HUMAN PAPILLOMAVIRUS INFECTION AFTER TREATMENT FOR HIGH-Grade Cervical Intraepithelial Neoplasia

E. Shikova¹, Z. Ivanova¹, A. Zvirbliene², G. Ganchev³
¹Institute of Experimental Morphology, Pathology and Anthropology with Muzeum at Bulgarian Academy of Sciences, Sofia, Bulgaria
²Institute of Biotechnology of Vilnius University, Vilnius, Lithuania
³National Specialized Hospital for Active Treatment in Oncology, Sofia, Bulgaria
Correspondence to: Evelina Shikova
E-mail: evelina_sh@abv.bg

ABSTRACT

Cervical carcinoma is a leading cause of mortality from cancer among women worldwide. Oncogenic types of human papillomavirus (HPV) are recognised as causative agents of cervical cancer and its precursor, cervical intraepithelial neoplasia (CIN). It was shown that successful treatment of CIN results in clearance of HPV infection and treatment failure is accompanied by the detection of viral persistence. Therefore, follow-up testing for HPV of CIN treated patients is now accepted as an option for monitoring for recurrent disease.

The aim of this study was to investigate the prevalence of high risk HPV after treatment of Bulgarian women for high-grade CIN. Cervical specimens were obtained from 59 women treated for CIN by conization. They were subjected to Pap smear test and HPV DNA testing by PCR. Cytological abnormalities were found in 18.6% of all women. HPV DNA was detected in 91% of women with abnormal cytology. Only 8.3% of women with normal cytology were HPV positive. HPV genotyping showed that the most prevalent HPV type was HPV16 accounting for 80% of HPV positive samples with abnormal cytology and for 25% of HPV positive samples with normal cytology. HPV18 was detected only in one specimen with abnormal cytology. HPV31 was found in 50% of all HPV positive materials. We were not able to detect HPV33. Our study indicates that the posttreatment high risk HPV infection detected in Bulgarian women is a risk factor for subsequent cervical dysplasia and that the high risk HPV testing after conization is important for predicting the risk of disease recurrence.

Keywords: human papillomavirus, cervical intraepithelial neoplasia (CIN), PCR, recurrent disease

Introduction

Persistent infection with high risk human papillomavirus (HPV) is the major cause of cervical cancer and its precursor, cervical intraepithelial neoplasia (CIN) (12). HPV is found in close to 100% of cancers (30, 31). Therefore, HPV testing has been introduced into cervical cancer screening and management algorithms.

Women with untreated high-grade CIN are at risk of cervical cancer. As it is not possible to predict which CIN will regress and which will progress, every higher CIN grade (CIN 2 and CIN 3) is treated to remove the altered tissue. One of the recognized treatment methods of CIN is conization. Numerous studies have shown that successful CIN treatment results in clearance of HPV infection and treatment failure is accompanied by the detection of viral persistence during post-treatment follow-up (1, 3, 4, 9, 13, 14, 18, 23). On the basis of these data, follow-up testing for high risk HPV of CIN treated patients is now accepted as an option for monitoring for recurrent disease. Moreover, according to Nobbenhuis et al. a positive high risk HPV DNA test 6 months after treatment of CIN 2/CIN 3 is more predictive for recurrence than an abnormal cytology (19).

Now it is known that women who have been treated for high-grade CIN still have an increased risk of acquiring invasive cancer compared with the general female population (27). HPV persistence after treatment for high-grade CIN may be associated with the risk of treatment failure as the high risk HPV types are often found in CIN recurrences. Although various studies show the presence of HPV after CIN treatment in 0-92% of women (7), in recent years a high incidence of HPV persistence during post-conization is reported.

There are limited data in Bulgaria on the persistence of HPV infection after conization. Therefore, we studied the prevalence of high risk HPV after treatment of Bulgarian women for high grade CIN.

Materials and Methods

Clinical specimens

Cervical specimens were obtained from the National Specialized Hospital for Active Treatment in Oncology, Sofia. They were collected from 59 women treated for high-grade CIN lesions by conization. Three to six months after the treatment an endo/ectocervical sample was taken with a cytobrush and used for cytology and HPV testing. After a smear was prepared on a glass slide the brush was placed in 1.0 ml 0.9% NaCl and sent to the laboratory for HPV detection and typing.
Cytology
All cervical smears were stained using the Papanicolaou method and cytomorphologically classified as Pap 1 (normal), Pap 2 (inflammation), Pap 3a (atypical cells of undetermined significance), Pap 3 (mild dysplasia), Pap 3D (moderate dysplasia), Pap 4A (severe dysplasia), Pap 4 (carcinoma in situ), Pap 5 (invasive carcinoma).

Sample processing for DNA analysis
The samples were vigorously stirred and centrifuged at 4000 x g for 10 min at room temperature. The cell pellet was resuspended in 500 μl of detergent buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.45% Triton X100, 0.45% Tween 20) and digested with 100μg/ml proteinase K (Promega) for 2h at 56°C (26). The proteinase was inactivated by incubation at 95°C for 10 minutes. The processed samples were stored at -20°C until analysis was performed. 10μl of processed samples was used in each PCR assay.

HPV DNA detection and genotyping by PCR
Each sample was subjected to 6 parallel PCR reactions using the following primer sets: consensus primers MY09/MY11 and type-specific primers for HPV16, 18 (L1 and E6 gene fragments), 31 and 33 (15, 28). All oligonucleotide primers were synthesized by LKB Vertriebs GmbH. The final 50μl PCR mixture contained 10-μl sample, 25 μl PCR Master Mix (Promega), 3 mM MgCl₂, 20 pmol of each primer. Amplifications were performed with the following cycling profile: incubation at 94°C for 5 min followed by 40 cycles of 1-min denaturation at 95°C, 1-min annealing at 55°C, and 1-min elongation at 72°C. The last cycle was followed by a final extension of 10 min at 72°C.

All specimens were also amplified with β-globin primers PC04 and GH20 to check the DNA quality. During amplification positive and negative control samples were included. As positive controls were used: CaSki cell DNA (HPV16 positive); HeLa cell DNA (HPV18 positive); HPV31 and HPV33 positive samples obtained from the Faculty of Medicine at TU Dresden.

PCR products were analysed on a 2% agarose gel stained with ethidium bromide and visualized by UV-transillumination.

Results and Discussion
Cervical smear test and testing for HPV DNA were performed 3-6 months after treatment by conization for high-grade CIN. The mean age of the women was 35 years (range 27-61 years). Cytological abnormalities were found in 11 women (18.6%). Of them 7 women were diagnosed with Pap 3D and 4 women- with Pap 3a (atypical cells of undetermined significance).

PCR analysis using MY09/11 general primers detected HPV in cervical samples of 14 women, representing 23.7% of all women. Fig. 1 shows amplification of 450 bp fragment in HPV positive material. All except one of the women with posttreatment cytological abnormalities were HPV positive. Four women with normal Pap tests were also HPV positive.

PCR was also used for genotyping to detect the high risk HPV DNA types 16, 18, 31 and 33. The type specific PCRs amplified fragments of 405bp and 216 bp in HPV 18 positive sample, 151 bp and 514 bp in HPV16, and HPV31 positive cervical samples, respectively (Fig. 1). The most common HPV type was HPV16 - found in 8 women with cervical abnormalities and in 1 woman with normal Pap test. Seven women were positive for HPV31, of them 4 women were with abnormalities and 3 with normal Pap tests. Although we used two primer pairs, targeted L1 and E6 gene fragments, we were able to detect HPV18 only in one woman with posttreatment cytological abnormalities. We were not able to find any specimens positive for HPV33. Three women with abnormal Pap tests were infected with both HPV16 and HPV31. The HPV status according to the cytological diagnosis is detailed in Table 1. Our results show that most women (48/59) treated by conization for high-grade CIN have normal cytology indicating that the treatment was successful. Only 11 cases were considered treatment failures. Failure rates between 5% and 15% have been reported by other authors (1, 2, 5, 11, 16, 29). The rate of treatment failure in our study is higher (18.6%). However, in our case 4 of women had Pap 3a (atypical cells of undetermined significance) cytology and further verification is needed in order to rule out false positive results.
According to the literature incomplete removal of the CIN lesion, development of a new CIN lesion due to the HPV persistence and the revival of occult HPV infections could be a possible explanation (6, 20). Moreover, in addition to HPV persistence during follow-up after conization (22) a significant relationship was also found between high HPV load before treatment and recurrent disease (17, 24).

<table>
<thead>
<tr>
<th>Cytology</th>
<th>HPV</th>
<th>HPV16</th>
<th>HPV18</th>
<th>HPV31</th>
<th>HPV33</th>
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<td>Pap test</td>
<td></td>
<td></td>
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<tr>
<td>Normal cytology</td>
<td>4</td>
<td>1</td>
<td>-</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>(n=48; 8.3%)</td>
<td>(n=4; 25%)</td>
<td></td>
<td></td>
<td>(n=4; 75%)</td>
<td></td>
</tr>
<tr>
<td>Abnormal cytology</td>
<td>10</td>
<td>8</td>
<td>1</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>(n=10; 80%)</td>
<td>(n=10; 10%)</td>
<td>1</td>
<td>(n=10; 40%)</td>
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<tr>
<td>All</td>
<td>14</td>
<td>9</td>
<td>1</td>
<td>7</td>
<td>-</td>
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<tr>
<td>(n=59; 23.7%)</td>
<td>(n=14; 64.3%)</td>
<td>1</td>
<td>(n=14; 7.1%)</td>
<td>(n14; =50%)</td>
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</table>

It is assumed that successful treatment for CIN is associated with HPV clearance (25). There are many studies on the clearance of HPV infection after CIN treatment, however the results are quite variable. Dannecker et al. reported the results of an meta-analysis of the HPV persistence after treatment of CIN (8). Most of the studies indicated that HPV disappears or an extremely low HPV persistence is found after successful CIN treatment. A systematic review of studies concerning HPV DNA testing in the follow-up period after treatment for cervical intraepithelial neoplasia indicates that among women in whom the treatment was considered to be successful, 84.2% had a negative posttreatment HPV DNA test and 15.8% a positive one. The corresponding rates for cases with treatment failures were 17.2% and 82.8%, respectively(21). Furthermore, in a study Verguts et al reported that all women with recurrent CIN were high risk HPV positive (29).

In our case 23.7% of all women had a positive HPV test, indicating that in most cases treatment resulted in eradication of HPV. However, in women with posttreatment abnormal Pap test this percentage is much higher (approx. 91%). This indicates that HPV was a significant risk factor for the recurrent cytological abnormality.

HPV type 16 infection has been found to increase the risk of recurrence after treatment for CIN (10). It was shown that the 2-year risk associated with HPV 16 positivity after treatment was 37.0%, which was significantly higher than for other carcinogenic HPV types (10.8%), noncarcinogenic types (1.5%), or testing HPV negative (0%) (13). We confirmed that the type of HPV, specifically HPV type 16, is very important factor for the recurrent cervical abnormalities after CIN treatment. In our study HPV16 was the most prevalent genotype accounting for 80% of HPV positive specimens with abnormal cytology. It should be taken into account, however, that HPV testing was not done before conization and therefore a possible role of reinfection after treatment cannot be excluded. In addition, our findings must be interpreted with caution due to the limited sample size.

**Conclusions**

Our study confirms the role of the posttreatment high risk HPV infection, especially HPV type 16 infection, as a risk factor for subsequent cervical dysplasia and the relevance of HPV testing for predicting the risk of disease recurrence.

**Acknowledgments**

This work was supported by the Lithuanian Science Council (grant No. AUT-16/2010) and the Agency for Science, Innovations and Technology (grant No. 31V-116).

**REFERENCES**