffin sections of lung tumors were used to assess protein expression in vivo. p53 protein was not detected in tumors positive for HPV16 or HPV18 E6; conversely, HPV16 or HPV18 E6–negative tumors had positive p53 protein expression (Fig. 2). The reverse correlation between HPV16/18 E6 and p53 expression in vivo clearly revealed the possibility that HPV16/18 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 p21WAF1/CIP1 and mdm2, which function downstream of p53, in lung tumors were measured by real-time RT-PCR. The mRNA levels of p21WAF1/CIP1 and mdm2 in HPV16 E6–, HPV18 E6–, and HPV16/18 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 HPV16/18 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 in 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 non–HPV16-infected lung adenocarcinoma cell lines from patients’ pleural effusions. The HPV16 DNA copy numbers of these three cell lines were evaluated by FISH showing that two to three, one, and four to five of HPV16 DNA copies were revealed in TL-1, TL-2, and

### Table 1. Relationships between HPV16 E6, HPV18 E6, and HPV16/18 E6 immunostaining and clinicopathologic parameters in lung tumors

<table>
<thead>
<tr>
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<th>HPV18 E6</th>
<th>HPV16/18 E6</th>
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<td>1 + 2 + 3</td>
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<td>HPV DNA</td>
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<td>HPV16</td>
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<td>HPV18</td>
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<tr>
<td>HPV16/18</td>
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**NOTE:** –, negative immunostaining; +, positive immunostaining. χ² test was used for the statistical analysis. HPV16 and HPV18 viral DNAs and E6 proteins were detected by nested PCR and immunohistochemistry. Abbreviations: AD, adenocarcinoma; SQ, squamous cell carcinoma.
TL-3 cells, respectively. Thus, these results clearly showed that HPV16 DNA integrated into the chromosomes of these cells (Supplementary Fig. S1). The tumorigenicity of these established lung cancer cell was shown by soft agar assay. HPV16-infected cells had the ability to form larger colonies in soft agar assay compared with the noninfected cells after 14 days of 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 derepressed by E2 splicing when HPV16 DNA integrated into host chromosomes (19). E6 protein was then evaluated by Western blotting showing that different levels of HPV16 E6 proteins were expressed in HPV16-infected TL-1, TL-2, and TL-3 cells. As expected, E6 was not detected in non–HPV16-infected TL-4 cells. Our data also revealed that p53 protein levels in E6-positive TL-1, TL-2, and TL-3 cells were significantly lower than in E6-negative TL-4 cells (Fig. 3A). Immunoprecipitation assay clearly showed that E6 protein interacted with p53 protein in E6-positive cells but not in E6-negative cells (Fig. 3B). To further verify whether the interaction between E6 and p53 could be responsible for p53 inactivation, TL-1 E6 was knocked down by two RNAis. Western blot showed that E6 protein in siE6-1 (the first RNAi) and siE6-2 (the second RNAi) transient cells was reduced compared with negative control cells; however, E6 was more efficiently reduced in siE6-2 cells than in siE6-1 cells (Fig. 3C). Conversely, p53 protein levels were markedly increased in both siE6 cells (Fig. 3C). The levels of p21WAF1/CIP1 and mdm2 mRNA evaluated by real-time RT-PCR were significantly restored in siE6-2 cells but relatively renovated in siE6-1 cells compared with negative 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 (Supplementary Fig. S2A). As shown in Fig. S2, the doubling time of TL-1 cells with siE6-1 and siE6-2 was extended to 28 to 32 h and 36 to 38 h compared with 24 to 26 h of doubling time for parental TL-1 cells, respectively. The plating efficiency of TL-1 cells was decreased from 42% to 22% (siE6-1) and 15% (siE6-2). The cloning efficiency was also reduced from 95% to 46% (siE6-1) and 25% (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. S2B). These results clearly indicated that p53 inactivation by E6 may increase cell proliferation and colony formation.

### Discussion

One of the key events of HPV-induced carcinogenesis is the integration of the viral genome into a host chromosome (20). HPV

<table>
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<tr>
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<th>p53 immunostaining</th>
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<tr>
<td></td>
<td>Negative (n = 78)</td>
<td>Positive (n = 44)</td>
</tr>
<tr>
<td>E6 immunostaining</td>
<td>HPV16</td>
<td></td>
</tr>
<tr>
<td>Negative (n = 88)</td>
<td>50</td>
<td>38</td>
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<tr>
<td>Positive (n = 34)</td>
<td>28</td>
<td>6</td>
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<tr>
<td>HPV18</td>
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<td>Negative (n = 91)</td>
<td>54</td>
<td>37</td>
</tr>
<tr>
<td>Positive (n = 31)</td>
<td>24</td>
<td>7</td>
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<tr>
<td>HPV16 or HPV18</td>
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<tr>
<td>Negative (n = 67)</td>
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<td>32</td>
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<tr>
<td>Positive (n = 55)</td>
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<td></td>
<td>62</td>
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<td>0.502</td>
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NOTE: p53 mutations in lung tumors were determined by direct sequencing.

Table 2. Correlation of p53 immunostaining with HPV16 E6, HPV18 E6, and HPV16/18 E6 immunostaining in lung tumor tissues

HPV16 E6 mRNA expression by in situ four had p53 deletion mutations. The other four tumors showed eight tumors negative for HPV16/18 E6 and p53 protein expression, the increased protein stability by p53 missense mutations. Among previous study (25) showing that positive p53 expression was due to 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 (25) showing that positive p53 expression was due to the increased protein stability by p53 missense mutations. Among eight tumors negative for HPV16/18 E6 and p53 protein expression, four had p53 deletion mutations. The other four tumors showed HPV16 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 HPV16/18 E6 and p53 expression, four were detected with HPV16 or HPV18 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, 26) reported that HPV16 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 HPV16 or HPV18. Recently, microarray analysis indicated that a distinct and large subset of cell cycle and cell proliferation genes were up-regulated in HPV-positive head and neck cancer as well as cervical cancer compared with that observed in HPV-negative head and neck cancer, such as cyclin E2, cyclin B1, P53, E2Fs, and cdc2 (27). 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 (Supplemental Fig. S2B). Malanchi et al. (12, 26) also showed that E6 may strongly down-regulate p21WAF1/CIP1. Overexpression of p21WAF1/CIP1 decreases E6-induced proliferation, indicating that the observed down-regulation of endogenous p21WAF1/CIP1 in E6-expressing cells is a key mechanism for cell cycle dysregulation. Interestingly, all these events seem to be independent of p53 inactivation. This finding may support the present study showing that the decrease in p21WAF1/CIP1 mRNA levels by HPV16/18 E6 through the p53-independent pathway was more pronounced than the decrease through the p53-dependent pathway on p53 mutation. The inactivation of p53 by a high-risk HPV E6 oncoprotein is a crucial event during cervical carcinogenesis (10, 24). 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 the E6-negative tumors were compared with that observed in HPV16/18 E6–positive lung tumors as compared with E6-negative tumors. Collectively, our data show that most lung tumors that expressed HPV16/18 E6 were negative for p53 immunostainings. Moreover, E6 seems to down-regulate p21WAF1/CIP1 and mdm2 mRNA expression, which strongly suggests that HPV16/18 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 proapoptotic protein Bak, which results in

<table>
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<th>E6 protein</th>
<th>mRNA level (C T/104)</th>
<th>p21WAF1/CIP1</th>
<th>mdm2</th>
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<th>P</th>
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<tr>
<td>Negative (n = 56)</td>
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<td>189.03 ± 625.74</td>
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<td>Positive (n = 34)</td>
<td>16.81 ± 51.68</td>
<td>43.59 ± 200.31</td>
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</tr>
<tr>
<td>HPV18</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Negative (n = 64)</td>
<td>133.35 ± 824.04</td>
<td>123.27 ± 452.53</td>
<td>0.009</td>
<td>0.062</td>
<td></td>
</tr>
<tr>
<td>Positive (n = 26)</td>
<td>20.54 ± 88.33</td>
<td>16.07 ± 64.43</td>
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</tr>
<tr>
<td>HPV16/18</td>
<td></td>
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<tr>
<td>Negative (n = 40)</td>
<td>199.16 ± 104.06</td>
<td>161.79 ± 541.64</td>
<td>0.003</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Positive (n = 50)</td>
<td>22.05 ± 75.32</td>
<td>11.91 ± 49.09</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>p53 protein</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Negative (n = 63)</td>
<td>34.47 ± 115.81</td>
<td>167.27 ± 603.46</td>
<td>0.489</td>
<td>0.287</td>
<td></td>
</tr>
<tr>
<td>Positive (n = 27)</td>
<td>255.44 ± 1,262.73</td>
<td>566.57 ± 1,383.17</td>
<td></td>
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<tr>
<td>p53 mutation</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Negative (n = 69)</td>
<td>122.08 ± 794.09</td>
<td>151.23 ± 574.39</td>
<td>0.625</td>
<td>0.147</td>
<td></td>
</tr>
<tr>
<td>Positive (n = 21)</td>
<td>30.71 ± 99.36</td>
<td>77.73 ± 198.14</td>
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<td></td>
</tr>
</tbody>
</table>

NOTE: E6 and p53 expression in lung tumors was determined by immunohistochemistry.
resistance to apoptosis and increased chromosomal instability (20, 28). Apart from resistance to apoptosis, many other functions for HPV16/18 E6 in human carcinogenesis have been reported (11). For example, the activation of telomerase and the postulated inhibition of degradation of Src family kinases seem to fulfill important functions in stimulating tumor growth (28, 29). Nevertheless, these results provide crucial evidence in support of our previous reports showing that HPV16/18 infection may be associated with lung tumorigenesis, especially for Taiwanese female nonsmokers.

**Acknowledgments**

Received 4/20/2007; revised 8/7/2007; accepted 9/11/2007.

**Grants support:** National Health Research Institute (NHRI93A1-NSCLC07-5; NHRI-EX93-9125BI), National Science Council (NSC91-3112-P-040-002; NSC92-2314-B-040-023; NSC93-2320-B-040-056) and Department of Health (DOH 91-7D-1083) of Taiwan, Republic of China.

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We thank Dr. J.H. Tsai for her valuable suggestions and critical reading of the manuscript.

**References**


**Figure 3.** A, detection of HPV16 E6 and p53 protein expression in lung adenocarcinoma cell lines established from pleural effusion. Cervical cancer Caski cells were used as positive control and β-actin was used as internal control. B, correlation between HPV E6 status and p53 protein expression in established lung adenocarcinoma cell lines. The immunoprecipitation results with E6 antibodies followed by immunoblotting of p53 protein were shown in p53/HPV16 E6. Caski cells were used as positive control. C, HPV16 E6 and p53 protein expression in HPV16 E6–knockdown TL-1 cells. SiHa cells were used as positive control and β-actin was used as internal control. D, mdm2 and p21 mRNA expression in HPV16 E6 siRNA–transfected cervical cancer cell line SiHa (data not shown) and lung cancer cell line TL-1 compared with parental control.
p53 Inactivated by HPV 16/18 E6 in Lung Cancer


Human Papillomavirus 16/18 E6 Oncoprotein Is Expressed in Lung Cancer and Related with p53 Inactivation

Ya-Wen Cheng, Ming-Fang Wu, John Wang, et al.


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