Expression of human papillomavirus E7 mRNA in human oral and cervical neoplasia and cell lines

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Abstract

Human papillomaviruses (HPVs) have been strongly linked to progression of human cancers, such as cervical and oral cancers. Two HPV oncoproteins, E6 and E7, can inhibit the tumor suppressor proteins, p53 and pRB, respectively, resulting in a deregulation of the cell cycle. In order to further test the significance of HPV expression in oral and cervical carcinogenesis, we analyzed HPV E7 mRNA in oral and cervical neoplasia and cell lines by reverse transcriptase-polymerase chain reaction (RT-PCR). We found that HPV E7 mRNA was present in 90% of patients with oral neoplasia and 100% of patients with cervical neoplasia. Quantitative RT-PCR and western blot analysis on both transformed cervical and oral epithelial cell lines demonstrated that the mRNA level of HPV-16 E7 corresponded to E7 protein level, suggesting that HPV oncogene expression is primarily regulated at the transcriptional or post-transcription level. The potential clinical application of quantitative RT-PCR for HPV E7 mRNA expression in cancer screening and treatment evaluation requires further investigation. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Human papillomaviruses (HPVs) have been strongly linked to progression of several human cancers. In most studies, about 90% of cervical carcinomas are HPV positive [1], where the two high risk types, HPV-16 and HPV-18, are found in 40–60% and 10–20% of the tumors, respectively. HPV is also associated with other cancers, such as vulvar [2], lung [3], and oral cancers [4,5].

HPV is a small DNA virus encoding two major oncoproteins, E6 and E7. The E6 protein appears to alter cell growth through its effect on p53, a cell-derived tumor-suppressor protein. Binding of E6 to p53 stimulates degradation of cellular p53. The E7 protein alters cellular growth control by binding to pRb, another tumor-suppressor protein, to inhibit its function. In general, the E6/p53 and/or E7/Rb interaction result in a deregulation of the cell cycle with loss of control on crucial events, such as DNA replication, DNA repair and apoptosis [6]. E6 and E7 can act together to produce efficient immortalization of primary human epithelial cells [7], providing further evidence for the role of HPV in tumorigenesis.

However, HPV infection does not always result in histological changes. Many reports have found HPV DNA in normal oral and cervical tissues [8,9]. Current methods for diagnosis of HPV infection are mainly based on DNA detection including Southern blot, polymerase chain reaction (PCR), in situ hybridization, and hybrid capture [10]. Although most experts currently regard HPV DNA testing as investigational technology best used for guiding therapy of women with cervical lesions, it is difficult to predict progression to HPV-associated cervical cancer because of the high frequency of subclinical infection and lack of a sensitive and specific assay for HPV gene expression in cervical tissues.

It has been proposed that a HPV mRNA assay may provide higher specificity than HPV DNA detection since HPV DNA fragments can derive from dead cell
debris sequestered in the lymph node, while HPV mRNA is actually more closely associated with proliferating tumor cells [11,12]. Current HPV mRNA detection methods have focused on E6/E7 bicistronic mRNA [12]. Quantification of a relative ratio between two E6 transcripts, E6*I and E6*II, has also been developed [13]. Since these methods cannot determine copy numbers of each transcript, it is difficult to correlate HPV E6 mRNA with protein expression. A common method for mRNA quantification is competitive reverse transcriptase-polymerase chain reaction (RT-PCR) which can quantitate target mRNA at a molecular level and may be utilized for HPV mRNA analysis [14].

In order to further test the significance of HPV mRNA expression in carcinogenesis, we analyzed HPV E7 transcripts in both oral cancer and cervical neoplasia cells by a RT-PCR assay. We found that HPV E7 mRNA was present in 100% of patients with cervical neoplasia and 90% of patients with oral neoplasia. Quantitative RT-PCR and western blot analysis on both transformed cervical and oral epithelial cells demonstrated that the mRNA level of HPV-16 E7 corresponded to the E7 protein level, suggesting that HPV oncogene expression is primarily regulated at the transcriptional or post-transcriptional level.

2. Materials and methods

2.1. Tissue samples and cell lines

Cervical neoplasia biopsies were collected from 11 patients. Oral neoplasia biopsies were collected from 10 patients. Biopsies were also obtained from adjacent histologically normal tissues of the same anatomic site in each of the patients.

The cervical cancer cell lines, HeLa and CaSki, were purchased from American Type Culture Collection (Rockville, MD). HeLa cells express HPV-18 mRNA and CaSki cells express HPV-16 mRNA. Both cell lines were grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 with 10% fetal bovine serum. The oral cancer cell lines, 1438, 686LN, and HPV-16 immortalized human oral keratinocyte cell lines, HOK-16B, 16BNNK, and normal human oral keratinocytes (NHOK) were described in our previous publication [15]. 16BNNK cells were derived from non-tumorigenic HOK-16B by treatment with tobacco-related carcinogens and subsequently became tumorigenic in nude mice.

2.2. Total RNA, mRNA and cDNA preparation from tumor samples and tissue culture cells

Total RNA was prepared using Trizol RNA isolation reagent following the manufacturer’s protocol (Gibco BRL Life Technologies, Gaithersburg, MD). Of total RNA, 5 μg was then used for mRNA isolation and subsequently for ss-cDNA preparation using the MicroFastTrack Kit and the cDNA Cycle Kit (Invitrogen Corporation, San Diego, CA). The final volume of ss-cDNA product was adjusted to 100 μl. Thus, 1 μl of ss-cDNA represented the amount of ss-cDNA transcribed from 50 ng of total RNA.

2.3. Construction of the internal standards and PCR quantification of HPV-16 and -18 E7 mRNAs

Strategies for construction of internal standards and detailed methods for performing the quantitative PCR were described in our previous report [16]. Primers designed for construction of an internal standard for HPV-16 and -18 E7 were:

**HPV-16:**
- For HPV-16 E7 PCR: 5'-atgacagctcagagggagtagaatcagattttggagggatctcgc-3'
- For 278 bp internal standard fragment in the same PCR tube: 5'-cagatgacacacaatcctacgtttgagggatctcgc-3'

**HPV-18:**
- For HPV-18 E7 PCR: 5'-cagacagactaagcagacagagttctattggtcgtattgg-3'
- For 226 bp internal standard fragment of HPV-16 E7 cDNA (nt 647–nt 842): 5'-atgacagacctcagacacacaaagtagttttggagggatctcgc-3'

Primers used for HPV-16 E7 PCR were 5'-atgacagctcagagggagtagaatcagattttggagggatctcgc-3' and 5'-cagatgacacacaatcctacgtttgagggatctcgc-3'. They were designed to amplify a 196 bp fragment of HPV-16 E7 cDNA (nt 647–nt 842) and a 278 bp internal standard fragment in the same PCR tube. Primers for HPV-18 E7 PCR were 5'-atgacagctcagagggagtagaatcagattttggagggatctcgc-3' and 5'-cagacagactaagcagacagagttctattggtcgtattgg-3' which amplified a 226 bp fragment of HPV-18 E7 cDNA (nt 671–nt 896) and a 279 bp internal standard fragment generated in the same reaction tube. Reaction contamination by exogenous HPV DNA or RNA was ruled out by the inclusion of a reaction tube containing all reagents but no sample RNA.

Data analysis for the quantitative RT-PCR was performed as described by Ke et al. [16]. The HPV-16 and -18 E7 signals were corrected by a factor of 1.18 for HPV-16 and 1.05 for HPV-18 E7, which is the ratio of dC residues in the internal standard versus the target E7 mRNA. The HPV-16 and -18 E7 mRNA counts were determined.

To perform a semi-quantitative RT-PCR, the internal standards were sequentially diluted into standard solution at a range of 1×10⁻⁵ to 1×10⁰ attomole/μl for use as PCR templates. The PCR was performed at the same condition as used for all of the samples. A standard curve was then generated from the PCR data by plotting the logarithm of standard derived signal (y) against the logarithm of the initial number of standard molecules (x). Linear regression was used to calculate slope (a) and intercept (b) in an equation, y = ax + b, from which the numbers of target molecules in the test samples were determined.
2.4. Western blot analysis of HPV-16 E7 protein

HPV-16 E7 monoclonal antibody was a commercially available preparation (Zymed Laboratories Inc., South San Francisco, CA). Western blot analysis was performed according to the manufacturer’s protocol. Total cellular protein was extracted from CaSki, HeLa, 686LN, HOK-16B, and 16BNNK cells and 150 μg protein was subjected to electrophoresis in a 15% sodium dodecyl sulfate–polyacrylamide gel, followed by transfer to a nitrocellulose membrane. The membrane was probed overnight at 4°C with 1:100 diluted primary antibody to HPV-16 E7. After washing, the membrane was incubated at room temperature with 1:1000 horseradish peroxidase-labeled goat anti-mouse IgG (Amersham Life Science, Buckinghamshire, UK). The HPV-16 E7 signal was visualized by enhanced chemiluminescence (ECL, Amersham) and quantified using an Alpha Imager® 2000 (Alpha Innotech Corp., CA).

3. Results

3.1. Expression of HPV E7 mRNA in oral and cervical biopsies

Tables 1 and 2 illustrate results of duplicate RT-PCR analysis of both oral and cervical tumors, and adjacent histologically normal tissues. HPV-16 E7 mRNA was detected in biopsies from 9 out of 10 oral tumor patients (90%). Six (60%) oral tumors also expressed HPV-18 E7 mRNA. HPV-16 E7 mRNA was detected in biopsies from 11 out of 11 cervical tumor patients (100%), with six (55%) co-expressing HPV-18 E7.

Table 2 also shows the result of semi-quantitative analysis of HPV-16 E7 mRNA in cervical neoplasia. Similar analysis was not performed on the oral tumors due to insufficient sample mRNA. The HPV E7 mRNA level approximately ranged from 1 to 9000 copies/ng total RNA in these patients. Three of them (27%) contained <33 copies/ng total RNA in both normal and tumor tissues. Since the amount of total RNA in one epithelial cell is 30 pg [17], there was less than one copy of HPV E7 mRNA per cell in these three specimens.

3.2. Levels of HPV E7 mRNA in oral and cervical cancer cells

Quantitative RT-PCR has been used successfully to detect expression of many genes [18]. We have developed a quantitative RT-PCR to measure HPV-16 and -18 E7 mRNA expression in cancer cells. The method was adapted from the system we developed to analyze epidermal growth factor receptor (EGFR) mRNA [16]. To confirm the specificity of the PCR primers, they were used to amplify ss-cDNA isolated from HPV-18 positive HeLa cells and HPV-16 immortalized HOK-16B cells. Amplified PCR products were confirmed as expected fragments of either HPV-18 E7 or HPV-16 E7 by Southern blot analysis using HPV-16 or HPV-18 specific DNA probes, respectively. No cross amplification was observed between the two HPV types, i.e. HPV-16 E7 primers did not amplify ss-cDNA isolated from HeLa cells, and vice versa (data not shown).

Fig. 1 shows the quantitative RT-PCR analysis of HPV-16 E7 in 16BNNK cells and HPV-18 E7 in HeLa cells. Expression levels of HPV E7 mRNAs in 1483, 686LN, 16BNNK, HOK16B, HeLa and CaSki cells are summarized in Table 3. The results show that the oral cancer cell line, 1483, expressed an 18-fold higher level of HPV-18 E7 mRNA than HeLa cells, although HeLa cells harbor 50 copies of HPV-18 and 1483 cells contain only 1 copy of HPV-18 [19].

The tumorigenic 16BNNK cells expressed twice as much HPV-16 E7 mRNA than its non-tumorigenic counterpart, HOK16B, which is consistent with a previous observation [20]. E7 mRNA in CaSki cells was detected at a 17-fold higher level than in HOK16B cells. In general, the level of HPV-16 E7 mRNA varies from

<table>
<thead>
<tr>
<th>Patient</th>
<th>Primary tumor site</th>
<th>Clinical stage</th>
<th>HPV-16 E7 Normal</th>
<th>Tumor</th>
<th>HPV-18 E7 Normal</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Maxilla, buccal mucosa</td>
<td>T4N0M0</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Epiglottis, vallecula</td>
<td>T4N1M0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Floor of mouth, tongue</td>
<td>T4N1M0</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Tonsil, pharynx, tongue</td>
<td>T3N1M0</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Epiglottis, supraglottis</td>
<td>T4N0M0</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Pyriform sinus, vocal cord</td>
<td>T3N0M0</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Floor of mouth, tongue</td>
<td>T3N0M0</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Mandibular alveolus</td>
<td>T4N0M0</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Floor of mouth</td>
<td>T4N0M0</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>Glottis</td>
<td>T3N0M0</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* -., HPV negative; +., HPV positive; ++., the expression level is approximately 10-fold higher than ‘+’, etc.
very low levels in 686LN to much higher levels in CaSki cells.

3.3. Expression of HPV-16 E7 protein in oral and cervical cancer cells

The relatively higher HPV-16 E7 mRNA expression in CaSki cells was also observed at the protein level. Fig. 2 shows that the E7 protein level in CaSki cells is 5.5-fold higher than in HOK16B cells, and the E7 protein in 16BNNK cells is 1.4-fold higher than in HOK16B cells. The E7 protein in 686LN cells was barely detectable (data not shown). The HOK16B and 16BNNK cells were used as models for oral tumors

Table 2
Expression of HPV-16 and -18 E7 mRNA in cervical neoplasia

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clinical stage</th>
<th>HPV-16 E7 (Copy No./ng total RNA)</th>
<th>HPV-18 E7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal</td>
<td>Tumor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ (1.4)</td>
<td>+ (36.1)</td>
</tr>
<tr>
<td>1</td>
<td>CIN-I</td>
<td>+ + (158.1)</td>
<td>+ + + (5541.1)</td>
</tr>
<tr>
<td>2</td>
<td>CIN-II</td>
<td>+ (3.2)</td>
<td>+ (96.6)</td>
</tr>
<tr>
<td>3</td>
<td>CIN-0</td>
<td>+ + (14.2)</td>
<td>+ (2.0)</td>
</tr>
<tr>
<td>4</td>
<td>CIN-I</td>
<td>+ (7.0)</td>
<td>+ + (36.1)</td>
</tr>
<tr>
<td>5</td>
<td>CIN-II</td>
<td>+ (7.4)</td>
<td>+ + + (150)</td>
</tr>
<tr>
<td>6</td>
<td>CIN-III</td>
<td>+ (7.4)</td>
<td>+ + + + (8591.3)</td>
</tr>
<tr>
<td>7</td>
<td>CIN-II</td>
<td>+ + + (102.1)</td>
<td>+ + + + (3395.5)</td>
</tr>
<tr>
<td>8</td>
<td>CIN-II</td>
<td>+ + (56.0)</td>
<td>+ + (96.6)</td>
</tr>
<tr>
<td>9</td>
<td>CIN-III</td>
<td>+ (3.2)</td>
<td>+ (7.0)</td>
</tr>
<tr>
<td>10</td>
<td>CIN-II</td>
<td>+ + (14.2)</td>
<td>+ (2.0)</td>
</tr>
<tr>
<td>11</td>
<td>CIN-III</td>
<td>+ + + (102.1)</td>
<td>+ + + + (3395.5)</td>
</tr>
</tbody>
</table>

- HPV negative; +, HPV positive; ++, the expression level is approximately 10-fold higher than ‘+’, etc.

b The copy number of HPV-16 E7 was calculated from a semi-quantitative equation, \( y = 40.94x - 9.46 (R^2 = 0.98) \), as described in Section 2.

Table 3
Expression of HPV-16 and -18 E7 mRNA in oral epithelial cells and cervical cancer cells detected by quantitative reverse transcriptase-polymerase chain reaction

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>HPV type</th>
<th>E7 mRNA (copy No./ng total RNA + SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHOK</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>1483</td>
<td>18</td>
<td>17065 ± 1068</td>
</tr>
<tr>
<td>HeLa</td>
<td>18</td>
<td>958 ± 156</td>
</tr>
<tr>
<td>686LN</td>
<td>16</td>
<td>292 ± 35</td>
</tr>
<tr>
<td>HOK-16B</td>
<td>16</td>
<td>661 ± 93</td>
</tr>
<tr>
<td>16BNNK</td>
<td>16</td>
<td>5082 ± 636</td>
</tr>
</tbody>
</table>

* SD was not determined.
It has been suggested that the expression level of HPV E7 is closely correlated with transforming potential [21], and that detection of HPV E6/E7 mRNA may be more significant than that of viral DNA sequences alone [12]. Using RT-PCR analysis, we found approximately 90% of oral and 100% of cervical neoplasia patients were infected by high risk HPVs (HPV-16 and -18). A previous DNA dot blot analysis on the same cervical tissues showed only 45% of them to contain HPV DNA sequences (data not shown). Since mRNA was used for the analysis, DNA contamination during RT-PCR can be ruled out. Furthermore, a single form of HPV-16 E7 mRNA has been identified in cervical carcinoma, while the E6 mRNA exists in three different forms [22], with the ratios among the forms varying among samples [13]. Thus, analysis of E7 mRNA is more straightforward than analysis of E6 mRNA.

It is interesting to notice that high levels of HPV E7 mRNA were observed in oral tumors from the tonsil and epiglottis. This is consistent with other publications showing that tonsil-region cancers have higher frequency of HPV infection than other areas in oral cavity [23,24].

While we found that 100% of the cervical specimens carried HPV RNA, 27% of them had very low levels of E7 mRNA (<1 copy/cell). These data suggest that HPV oncogene expression may not be necessary for the maintenance of the malignant phenotype in every case where HPV DNA is detected. Therefore, quantification of HPV E7 mRNA may be more accurate reflective of HPV status in patient biopsies, thereby providing information for cancer screening and treatment evaluation. Though semi-quantitative RT-PCR is a less accurate reflection of mRNA levels than the competitive RT-PCR based quantitative analysis, it provides useful information regarding HPV E7 expression when samples are limited. To establish a causal relationship between HPV expression and cancer will require case control studies demonstrating that the HPV expression level correlates with tumor progression in patients who carry the virus.

HPV E7 mRNA was also found in histologically normal tissues adjacent to the tumors. In oral biopsies, HPV-16 E7 mRNA was detected in both normal and tumor tissues from five patients (56%), and in tumor only from two patients (22%). There were two oral tumor patients (22%) for whom HPV-16 E7 mRNA was not detected in tumor tissues but was detected in histologically normal tissues. This also suggests that HPV E7 expression may not be necessary for maintenance of the malignancy in some cases. In our previous study, on EGFR, we found that the ratio of EGFR mRNA levels between tumor and normal tissues was 10 times higher in these two cases than in others ($p < 1 \times 10^{-6}$, Student’s $t$-test) [16], suggesting that other factors, such as EGFR, may play a major role in cancer progression.

HPV-16 E7 mRNA was also found in both normal and tumor tissues from all HPV positive patients with cervical neoplasia (100%) though in most cases the E7 mRNA level was lower in the normal tissue than in the tumors, raising the possibility that the histologically normal tissues are at risk for subsequent malignant transformation. This is supported by our observation that HPV-16 E7 mRNA was detected in both HPV-16 immortalized tumorigenic and non-tumorigenic oral cells, HOK-16B and 16BNNK, respectively. These two cell lines may serve as a tumor progression model for both pre-malignant and malignant tissues in which HPV E7 mRNA was detected. A similar result was also observed by Choo et al. [25] in cervical cells, CaSki, and ECE16-1.

We have suggested that overexpression of HPV E6 and E7 oncogenes in oral cancer may be influenced by mutations in the long control region [15]. We found a 193 bp deletion in the HPV LCR isolated from 1483 cells which had higher level of HPV E7 mRNA than
HeLa cells. The deleted region involves several putative binding sites of *trans*-regulatory proteins and several histone H1 binding sites, the deletion of which might alter chromatin structure to activate HPV E6 and E7 gene expression. In cervical cancer, mutations and deletions at binding sites of the *trans*-regulatory factor YY1 were found in tumor tissues and resulted in activation of HPV E6 and E7 gene expression [26].

Our results demonstrated that HPV E7 mRNA expression correlated with E7 protein expression, suggesting that HPV oncogene expression is primarily regulated at the transcriptional or post-transcriptional level. We also found that the protein detection was not as sensitive as mRNA detection, based on the comparison of data obtained from western blot and quantitative RT-PCR analysis. A large amount of protein (150 μg) was needed for detecting HPV-16 E7 expression by western blot analysis. Since it is difficult to procure large tissue samples in most clinical situations, mRNA detection may be a better choice for quantification of HPV expression. The potential clinical application of quantitative RT-PCR for HPV mRNA expression in cancer screening and treatment evaluation requires further investigation.

References