Human Telomerase Reverse Transcriptase Activated by E6 Oncoprotein Is Required for Human Papillomavirus-16/18-Infected Lung Tumorigenesis

Ya-Wen Cheng, Tzu-Chin Wu, Chih-Yi Chen, Ming-Chih Chou, Jiunn-Liang Ko, and Huei Lee

Abstract

Purpose: Our recent report indicates that human papillomavirus (HPV)-16/18 E6 oncoprotein is expressed in lung tumors and is related to p53 inactivation. We further explored whether human telomerase reverse transcriptase (hTERT) transcription is up-regulated by E6 and contributes to lung tumor development.

Experimental Design: Immunohistochemistry detected HPV-16 E6 oncoprotein in 135 lung tumors, and hTERT mRNA was evaluated by real-time reverse transcription-PCR and in situ hybridization, respectively. A small RNA interference (RNAi), Western blotting, and chromatin immunoprecipitation analysis were used to clarify whether hTERT transcription was regulated by c-Myc and Sp1. The telomerase activity and oncogenic potential of TL-1 with or without E6- or hTERT-RNAi was determined by real-time quantitative telomeric repeat amplification protocol analysis and soft-agar assay, respectively.

Results: hTERT mRNA levels in E6-positive tumors, which were prevalent in females, nonsmokers, and adenocarcinomas, were significantly higher than in E6-negative tumors. In addition, hTERT mRNA levels in early tumors (stage I) were greater than levels in advanced tumors (stages II and III). Chromatin immunoprecipitation assay showed that Sp1 cooperated with c-Myc to activate hTERT transcription in TL-1 cells, which was similar to the SiHa cells. The telomerase activity of the TL-1 cells decreased concomitantly with the transfection of various doses of E6- or hTERT-RNAi. A soft-agar assay showed that the oncogenic potential of TL-1 cells was significantly reduced after being transfected with E6-RNAi. Moreover, a colony of TL-1 cells could not form after transfection with hTERT-RNAi.

Conclusion: Transcriptional activation of hTERT by E6 oncoprotein is required for HPV-16/18-infected lung tumorigenesis.

Telomerase activity is detected in >90% of immortalized and cancer cells but is absent in most normal somatic cells, suggesting that telomerase activation is an important event in the transformation process of malignancy (1–3). The key determinant of human telomerase activity is considered to be human telomerase reverse transcriptase (hTERT; refs. 1, 2).

Although various post-transcriptional and post-translational modifications can regulate hTERT function, transcriptional control of the gene is a major contributor to the regulation of telomerase activity in many human cancers, including lung cancer (1–3). The activation of hTERT transcription has been found in early genetic abnormalities of bronchial carcinogenesis (4). Additionally, elevated hTERT mRNA is associated with an increased relative risk in the prevalence and incidence of bronchial squamous cell carcinoma (5). hTERT overexpression has also been observed in 77% of high-grade atypical alveolar hyperplasia representing the lung adenocarcinoma precursor lesion in 97% of nonmucinous bronchioloalveolar hyperplasia but in only 27% of low-grade atypical alveolar hyperplasia (6). These results suggest that the activation of hTERT transcription may play a role in the initiation of squamous cell carcinomas of the lung and atypical alveolar lung adenocarcinomas.

Lung cancer is the most common malignancy worldwide and also in Taiwan (7–9). Cigarette smoking is the major cause of lung cancer; however, ~50% of Taiwanese lung cancer cases cannot be explained by active cigarette smoking, especially among Taiwanese women, 90% of whom have never smoked (8, 9). Thus, different etiologic factor(s) may be involved in lung carcinogenesis in Taiwan. We have shown previously that a human papillomavirus (HPV)-16/18 infection might be associated with lung cancer development in Taiwanese women.
Translational Relevance

In the present study, we provide the evidence to indicate that hTERT transcription activated by HPV-16/18 E6 oncoprotein is required for HPV-infected lung tumorigenesis. Inhibition of telomerase in cancer cells may be viable target for anticancer therapeutics. We thus strongly suggest that inhibition of hTERT transcription may be a feasible molecular targeting therapy for HPV-infected lung cancer.

(9, 10). In fact, our most recent study showed that the HPV-16/18 E6 protein is indeed expressed in about half of HPV-16/18 DNA-positive lung tumors and leads to p53 inactivation (11). p53 has been reported to repress hTERT expression by binding to Sp1 and preventing its access to the hTERT promoter (12). Human keratinocytes, transformed by HPV-16 E6, are partially mediated through hTERT transcriptional activation by promoting the binding of c-Myc and Sp1 to GC-rich sequences within the hTERT promoter (1, 13, 14). These results have led us to elucidate whether transcriptional activation of hTERT by E6 oncoprotein could play a crucial role in HPV-16/18-infected lung tumorigenesis.

Patients and Methods

Study subjects. Lung tumor specimens were collected from 135 patients with primary lung cancer. All of these patients, including 63 females and 72 males who were admitted to the Department of Thoracic Surgery at Taichung Veteran’s General Hospital, in Taichung, Taiwan between 2000 and 2003, were asked to submit a written informed consent based on a biology study approved by the institutional review board. None of the subjects had received radiation therapy or chemotherapy before surgery. Tumor types and stages were histologically determined according to the WHO classification system (1981). Pathology samples were processed for conventional histologic procedures.

Preparation of RNA and real-time quantitative reverse transcription-PCR. Total RNA from the lung tumors (100 mg) and from the tumor cell lines (1 × 10^6 cells) was extracted by homogenization in 1 mL TRIzol reagent (Invitrogen) followed by chloroform reextraction and isopropanol precipitation. The total RNA extracted from the 100 mg tissue ranged from 20 to 60 μg and only 3 μg total RNA was used for cDNA synthesis by SuperScript II Reverse Transcriptase (Invitrogen) according to the standard protocol (SuperScript II Reverse Transcriptase protocol). Real-time quantitative PCR was done in a final volume of 25 μL containing 1 μL of each cDNA template, 10 pmol hTERT gene-specific primer (up: 5′-GGCGGAAGA-CAGTGATGACA-3′ and down: 5′-AGCTGGAGTAGTCGCTCTGC-3′), and 12.5 μL SYBR Green master mix (Applied Biosystems). The primers were designed using ABI Prism 7500 SDS Software. The size of the PCR product was 146 bp. Quantification was carried out using the comparative threshold cycle (Ct) method, and water was used as the negative control. An arbitrary threshold was chosen based on the variability of the baseline. Ct values were calculated by determining the cycle number at which the fluorescence exceeded the threshold limit. The average Ct values for the target gene were normalized to an endogenous housekeeping gene encoding 18S rRNA.

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 that was used for all subsequent washes. For HPV-16 E6 detection, sections were heated in a microwave oven twice for 5 min in citrate buffer (pH 6.0) and then incubated with polyclonal anti-HPV-16 E6 antibody (Santa Cruz Biotechnology) for 90 min at 25°C. The conventional streptavidin peroxidase method (DAKO; LSAB Kit K675) was done to develop signals, and the cells were counterstained with hematoxylin. Negative controls were obtained by leaving out the primary antibody. Three observers independently evaluated the intensities of the signals. Negative immunostaining was defined as having 0% to 10% positive nuclei and cases with >10% positive nuclei were specified as positive immunostaining. The cervical cancer tumor tissues with HPV-16/18 were used as a positive control for HPV-16/18 E6. The antibody dilution buffer was used to replace antibodies to serve as a negative control.

In situ hybridization. In situ hybridization, for the detection of hTERT mRNA, was done using digoxigenin-labeled oligonucleotide probes and a commercially available hybridization kit (Boehringer Mannheim). Briefly, the hybridizing probe was prepared by PCR amplification using hTERT-specific primer (up: 5′-GGCGGAAGAACATCTGGATCAACT-3′ and down: 5′-AGCTGGAGTAGTCGCTCTGC-3′) with digoxigenin-ddUTP (Roche Diagnostics) as the substrate following the manufacturer’s instructions. The deparaffinized and rehydrated 5 μm sections were digested with proteinase K (Roche Diagnostics) and DNase (Promega), rinsed with PBS, and dehydrated. The hybridization was done in a humidified chamber at 48°C for 16 h followed by a washing with sodium chloride-sodium citrate. Thereafter, the detection reagent anti-digoxigenin horseradish peroxidase (NEB Life Science Products) was applied to the sections and the sections were incubated with DAB solution (DAKO) to allow the signals to develop. After the signal development, the sections were counterstained with hematoxylin (DAKO), rinsed briefly in absolute ethanol, mounted, and observed for signals under a microscope.

Establishment of HPV-16-infected and noninfected lung cancer cell lines from patients’ pleural effusions. Lung tumor cells were isolated from the pleural effusions of lung cancer patients by the Ficoll-Paque density-gradient centrifugation method. The clinical characteristics of these patients and the identification of these cell lines according to the lung adenocarcinoma cell type are recorded as described previously (11).

Protein extraction and Western blotting. Total protein was extracted from cells with a lysis buffer [100 mMol/L Tris (pH 8.0), 1% SDS], and recovered protein concentrations were determined using the Bio-Rad protein assay kit followed by separation with SDS-PAGE (12.5% gel). After the electrophoretic transfer to a polyvinylidene difluoride membrane, nonspecific binding sites were blocked with 5% nonfat milk in TBS containing 0.1% Tween 20. HPV-16 E6, c-Myc, Sp1, and β-actin were detected by incubating the membrane with anti-HPV-16 E6 (Santa Cruz Biotechnology and Chemicon), anti-c-Myc (Santa Cruz Biotechnology), anti-Sp1 (Santa Cruz Biotechnology), or anti-β-actin (DAKO) for 60 min at room temperature followed by a subsequent incubation with a peroxidase-conjugated secondary antibody (1:5,000).
A method was used to detect telomerase activity according to a previous description. Real-time quantitative telomeric repeat amplification protocol analysis of tumor samples revealed that the presence of telomerase activity correlated with the presence of hTERT-RNAi. The experiment was carried out with a total of 100 samples, divided into two groups: one group with hTERT-RNAi and the other without. The results showed that the hTERT mRNA expression levels were markedly higher in HPV-16/18 E6-positive tumors compared with those in HPV-negative tumors.

### Results

**Table 2. Relationships between hTERT mRNA levels and tumor clinical variables**

<table>
<thead>
<tr>
<th>Variables</th>
<th>hTERT mRNA (Ct/10⁴)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65 (n = 66)</td>
<td>579.40 ± 875.99</td>
<td>0.163</td>
</tr>
<tr>
<td>&gt;65 (n = 69)</td>
<td>403.68 ± 771.57</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female (n = 63)</td>
<td>709.14 ± 968.88</td>
<td></td>
</tr>
<tr>
<td>Male (n = 72)</td>
<td>297.45 ± 622.41</td>
<td>0.003</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 95)</td>
<td>622.31 ± 934.65</td>
<td>0.002</td>
</tr>
<tr>
<td>+ (n = 40)</td>
<td>174.37 ± 305.43</td>
<td></td>
</tr>
<tr>
<td>Tumor type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenoma (n = 87)</td>
<td>604.04 ± 875.06</td>
<td>0.011</td>
</tr>
<tr>
<td>Squamous (n = 48)</td>
<td>282.14 ± 689.50</td>
<td></td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early (n = 52)</td>
<td>568.56 ± 806.21</td>
<td>0.03</td>
</tr>
<tr>
<td>Late (n = 38)</td>
<td>440.11 ± 839.02</td>
<td></td>
</tr>
<tr>
<td>T factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 + 2 (n = 109)</td>
<td>539.41 ± 880.27</td>
<td>0.035</td>
</tr>
<tr>
<td>3 + 4 (n = 21)</td>
<td>280.71 ± 502.42</td>
<td></td>
</tr>
<tr>
<td>N factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (n = 58)</td>
<td>560.59 ± 822.16</td>
<td>0.093</td>
</tr>
<tr>
<td>1 + 2 + 3 (n = 77)</td>
<td>436.10 ± 830.03</td>
<td></td>
</tr>
</tbody>
</table>

---

**hTERT mRNA expression levels were markedly higher in HPV-16/18 E6-positive tumors compared with those in HPV-negative tumors.** Real-time reverse transcription-PCR analysis indicated that hTERT mRNA levels in HPV-16 E6-positive tumors were significantly higher than in HPV-16 E6-negative tumors (P = 0.0001) but not in HPV-18 tumors (P = 0.1000); however, a difference between HPV-16 or HPV-18 E6-positive and E6-negative tumors (P = 0.001; Table 1) was noted. hTERT mRNA levels from tumors of females, nonsmokers, adenocarcinomas, and stage I tumors were higher than those of males, smokers, squamous cell carcinomas, and stage II + III tumors, respectively (Table 2). This association was not apparent for other clinical variables including age, tumor size (T value), and nodal micrometastasis (N value; Table 2). Additionally, immunohistochemistry and in situ hybridization were used, respectively, to detect the HPV-16/18 E6 oncoprotein and hTERT mRNA expression in serial sections of lung tumors. Our data showed that E6 oncoprotein was predominately expressed in tumors cells (Fig. 2A). Meanwhile, hTERT mRNA was concomitantly detected in the tumor cells of the serial section of lung tumor tissue array (Fig. 1B). This result strongly suggests that the HPV-16/18 E6 oncoprotein may contribute to the activation of hTERT transcription in HPV-infected lung tumors.

**Sp1 cooperates with c-Myc to activate hTERT transcription in HPV E6-positive lung cancer cells.** To examine whether c-Myc and Sp1 are involved in activating hTERT transcription in HPV E6-positive lung cancer cells, Western blotting was used to evaluate E6, Sp1, and c-Myc protein expression in HPV-16-infected TL-1 lung cancer cells, SiHa cervical cancer cells, HPV-noninfected TL4 lung cancer cells, and A549 lung cancer cells. As shown in Fig. 2, the c-Myc expression levels in E6-positive TL-1 and SiHa cells were higher than in E6-negative TL4 and A549 cells, but a similar Sp1 expression level was observed among the four cells (Fig. 2A). To clarify whether hTERT mRNA was up-regulated by E6 through the induction of c-Myc, E6 from TL-1 and SiHa cells were, respectively, knocked down by E6-RNAi. Western blotting showed that c-Myc expression decreased significantly in the E6-knockdown of TL-1 and SiHa cells compared with those with RNAi-negative
controls and parental cells (Fig. 2B). Consequently, hTERT mRNA expression levels reduced significantly in both E6-knockdown cells (Fig. 2C).

To elucidate whether the binding activity of c-Myc and Sp1 on the hTERT promoter is regulated by E6, chromatin immunoprecipitation analysis showed that the binding activity of c-Myc and Sp1 in E6-knockdown TL-1 and SiHa cells was almost eliminated compared with those of the RNAi-negative controls and parental cells, respectively (Fig. 2D). These results showed that c-Myc and Sp1 are involved in the transcriptional activation of the hTERT gene in HPV-16 E6-positive TL-1 lung cancer cells and SiHa cervical cancer cells.

Telomerase activity is related with hTERT mRNA up-regulated by E6 to confer oncogenic potential of TL-1 cells. To elucidate

![Fig. 1. Representative reciprocal relationships between HPV-16 E6 protein and hTERT mRNA expression in serial tissue array paraffin sections of lung tumors analyzed by immunohistochemistry and in situ hybridization. Magnification, ×200 (A and B).](https://example.com)

![Fig. 2. A, HPV-16 E6, c-Myc, and Sp1 protein expressions in HPV-16-infected TL-1 lung cancer cells. SiHa cervical cancer cells were used as a positive control, and A549 and TL-4 lung cancer cells were used as HPV-noninfected controls. β-Actin protein was as a protein loading control. B, HPV-16 E6 of TL-1 lung cancer cells were knockdown by E6-RNAi. The human small interfering RNA-negative control duplex for nontargeting RNA was used for negative controls. c-Myc, Sp1, and HPV-16 E6 protein in E6-knockdown TL-1 cells were evaluated by Western blotting to verify the efficiency of RNAi for E6 compared with TL-1 parental cells. C, hTERT mRNA levels in E6-knockdown TL-1 cells evaluated by real-time reverse transcription-PCR were compared with that of TL-1 parental cells. D, binding activity of c-Myc and Sp1 to the hTERT promoter evaluated by chromatin immunoprecipitation analysis in HPV-positive (TL-1) and HPV-negative (TL-4) cells. Chromatin was isolated and immunoprecipitated with an antibody specific for c-Myc and Sp1. SiHa cells were used as a positive control.](https://example.com)
c-Myc overexpression in HPV-positive lung cancer could be related to HPV DNA integration. Therefore, transcriptional activation of the hTERT gene by c-Myc, which is through p53 inactivated by E6 or HPV DNA integration, may play a role in HPV-associated lung tumorigenesis.

hTERT mRNA detected by real-time reverse transcription-PCR and in situ hybridization showed that E6-positive tumors had higher hTERT mRNA expression than in E6-negative tumors. The transcriptional activation of the hTERT gene by E6 in lung cancer cells was similar to previous studies of HPV-16 E6-immortalized keratinocytes showing that c-Myc expression induced by E6 promotes its binding onto the hTERT promoter to activate hTERT transcription (13, 14). Although Sp1 expression was not affected by HPV-16 E6 in SiHa and TL-1 cells (Fig. 2C), the binding activity of Sp1 on the hTERT promoter decreased significantly in the E6 knockdown of the SiHa and TL-1 cells (Fig. 2D). These results are consistent with previous reports indicating that Sp1 cooperates with c-Myc to activate transcription of the hTERT gene (13, 14, 20). In addition, the Sp1 protein expression level in HPV-16-infected TL-1 lung cancer cells was relatively higher than in HPV-16-infected SiHa cervical cancer cells (Fig. 2B), suggesting that the transcriptional activation of the hTERT gene by Sp1 in cooperation with c-Myc may be more notable in TL-1 cells than in SiHa cells. This observation in TL-1 cells seems to support the finding that E6-positive tumors have higher hTERT mRNA levels than E6-negative tumors. These results were consistent with previous studies showing that c-Myc and Sp1 are involved in the transcriptional activation of the hTERT gene in HPV-16 E6-transfected keratinocytes (13, 21–23), cervical cancer cells (24), and HPV-16 E6-positive TL-1 lung cancer cells. In this tumor set, E6 expression in female, adenocarcinoma, and nonsmokers was higher than male, squamous cell carcinomas, and smokers (64% versus 36% for genders, P = 0.016; 62% versus 27% for tumor type, P = 0.019; 58% versus 26% for smoking status, P = 0.004), and this is consistent with results reported in our previous report (11). Therefore, higher hTERT mRNA levels in tumors from females, nonsmokers, and adenocarcinomas was due to the common expression of the E6 oncoprotein in these categorized tumors (Table 2), although E6 expression was not associated with the tumor stages of lung tumors (data not shown). More interestingly, hTERT mRNA levels in early tumors (stage I) was markedly higher than in those of advanced tumors (stages II and III). Early hTERT mRNA expression has been reported in lung carcinogenesis, including preneoplastic bronchial lesions (5), high-grade atypical alveolar hyperplasia (6), and non-mucinous bronchioloalveolar carcinomas (7). A significant increase in hTERT mRNA expression has been observed with increasing degrees of cervical dysplasia including atypical squamous cell of undetermined significance, low-grade squamous intraepithelial lesion, and high-grade squamous intraepithelial lesion (25–27). We thus suggest that transcriptional activation of the hTERT gene by E6 may play an important role in the early stage of lung tumor development.

The telomerase activity of TL-1 cells decreased gradually with various doses of E6- and hTERT-RNAi during a 96 h treatment, suggesting that hTERT transcription up-regulated by
E6 may activate telomerase activity and contribute to lung tumor development (Figs. 2 and 3). Moreover, a colony of hTERT-knockdown TL-1 cells was not observed in a soft-agar assay (Fig. 4), and stable clones of E6-knockdown TL-1 cells could not be established. These observations strongly suggest that transcriptional activation of the hTERT gene by E6 is required for HPV-infected lung tumorigenesis. In summary, we analyzed a panel of tumors from Taiwanese lung cancer patients and identified a marked increase in hTERT mRNA expression in lung cancer cells and in lung tumor tissues expressing HPV-16/18 E6. The transcriptional activation of the hTERT gene by E6 is required for the oncogenic potential of HPV-16 E6-positive TL-1 lung cancer cells. Thus, we strongly suggest that the hTERT gene may be as a molecular target for HPV-infected lung cancer therapy using specific telomerase inhibitors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

17. Kim NW, Wu F. Advances in quantification and
Human Telomerase Reverse Transcriptase Activated by E6 Oncoprotein Is Required for Human Papillomavirus-16/18-Infected Lung Tumorigenesis


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/14/22/7173

Cited articles
This article cites 26 articles, 9 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/14/22/7173.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/14/22/7173.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/14/22/7173.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.