Detection and Typing of Human Papillomavirus in Cervical Carcinomas in Russian Women

A Prognostic Study

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BACKGROUND. The correlation between human papillomavirus (HPV) infection and tumor prognosis in 159 Russian women with cervical carcinoma was investigated. The presence of various HPV types was correlated with the histologic parameters of the carcinomas and with their immunoreactivity with antibodies to p53, Ki-67-Ag, and bcl-2.

METHODS. Formalin fixed, paraffin embedded tissue specimens representing 159 cases of International Federation of Gynecology and Obstetrics Stage I and II were used. HPV DNA was detected by polymerase chain reaction (PCR) using a general primer set that targets the L1 region and synthesizes a product of only 65 base pairs. The HPV types were determined by direct sequencing and compared with known HPV types.

RESULTS. All 159 carcinomas were positive for HPV. HPV 16 (64.8%) was most frequently found, followed by HPV 18 (10.7%) and HPV 45 (8.2%). In 6 patients (3.8%), HPV types could not be further classified, and these cases were therefore categorized as HPV X. Although a trend was noted toward poorer prognosis for women with carcinomas harboring HPV types 16, 18, and 45 than for patients with carcinomas harboring HPV types 31, 33, 35, 52, 56, 58, and 68, the differences were not statistically significant. The prevalence of adenocarcinoma and adenosquamous carcinoma was higher among HPV 18 positive patients than among patients with the other known HPV types (P = 0.0002).

CONCLUSIONS. The rate of HPV positivity in these 159 cervical carcinomas was 100%. These findings challenge the assumption that HPV negative cervical carcinomas exist. This high rate might be attributed to the use of a new broad-spectrum HPV PCR test. HPV typing in cervical carcinoma was not significantly related to clinical outcome. HPV 18 was significantly more frequently found in adenocarcinoma and adenosquamous carcinoma. The possibility of classifying HPV 45 as an oncogenic high risk type should be considered. Cancer 1999;85:2011–6.

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KEYWORDS: human papillomavirus type, cervical carcinoma, tumor stage, relapse risk, survival, bcl-2, Ki-67-Ag, p53.

The role of human papillomaviruses (HPV) in the development of (pre)malignant lesions of the cervix has been firmly established.¹⁻³ Phylogenetic analyses of full-length HPV DNA genomes revealed that HPV can be classified into different groups (A1–A11), each group containing a number of HPV types with a certain DNA sequence similarity.⁴⁻⁵

On the basis of their oncogenic potential, the HPV viruses can be subdivided into a low risk group containing types 6, 11, 42, and 44 (among other types) and a high risk group containing types 16, 18, 31,
This clinical differentiation is based on the observation that only the high risk HPV types are found in cervical carcinoma.6 A slightly different classification of HPV types, based on the ratio of the percentages of specific HPV types in cervical intraepithelial neoplasia (CIN) and cervical carcinoma, places only HPV 16 and 18 in the high risk group, whereas types 31, 33, 35, 45, 52, 56, and 58 are classified as viruses with an intermediate risk factor.7,8 However, there has been discussion regarding the oncogenic potential of HPV 45 and 56, which are considered high risk viruses by Lorincz and intermediate risk by Bergeron.7,8

Various studies have demonstrated that a high percentage of women with normal cervical smears containing oncogenic HPV may rapidly develop CIN, in contrast to HPV negative women or women infected with HPV with low oncogenic potential.9 Furthermore, in women with CIN accompanied by infection with high risk types of HPV, swift progression to a higher grade of CIN or cervical carcinoma has been described.10–12 Also, the duration of the infection and/or the load of HPV with oncogenic potential have been identified as important risk factors for progression of CIN.13

The literature to date presents a number of controversial issues concerning HPV prevalence, HPV type, and prognosis of cervical carcinoma. HPV negative carcinomas have been described, and it was even emphasized that this group had a considerably worse prognosis than patients with HPV positive carcinomas.14–16 Lymph node metastases and recurrences were shown to occur more frequently in the HPV negative group. Later studies that used more sensitive HPV detection techniques17 also described cases of HPV negative cervical carcinoma but failed to confirm the correlation with poorer prognosis.17

More recently it was shown that women with cervical carcinoma associated with HPV 18 are younger and have a poorer prognosis than those with cervical carcinoma associated with other oncogenic HPV types, e.g., HPV 16.17–19 Furthermore, a higher prevalence of HPV 18 in adenocarcinomas has been described, whereas HPV 16 is more frequently found in squamous cell carcinomas.20

In this study the correlation between HPV type and tumor prognosis in Russian women with cervical carcinomas was investigated. These women had not participated in a cervical screening program. We compared HPV type with clinical parameters, such as stage of disease, lymph node status, histologic classification, tumor dimensions, depth of infiltration, and patient age, p53, Ki-67-Ag, and bcl-2 status of each tumor were also correlated with the presence of various HPV types.

MATERIALS AND METHODS

Formalin fixed, paraffin embedded tissue specimens representing 159 cases of International Federation of Gynecology and Obstetrics (FIGO) Stage I and II cervical carcinoma, diagnosed and primarily surgically treated in the period between 1988 and 1994, were taken from the files of the Russian Cancer Centre in Moscow.21

For each patient, a standardized questionnaire based on the medical records was completed by the supervising clinician (S.P.). This included general information, i.e., age and parity, and information concerning the cervical lesion, including tumor size and depth of infiltration measured in the macroscopic specimen and/or in histologic slides. Extension of carcinoma beyond the cervix was clinically evaluated. Lymph node status and histologic classification were histologically determined. Furthermore, information was obtained regarding therapy, i.e., surgery, radiotherapy, and/or chemotherapy. Patients gave consent to all of these means of obtaining data. Follow-up information consisted of disease free interval and survival status.

An average of 10 paraffin blocks were available on each case (a minimum of 4 and a maximum of 20). From a tissue block most representative of the carcinoma, four consecutive 4-μm-thick sections were cut for conventional diagnosis according to World Health Organization (WHO) criteria and one 10 μm section was taken for DNA isolation and PCR analysis. Each block was separately cut, then the microtome was thoroughly cleaned with alcohol. The technician used new surgical gloves when cutting a new block and also used new gloves to clean the microtome.

Sections were stained for H & E and tested for the presence of mucins with PAS after treatment with diastase and Alcian blue. Histology was read blind to HPV test results.

The maximum depth of infiltration of each carcinoma was, where possible, again established using a calibrated ocular. In cases of discrepancy, the last measure of infiltration depth was used.

DNA Isolation

DNA isolation was performed in Delft, The Netherlands, according to a modified version of the method described by Claas et al.22 Briefly, the 10-μm-thick section was collected in a 1.5 mL tube and deparaffinized in 500 μL xylene. After gentle shaking for 2 minutes and centrifugation for 5 minutes the pellet was again extracted in 500 (l xylene, then washed
twice in 500 \( \mu \text{L} \) alcohol 96% and once in 500 \( \mu \text{L} \) acetone. Subsequently, the pellet was air-dried, dissolved in 200 \( \mu \text{L} \) 5mM Tris-HCl pH 9.0 containing 1 mg/mL proteinase K (Merck, Gibbstown, NJ), and incubated overnight at 37°C.

**Polymerase Chain Reaction**

For detection of HPV DNA, a novel set of broad-spectrum HPV primers, SPF1 and SPF2, were used.\(^{23}\) This primer set targets the L1 region and directs the synthesis of a product of only 65 base pairs. PCR was performed essentially as described by Saiki.\(^{24}\) Briefly, the final volume of 100 \( \mu \text{L} \) contained 10 \( \mu \text{L} \) of the isolated DNA, 10 mM Tris-HCl pH 9.0, 50mM KCl, 2.5 mM MgCl\(_2\), 0.1% Triton X-100, 0.01% gelatin, 200 \( \mu \text{M} \) of each deoxynucleoside triphosphate, 100 pmol of forward and reverse primer, and 0.25 units of Super-Taq (Sphaero Q, Cambridge, UK). PCR conditions were as follows: a preheating step for 1 minute at 94°C followed by 40 cycles for 1 minute at 94°C, 1 minutes at 52°C, and 1 minute at 72°C. As a control for successful DNA isolation, PCR was performed using \( \beta \)-globin primers as described by Saiki.\(^{25}\) In each PCR experiment, containing 20 specimens, negative controls were tested together with the clinical sample to exclude cross-contamination.

**HPV Genotype**

PCR products were electrophoresed on 3% agarose gel and detected by ethidium bromide staining. Amplicons were isolated from the gel and analyzed by direct sequencing to determine the HPV genotype. Sequence reactions were performed with both SPF primers using a cycle-sequencing kit (Perkin Elmer Corporation, Norwalk, CT). The obtained sequences were analyzed with PC-Gene software and compared with those of known HPV types (IntelliGenetics, Oxford, UK).

**Immunohistochemistry**

HPV type was compared with the following biomarkers: Ki-67-Ag as a marker of proliferation, \( bcl-2 \) as a marker of protection from apoptotic cell death, and p53 as a marker of cell cycle control. Results of the expression of the aforementioned immunohistochemical markers in this series of cervical carcinomas are described elsewhere.\(^{26}\)

**Statistical Analysis**

For statistical evaluation, the cases were divided into three groups consisting of HPV 16 (I) and 18 (II) (high risk types) and all other characterized HPV types (III) (intermediate risk) taken together. Furthermore, a di-
vision was made into three groups (A6, A7, and A9) according to their phylogenetic relation8 (Table 1).

Curves for survival analysis and relapse risk were calculated using the Kaplan–Meier method; comparison of curves was done with the log rank test.

Time to event was calculated as the period between surgery and death (survival) or as the period between surgery and relapse of disease (relapse risk).

Cross-tables were analyzed with the chi-square test.27

RESULTS

Patients were classified as Stage I (n = 74) and Stage II (n = 85), according to FIGO staging, with no further subdivision.21 The median follow-up time was 35 months (range, 1–131 months), whereas 38 patients relapsed, of whom 23 died. Histologically the carcinomas were classified, according to WHO criteria, into nonkeratinizing squamous cell carcinoma (n = 94), keratinizing squamous cell carcinoma (n = 19), adenocarcinoma (n = 27), and adenosquamous carcinoma (n = 19). There was no significant correlation between histologic classification and stage.

HPV Characterization

HPV was detected in all 159 carcinomas. The majority of cases was positive for the high risk HPV types 16 and 18, i.e., 65% and 11%, respectively. Surprisingly, of these 159 carcinomas, 8% were positive for HPV 45 and 3% for each of HPV types 31, 33, and 56. Types 35, 52, 58, and 63 were each positive in 1%. In 6 patients (4%), unknown HPV types were detected, and these cases were therefore categorized as HPV type X. These six patients harbored one of five different HPV sequences not described to date (Table 2).

HPV Type versus Survival

Five-year survival in women with cervical carcinomas infected with HPV 16 was 69%, with HPV 18 82%, and with HPV 45 83%. These differences were not significant. Survival data on women with cervical carcinomas infected with HPV 16 did not differ significantly from the results obtained for women infected with HPV 31, 33, 35, 52, 56, 58, and 68.

Although there was a trend toward poorer survival for patients infected with the HPV types 16, 18, and 45 as compared with women who had carcinomas harboring HPV types 31, 33, 35, 52, 56, 58, and 68, the differences were not statistically significant.

HPV Type versus Stage, Depth of Infiltration, Tumor Dimension, and Lymph Node Status

There was no significant correlation between any of the HPV types and stage of disease, nor was there a correlation between viral type and depth of tumor infiltration in the cervix, tumor dimensions, or lymph node status. HPV positivity was not separately tested in lymph nodes.

HPV Type versus Histology

The distribution of HPV among different histologic subtypes is shown in Table 1. There was a significant correlation between HPV 18 and histologic classification (Table 1). In the group of adenocarcinomas comprising adenocarcinoma and adenosquamous carcinomas, HPV 18 was found in 26% of patients, whereas only 4% squamous cell carcinomas harbored HPV 18; this correlation was statistically significant (P = 0.0002). Histologic typing indicated that even more adenocarcinomas contained HPV 18 than the adenosquamous carcinomas (33% vs. 16%, respectively) (P = 0.001). HPV 16 and the other types did not have a comparable correlation to histologic classification.

HPV 16 infection was predominant. The distributions of this HPV type in squamous and adenocarcinoma were 69% (78 of 113 cases) and 54% (25 of 46 cases), respectively. The ratio of HPV 16 positive squamous versus adenocarcinomas was 3:1 (78/113:25/46) (Table 1).

According to the phylogenetic classification, HPV types 18, 45, and 68 (= A7) together are found significantly more frequently in adenocarcinoma and adenosquamous carcinoma than in squamous cell carcinoma (P = 0.017). This correlation was also observed for adenocarcinomas alone (P = 0.017). However, HPV type 45 taken alone showed more similarity in its histologic distribution to HPV 16 (A9) than to HPV 18/68 (A7) (Tables 1 and 3).

TABLE 2

Distribution of HPV Types in Russian Cervical Carcinomas

<table>
<thead>
<tr>
<th>HPV type</th>
<th>Phylogenetic group</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>A9</td>
<td>103</td>
<td>64.8</td>
</tr>
<tr>
<td>18</td>
<td>A7</td>
<td>17</td>
<td>10.7</td>
</tr>
<tr>
<td>31</td>
<td>A9</td>
<td>4</td>
<td>2.5</td>
</tr>
<tr>
<td>33</td>
<td>A9</td>
<td>4</td>
<td>2.5</td>
</tr>
<tr>
<td>35</td>
<td>A9</td>
<td>2</td>
<td>1.3</td>
</tr>
<tr>
<td>45</td>
<td>A7</td>
<td>13</td>
<td>8.2</td>
</tr>
<tr>
<td>52</td>
<td>A9</td>
<td>2</td>
<td>1.3</td>
</tr>
<tr>
<td>56</td>
<td>A6</td>
<td>4</td>
<td>2.5</td>
</tr>
<tr>
<td>58</td>
<td>A9</td>
<td>2</td>
<td>1.3</td>
</tr>
<tr>
<td>68</td>
<td>A7</td>
<td>2</td>
<td>1.3</td>
</tr>
<tr>
<td>X</td>
<td></td>
<td>6</td>
<td>3.8</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>159</td>
<td>100</td>
</tr>
</tbody>
</table>

HPV: human papillomavirus.
TABLE 3
HPV Types 16, 18, 45, and 56 According to Histologic Type

<table>
<thead>
<tr>
<th>Histologic type</th>
<th>HPV type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratinizing ca</td>
<td>13</td>
</tr>
<tr>
<td>Nonkeratinizing ca</td>
<td>65</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>11</td>
</tr>
<tr>
<td>Adenosquamous ca</td>
<td>14</td>
</tr>
<tr>
<td>n</td>
<td>103</td>
</tr>
</tbody>
</table>

HPV Type versus Biomarkers
There was no correlation between HPV type and the immunoreactivity of p53, Ki-67-Ag, and bcl-2.

HPV Type versus Age
In the comparison between average age in patients infected with high risk and intermediate risk type viruses, a correlation between age and virus type was not attained. Investigation of the age groups of 40 years and younger and 50 years and younger compared with the respective age groups of 40 and 50 years and older showed no correlation between age and the group’s viral type.

DISCUSSION
To date, most studies investigating the correlation between cervical carcinoma and HPV have invariably found a number of HPV negative carcinomas. Recent studies of HPV DNA detection have shown that the overall prevalence of HPV is underestimated when a single method is used.28,29 The sensitivity of the PCR test is strongly related to the size of the PCR product, especially in paraffin embedded tissues.28 In this study, the primer set SPF1/2, which amplified a sequence of only 65 bp, was able to detect HPV DNA in all 159 formalin fixed, paraffin embedded cervical carcinomas. Mixed infections were not found in this group of cervical carcinomas. We have no conclusive explanation for this. Premalignant cervical lesions harbor more than 1 type of HPV in 21% of cases.30 However, a single cell from such a lesion is probably infected with only one type of HPV, and if such a cell transforms into a cancer cell it should be monoclonal for that HPV type. If more than one type of HPV occurred in a cervical carcinoma, one would expect that this carcinoma could have originated from more than one premalignant cell, each infected with a different type of HPV. Another explanation is of a more technical nature. The amplicon containing the predominant HPV DNA will be characterized by sequence analysis, meaning that other HPV types, if present, will not be observed.

The distribution of HPV in this population of Russian women is roughly similar to the prevalence of HPV in cervical carcinoma worldwide, as described by Bosch and Manos.20

The prevalence of HPV 16 and 18 in our study corresponds to what is reported in other European studies; on the other hand, the distribution of HPV 45 is quite different (8%), comparable to observations in Central and South American countries.20 The frequency of this HPV type in our study is approximately equal to the prevalence of high risk HPV 18. Strangely, HPV 45 has a very low prevalence in premalignant lesions. Therefore we think that HPV 45 should be classified as a high risk virus. The underestimated risk of type 45 in European studies might be due to the extremely low prevalence of HPV 45 in the population, as has been reported by Lorincz et al.5 In contrast, we only found a small number of cervical carcinomas containing HPV 56, which Lorincz also considers a high risk HPV type.3 This may reflect the low prevalence of this virus in the population studied.

Our study confirms that HPV 16 and 18 are related to squamous cell carcinoma and adenocarcinoma, respectively. HPV 18 is significantly more frequently found in adenocarcinomas and adenosquamous carcinomas.

HPV 45 phylogenetically related to HPV 18 (A9) could also be expected to be found more frequently in adenocarcinomas. In our study, the distribution of HPV type 45 mimics the distribution of HPV 16 (A7), because HPV 16 was detected in approximately 75% of squamous cell carcinomas and 25% of adenocarcinomas, including adenosquamous carcinomas. From this, we conclude that HPV 45 is not restricted in a specific carcinoma subtype.

The average age of women with a cervical carcinoma infected with HPV 18 is not lower than that of women infected with other HPV types. This does not support the hypothesis that cervical carcinomas infected with HPV 18 develop at an earlier age and progress more rapidly than cervical carcinomas infected with other HPV types.19 Furthermore, based on our observations, we are not able to confirm a study indicating that HPV 18-associated carcinomas follow a more aggressive course.17 Although there was a trend toward poorer prognosis in women with carcinomas infected with HPV 16, the differences were not statistically significant. However, it is important to note that in all studies, relatively high percentages of cervical carcinomas were HPV negative. For this reason, it could well be possible that the worse prognosis noted by some investigators in HPV 18-infected adenocarcinoma reflects a technical flaw in these studies.
We found that all 159 cervical carcinomas studied contained HPV DNA. This might have been due to the primer set we used, which amplifies a sequence of 65 base pairs only. The use of the short amplicons challenges the concept that HPV negative cervical carcinomas exist at all.

From our study, we conclude that HPV typing in cervical carcinoma has no prognostic value with regard to clinical outcome, as opposed to its prognostic value in normal cervical epithelia and premalignant lesions.

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