Human Papillomavirus Type 16, 18, and 33 Infection in Adenocarcinoma of the Uterine Cervix: Analysis of the p53 Gene Mutation and the Clincopathologic Correlation

Kwang-Sun Suh • Seong-Jun Cho
Sun-Young Na • Heung-Tae Noh
Sang-Ryun Nam1

Departments of Pathology and
1Obstetrics and Gynecology,
Chungnam National University School of Medicine, Daejeon, Korea

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Corresponding Author
Kwang-Sun Suh, M.D.
Department of Pathology, Chungnam National University School of Medicine, 6 Munwha-1-dong, Jung-gu, Daejon 301-745, Korea
Tel: 042-220-7199
Fax: 042-220-7189
E-mail: kssuh@cnu.ac.kr

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Human papillomaviruses (HPVs) are recognized as sexually transmitted agents, and are now considered to be carcinogens in humans. Epidemiologic and molecular biologic studies have established a causal relationship between HPV infection and cervical neoplasia. Current evidence has indicated that specific types of HPV, especially types 16 and 18, are involved in the development of cervical cancer, mostly of squamous cell carcinoma (SCC).1-4 Even though a number of HPV prevalence studies support a role for the same HPV types in the genesis of invasive cervical adenocarcinoma,7-13 controversy exists with regard to the predominant HPV type that causes cervical adenocarcinoma.

In addition to the infection with the high-risk strains of HPV, inactivation or mutation of the tumor suppressor gene p53 has been detected for cervical carcinoma including adenocarcinoma.14-16 Most previous reports have suggested an inverse correlation between HPV infection and mutation of p53 in the pathogenesis of cervical carcinomas.14-16 However, mutation of p53 is infrequently seen in cervical carcinomas and the relationship between p53 mutation and HPV infection is still controversial.14,16-19 Much less is known regarding a possible correlation in cervical adenocarcinomas.

In this study, we analyzed a series of 38 primary cervical adenocarcinomas to investigate the prevalence of p53 mutations and their correlation with the HPV status; we also attempted to determine the correlation of HPV infection with the clinicopathologic data. The HPV infections were analyzed by polymerase chain reaction (PCR) amplification, and the presence of p53 mutations was determined by the PCR-single strand conformation polymorphism (SSCP) method.

MATERIALS AND METHODS

Patients and Specimens

Tumor specimens were obtained from 38 patients with primary cervical adenocarcinomas at the time of either cone biop-
sy or hysterectomy at Chungnam National University Hospital from 1996 to 2001. The age of patients ranged from 31 to 71 years (mean, 44.1 years). The tumors were staged according to the 1995 modification of the FIGO staging system for carcinoma of the uterine cervix. Two cases were Stage 0, 13 cases were Stage Ia, 18 cases were Stage Ib, four cases were Stage IIa, and 1 case was Stage IIb. On histological examination, 32 cases (84.2%) were endocervical type (Fig. 1), 2 cases were endometrioid type, 2 cases were adenosquamous type, one case was intestinal type, and one case was mixed endocervical and intestinal type. Seven cases (18.4%) showed coexistent squamous epithelial lesions, one case showed a low-grade squamous intraepithelial lesion (LSIL), three cases showed SCC in situ, two cases showed microinvasive SCC, and one case showed SCC.

All the tumor samples were obtained from routine formalin-fixed, paraffin-embedded material. After a review of the pathology slides, the area of adenocarcinoma was circled with a marking pen. The tissue blocks were required to have at least 85% of neoplastic cells. To meet this requirement, adenocarcinoma areas were microdissected away from the surrounding normal tissues.

**DNA Extraction**

A total of 4 to 5 microdissected 5 μm sections were incubated at 52°C for 1 or 2 days in 400 μL DNA extraction buffer containing 0.25 μg/μL proteinase K (Roche, Mannheim, Germany), 20 mM Tris-HCl at pH 8.3, 5 mM MgCl2, 100 mM KCl, 1% Tween 20, and 1% Nonidet P-40. The mixture was boiled for 10 min to inactivate proteinase K and it was purified by phenol extraction, then it was concentrated by ethanol precipitation. The isolated DNA solution was quantified spectrophotometrically.

The positive controls were composed of purified, DNA of cloned HPV 16, 18, and 33 (100 pg of target DNA and 200 ng of carrier DNA). The negative controls were composed of 200 ng of carrier DNA, and they were run individually and simultaneously with the test samples.

**PCR Amplification**

The primers of HPV 16, 18, and 33 (Takara, Otsu, Japan) were as follows: HPVpF, the common forward primer was 5'-AAGGGCGTAACCAGAAATCGGT-3'; HPVp16R, the HPV 16 reverse primer was 5'-GTGTTCACATCTCTGATCA-3'; HPVp18R, the HPV 18 reverse primer was 5'-GTGTTCAGTTCGCGCACA-3'; HPVp33R, the HPV 33 reverse primer was 5'-GTCTCCAATGCTTGACACA-3'. Amplification was performed on 20 μL of DNA using 100 ng of template DNA, 0.5 units of ExTaq polymerase (Takara, Otsu, Japan), 2 μL of 10X reaction buffer, 1.6 μL of 10 mM dNTP, 10 pmol of forward and 10 pmol of reverse primer. The cycling parameters were 5 min at 94°C (one cycle), 30 s at 94°C, 2 min at 55°C, 2 min at 72°C (30 cycles), and 5 min at 72°C (one cycle). The PCR products were analyzed by 4% NuSieve 3:1 agarose (BMA, Rockland, USA) gel electrophoresis. The expected size of the amplified products for HPV 16, 18, and 33 were 140, 140 and 141 base pairs, respectively.

To verify the specificity of the method, appropriate positive controls (plasmids containing HPV DNA) and negative controls (buffer only) were involved in the PCR assays in each run.

PCR amplification of p53 exon 5-7 was performed in a total volume of 20 μL containing 500 ng of template DNA, 1 unit of ExTaq polymerase (Takara, Otsu, Japan), 1.25 mM dNTP, 15 pmole primers, and 2 μL of 1X reaction buffer. A PCR mixture for exon 8 was performed under the same conditions as for exon 5-7, except for the dNTP concentration (2.5 mM dNTP each). The PCR cycles consisted of 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C; this was followed by 1 cycle for 7 min at 72°C. The oligonucleotide primers used for amplification are shown in Table 1.

**SSCP analysis, silver staining, and direct sequencing**

Two μL of PCR product was mixed with 6 μL of sample loading buffer containing 95% formamide (deionized), 10 mM NaOH, 0.25% bromophenol blue, and 0.25% xylene cyanol. The samples were denatured for 3 min at 100°C and then quickly chilled on ice. The samples were next loaded onto 12% polyacrylamide
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gel containing 1X sample buffer (33 mM Tris-sulfate, 7% glycerol, pH 8.3), and they were electrophoresed at a constant voltage of 250 V. After electrophoresis, the gels were disassembled from the glass plate and stained using a Silver Stain Plus kit (BIO-RAD, USA), followed by air drying. The samples with abnormal bands were automatically sequenced on a Genetic analyzer (ABI, USA).

Statistical analysis
The association between HPV-DNA and clinical and histopathological findings was analyzed by Pearson’s chi-square analysis, Fisher’s exact test, and Student’s T-test. We considered results to be statistically significant when the p-value was less than 0.05.

RESULTS

HPV infection

The prevalence of HPV 16, 18 or 33 infection was 73.7% (28/38 cases). HPV 16 or 18 was present in 27 of 38 cases (71.1%): HPV 16 was present in 12 cases (44.4%) and HPV 18 was present in 15 cases (55.6%). HPV 33 was positive in one case (3.7%) (Fig. 2). The age of patients ranged from 31 to 71 years (mean, 44.1). The mean age of patients with HPV infection was 45.4 years and the mean age of HPV-negative patients was 52.5 years (p value=0.018). The mean age of patients with HPV 16 infection was 46.5 years and the mean age of those with HPV 18 infection was 45.6 years (p value=0.381). There was no correlation between HPV infection and the clinical stage or pathologic type (Table 2).

P53 gene mutation

Only one case (2.6%) that was positive for HPV 18 showed a p53 mutation in exon 6. The mutation was missense in codon 210 of exon 6 (AAC → AGC, asparagine → serine) (Fig. 3). p53 mutations were not present in all (10) of HPV-negative cases. There was no inverse correlation between HPV infection and p53 mutation.

DISCUSSION

Infection with HPV is the main cause of cervical cancers. Recently, it has been established that HPV DNA has been detected in 90.7% of cervical SCC patients and in 13.4% of control

Table 1. Primer sets for SSCP and direct sequencing analysis

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer</th>
<th>Sequence</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>p53-5F (21 mer)</td>
<td>5’-GTCTCCTCCCTCCTCCTAG-3’</td>
<td>243 bp</td>
</tr>
<tr>
<td></td>
<td>p53-5R (20 mer)</td>
<td>5’-CAACCAGCCCTGTCGCTCT-3’</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>p53-6F (21 mer)</td>
<td>5’-CTGATTCCTCACTGATT-3’</td>
<td>159 bp</td>
</tr>
<tr>
<td></td>
<td>p53-6R (21 mer)</td>
<td>5’-GACCCCAGTTGCAAACCAGAC-3’</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>p53-7F (20 mer)</td>
<td>5’-GGGCTGTGTTATCTCCTAG-3’</td>
<td>148 bp</td>
</tr>
<tr>
<td></td>
<td>p53-7R (18 mer)</td>
<td>5’-TGGCAAGTGGCTCCTG-3’</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>p53-8F (18 mer)</td>
<td>5’-TCCTATCCTGAGTAGTGG-3’</td>
<td>173 bp</td>
</tr>
<tr>
<td></td>
<td>p53-8R (21 mer)</td>
<td>5’-CTTCTGTCTGCTGTTAC-3’</td>
<td></td>
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</table>

Table 2. Clinicopathologic features according to HPV infection status

<table>
<thead>
<tr>
<th></th>
<th>HPV (+) (n=28)</th>
<th>HPV (-) (n=10)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Mean±SD) (years)</td>
<td>45.4±8.2</td>
<td>52.5±9.6</td>
<td>0.018*</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td>N.S.</td>
</tr>
<tr>
<td>Ia</td>
<td>13 (46.4)</td>
<td>2 (20.0)</td>
<td></td>
</tr>
<tr>
<td>Ib</td>
<td>13 (46.4)</td>
<td>6 (60.0)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>2 (7.1)</td>
<td>2 (20.0)</td>
<td></td>
</tr>
<tr>
<td>Histologic type</td>
<td></td>
<td></td>
<td>N.S.</td>
</tr>
<tr>
<td>Endocervical</td>
<td>20 (71.4)</td>
<td>8 (80.0)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>8 (28.6)</td>
<td>2 (20.0)</td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td>N.S.</td>
</tr>
<tr>
<td>1</td>
<td>9 (32.1)</td>
<td>3 (30.0)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>15 (53.6)</td>
<td>6 (60.0)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4 (14.3)</td>
<td>1 (10.0)</td>
<td></td>
</tr>
<tr>
<td>P53 mutation</td>
<td></td>
<td></td>
<td>N.S.</td>
</tr>
<tr>
<td>+</td>
<td>1 (3.6)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>27 (96.4)</td>
<td>10 (100.0)</td>
<td></td>
</tr>
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</table>

*statistically significant; N.S., not significant.

Fig. 2. PCR analysis of HPV infection in adenocarcinoma of the uterine cervix. The PCR product length for HPV 16, 18, and 33 is 140 bp, 140 bp, and 141 bp, respectively.
women. In Korea, the proportions of the detected HPV were 35.1% in the women with normal cytology, 92.7% in the patients with LSIL, 98.1% in those with HSIL, and 96.3% in cervical cancer patients. The absolute incidence of adenocarcinoma of the uterine cervix is increasing concomitantly with an increasing frequency of HPV infections, and this is especially so in young women. Some studies have also reported that there is an increasing absolute incidence of cervical adenocarcinoma linked to the widespread use of oral contraceptives. In this series the prevalence of HPV 16, 18 or 33 infection was 73.7%.

HPV 18 and less frequently HPV 16 are often detectable in cervical adenocarcinomas by using in situ hybridization and/or PCR techniques. The reported detection rate for the high-risk HPVs (16 and 18) is lower in cervical adenocarcinoma than in SCC, in which HPV 16 is the most commonly identified type. Previous studies have documented a relative predominance of HPV 18 in cervical adenocarcinomas, and those studies have suggested that the morphology of cervical carcinoma might be defined by a specific HPV type. Another study has reported that most of the HPV identified in cervical adenocarcinoma was type 16, 18 or 33, and that HPV 16 was the most predominant type. Therefore, the significance of HPV 18 in defining the glandular phenotype of cervical tumors has decreased. Either HPV 16 or 18 was present in 96.4% of the HPV positive cases (27 of 28 cases). Type 16 was present in 12 of the HPV positive cases and type 18 was present in 15 of the HPV positive cases.

Patients with HPV-positive carcinoma were significantly younger than those with HPV-negative carcinoma. In our series the mean age of patients with HPV infection was younger than the HPV-negative group (p=0.018). According to Kim et al., the inactivation of p53 protein by HPV type 16 E6 protein plays a greater role in carcinogenesis of the uterine cervix than the mutation of the p53 gene. Previous studies have shown that the overall rate of p53 mutations is approximately 5.6% in HPV-positive cervical cancer cases and 6.8% in HPV-negative cervical cancer cases (Table 3). In this study, only one case (3.6%) that was positive for HPV 18 showed a p53 mutation in exon 6. p53 mutations were not present in any of the HPV-negative cases. We observed no inverse correlation between the HPV infection and p53 gene mutation. These results fall in line with the results of previous studies.

It has been reported that patients with HPV-negative tumors have a worse overall survival rate than patients with HPV-positive adenocarcinomas. Previous reports have suggested that a poorer prognosis for patients with HPV-negative cancers reflects the higher prevalence of the p53 mutations. According to Tenti et al., the patients with HPV-negative tumors and the patients with p53 mutated neoplasms, irrespective of HPV infection, had a shorter survival time. However, an absence of HPV infection and presence of p53 mutations were not found to be independent risk factors for tumor-related death in their study after adjustment was made for clinicopathological parameters.

In conclusion, the detection rate for the high risk HPVs (16, 18 or 33) was high (73.7%) in cervical adenocarcinoma and HPV 18 was the type most commonly identified. The p53 gene was mutated in 2.6% of cervical adenocarcinoma cases. There was no correlation between HPV infection and clinical stage or patho-

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Table 3. Frequency of p53 mutations in cervical carcinomas (%)

<table>
<thead>
<tr>
<th>Carcinoma type</th>
<th>p53 mutation (+) cases</th>
<th>HPV (+) cases</th>
<th>HPV (-) cases</th>
<th>total cases</th>
<th>Reference No. (year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer*</td>
<td>1/30 (3.3)</td>
<td>2/16 (12.5)</td>
<td>3/46 (6.5)</td>
<td>Uchiyama et al. 10 (1997)</td>
<td></td>
</tr>
<tr>
<td>Cancer*</td>
<td>2/29 (6.9)</td>
<td>0/7 (0)</td>
<td>2/36 (5.6)</td>
<td>Paquette et al. 7 (1993)</td>
<td></td>
</tr>
<tr>
<td>Cancer*</td>
<td>2/133 (1.5)</td>
<td>0/3 (0)</td>
<td>2/136 (1.5)</td>
<td>Kim et al. (1997)</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>9/56 (16.1)</td>
<td>1/18 (5.6)</td>
<td>10/74 (13.5)</td>
<td>Tenti et al. 8 (1998)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14/248 (5.6)</td>
<td>3/44 (6.8)</td>
<td>17/345 (4.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>1/28 (3.6)</td>
<td>0/10 (0)</td>
<td>1/38 (2.6)</td>
<td>Our study (2004)</td>
<td></td>
</tr>
</tbody>
</table>

*Cervical cancer includes both squamous cell carcinoma and adenocarcinoma cases.
logic type of tumor. Other mechanisms that are independent of p53 inactivation may also be implicated for carcinogenesis of the uterine cervix without HPV infection.

REFERENCES


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