SIGNIFICANCE OF MOLECULAR DIAGNOSTICS IN HUMAN PAPILLOMA VIRUS (HPV) DETERMINATION

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Abstract - HPV infection is considered to be the most important etiologic factor in cervical cancer development. In this retrospective study, which included the period from 2000 to 2012, the results of two molecular techniques used in the detection of HPV infection among women of the South Bačka District were analyzed. By using the technique of in situ hybridization and the rPCR method, the proportion of high-risk HPV among women with normal cytology was determined to be 19.8% and 32.7%, respectively, and among women with abnormal cytology was determined to be 43.1% and 61%, respectively. Among the analyzed women, HPV type 16 was the most prevalent, followed by HPV types 31, 51 and 18. Application of molecular HPV diagnosis is valuable because it increases the sensitivity of the screening test, so that the application of both tests to detect cervical cancer is a true prevention of malignancy.

Key words: HPV, in situ hybridization, rPCR method.

Abbreviations: HPV – Human papillomavirus; PCR – polymerase chain reaction; rPCR – real-time polymerase chain reaction; HR-HPV – high risk human papillomavirus; LR-HPV – low risk human papillomavirus; LSIL – low-grade squamous intraepithelial lesion; HSIL – high-grade squamous intraepithelial lesion; CIN – cervical intraepithelial neoplasia

INTRODUCTION

Human papillomavirus (HPV) infections are the most common sexually transmitted diseases in the world. It is estimated that approximately 75-80% of sexually active people throughout their lives are exposed to infection by the HPV virus, the spread of which is not possible to prevent for the time being (Bosh et al., 2007).

Human papillomavirus (HPV) causes a variety of benign tumors and some types are associated with malignant disease in humans. HPV infection is considered to be the most important etiologic factor in cervical cancer development. The presence of HPV was detected in over 99% of cases of invasive cancer. The established virus is carcinogenic to humans and cervical cancer is considered a sexually transmitted disease (IARC, 1995).

Molecular methods have enabled the study of HPV and contributed to the development of diagnostic tests to confirm its presence. Hybridization methods were the first molecular methods for the detection of HPV, and have been in use since the 1980s (Hubbard, 2003). In situ hybridization, South-
ern blot, dot blot and digene hybrid capture differ in terms of performance, as well as sensitivity and specificity. Hybridization techniques are applicable in many laboratories because they do not require a separate space or expensive equipment to run (Villa at al., 2006.).

In the early 1990s, the PCR (polymerase chain reaction) was introduced and has been refined (Kroupis et al., 2011). DNA synthesis is catalyzed by thermostable DNA polymerase. Group-specific consensus primers MY9/MY11 and GP5+/GP6+ amplify part of the L1 gene allowing the reproduction of different genotypes in a single reaction (van den Brule et al., 2002). This test is necessary for further determination of the HPV genotype. The disadvantages of classical PCR techniques are overcome by rPCR that combines the classical technique with simultaneous PCR detection of amplified sequences by fluorescent technology. A large number of copies generated in real-time PCR reactions accumulate in microgram quantities that can be easily detected by the intensity of fluorescence emission (Molijin et al., 2005).

The cytological screening program is the most widely used method in the detection of cervical cancer. It is applied in developed and developing countries. The introduction of HPV diagnosis tests increases the sensitivity of cytological screening as reflected in the reduction of morbidity and mortality from this disease (Origoni et al., 2012).

The aim of the study was to assess whether different molecular techniques show the presence of high- and low-risk types of HPV among women from the South Bačka District in the period of one decade.

MATERIALS AND METHODS

A retrospective study was conducted at the Institute of Public Health of Vojvodina, Center for Virology, over ten years, from 2000 to 2012 (between 2001 and 2003 diagnostic methods were not carried out). The sample consisted of 842 women (19 to above 50 years old) with normal and abnormal cytology smears. Materials (endocervical swabs) for HPV diagnosis were sent by gynecologists from the Health Care of South Bačka District, Department of Gynecology and Obstetrics, Clinical Center of Vojvodina, and the Department of Gynecology, Oncology Institute of Vojvodina.

The technique of in situ hybridization was used on 111 bioptic samples and 275 endocervical swabs obtained from patients. In situ hybridization was performed using the Rembrandt In Situ Hybridization-Detection Kit, (Pan Path, Netherlands) for the detection of specific HPV types. It included a specific probe for the identification of HPV types 6 and 11, 16 and 18, 31 and 33. Tissue sections and endocervical swabs were placed on original glass slides coated with 3-aminopropyltriethoxysilane. The test was performed according to the manufacturer’s instructions.

Using real-time PCR, 487 endocervical swabs were analyzed. The swabs were collected in commercial transport media (Copan) and stored at -20° C until processed. HPV DNA isolation protocol was carried out according to the manufacturer’s instructions (Kit for the extraction of DNA, Sorb A, Sacace, Italy). In order to control the amplification of nucleic acid, 10 μl of internal control (β-globin) was added during isolation. From the endocervical swabs, 12 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) were determined with a HPV High Risk Typing Real-TM” kit (Sacace, Italy) and two low-risk HPV types, 6 and 11, with HPV 6/11 Real TM (Sacace, Italy). The process of amplification was performed in 45 cycles under the following conditions: 15 min at 95°C, 20 s at 95°C and 60 s at 60°C. The process of amplification is performed in 42 cycles under the following conditions: 15 min at 95°C, 20 s at 95°C and 60 s at 60°C.

Statistical analysis was performed with the \( \chi^2 \) test.

RESULTS

The presence of genital HPV infection was proven
SIGNIFICANCE OF MOLECULAR DIAGNOSTICS IN HUMAN PAPILLOMA VIRUS (HPV) DETERMINATION

Table 1. Frequency of HR-HPV and LR-HPV among women with different cytological findings

<table>
<thead>
<tr>
<th>Samples and methods</th>
<th>In situ hybridization technique</th>
<th>rPCR method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR-HPV</td>
<td>LR-HPV</td>
</tr>
<tr>
<td>Women with normal cervical smear</td>
<td>19.8%</td>
<td>18.3%</td>
</tr>
<tr>
<td>Women with abnormal cervical smear</td>
<td>43.1%</td>
<td>25.1%</td>
</tr>
</tbody>
</table>

Table 2. Results of HPV test and histopathological findings in biopsy

<table>
<thead>
<tr>
<th>Histological test</th>
<th>Number of biopsy</th>
<th>HPV positivity</th>
<th>HR-HPV</th>
<th>LR-HPV</th>
<th>Multiplex infections</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN 1</td>
<td>11</td>
<td>7 (63.6%)</td>
<td>5(63.6%)</td>
<td>1(9.0%)</td>
<td>3 (27.3%)</td>
</tr>
<tr>
<td>Basal hyperplasia</td>
<td>2</td>
<td>2 (100%)</td>
<td>2 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Genital warts</td>
<td>15</td>
<td>15 (100%)</td>
<td>5(33.3%)</td>
<td>6(40.0%)</td>
<td>4(26.6%)</td>
</tr>
<tr>
<td>CIN 2</td>
<td>10</td>
<td>8 (80%)</td>
<td>7(70.0%)</td>
<td>0 (0%)</td>
<td>1 (10.0%)</td>
</tr>
<tr>
<td>CIN 3</td>
<td>15</td>
<td>12 (80%)</td>
<td>9(60.0%)</td>
<td>0 (0%)</td>
<td>3 (20.0%)</td>
</tr>
<tr>
<td>Carcinoma in situ</td>
<td>1</td>
<td>1 (100%)</td>
<td>1 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

Fig. 1. Presence of genital HPV infection among women from the South Bačka District from 2000 to 2012 by two molecular methods

by different molecular methods in women from the South Bačka District (Fig. 1). In situ hybridization and rPCR revealed that high-risk HPV were present in 19.8% and 32.7% of women with normal cytology, respectively, and in 43.1% and 61% of women with abnormal cytology, respectively. Diagnosis of high-risk HPV types was statistically more frequently determined by the rPCR method ($\chi^2=6.05; p<0.05$), while diagnosis of low-risk HPV was significantly more frequently determined by in situ hybridization ($\chi^2=22.36; p<0.05$). Multiple HPV infections with HR-HPV and LR-HPV were statistically significantly more often observed by the rPCR method ($\chi^2=22.72; p < 0.05$) (Table 1).

Histological examination of biopsy and HPV diagnosis was performed in 111 patients. In 28 (25.2%), histologically analyzed biopsies were confirmed by the presence of low-risk lesions (L-SIL) relating to CIN1, genital warts and basal hyperplasia. High-risk lesions (H-SIL) related to CIN2, CIN3 and Ca in situ were found in 26 (23.4%) biopsies. Table 2 shows the results of histological examination in correlation to HPV test.

The results of rPCR and in situ hybridization used to examine the presence of genital HPV infection in different age groups are shown in Fig. 2. According to the results obtained by both methods, genital HPV infections were the most frequent in the women under 30 years of age. In older age groups, the percentage of HPV infections was lower.

Fig. 3 shows the presence of certain types of high-risk HPV as observed by the rPCR method
in endocervical swabs obtained from 487 women from the South Bačka District. The most prevalent type was HPV 16, found in about 79 (27.1%) HPV-positive patients. This was followed by types HPV31 (23.7%), HPV 18 (11.3%), HPV 51 (10.6%), HPV 58 (7.9%) and HPV 52 (7.2%). Other tested types (HPV 33, 59, 56, 45, 35, 39) were demonstrated in low percentages, i.e. less than 5%. In the group of patients younger than 30 years, the dominant types were HPV 16, 31, 18, 51 and 58. The same distribution of HPV types was also found in the age group of 30-39 years. Among women older than 40, HPV type 16 was dominant, followed by HPV51, and HPV 58.

DISCUSSION

In our retrospective study for the period from 2000 to 2012, the results of two molecular techniques used in the detection of HPV infection among women from the South Bačka District were analyzed. Since the association of HPV infection to cervical cancer and other cancers is well known, molecular diagnosis is justified in making an accurate diagnosis. Molecular methods for the detection of HPV infections at the Institute of Public Health of Vojvodina have been applied since 1999/2000 (Milošević, 2001). The first method was in situ hybridization, which was used from 2000 to 2011 to analyze 111 samples of biopsy and 275 endocervical smears. The method revealed that the proportion of HPV among women from the South Bačka District was 51% among women with normal cytology, and 38% among women with abnormal cytology. Results found were similar to previous results where the same diagnostic methods revealed that in a sample of women from Montenegro with symptoms of cervicitis, HPV infection was found in 44.3% of the cases (Mijović et al., 2002). In the project Decena that was conducted during one year, the frequency of
HPV infection was established to be 16% by Hybrid Capture methods (Rajović et al., 2007). A number of hybridization techniques cited in the literature differ in sensitivity and specificity (Hubbard, 2003; Kelesidis et al., 2011; Villa et al., 2006). Many authors have pointed out that the method of in situ hybridization can confirm the presence of specific HPV genotypes. It enables visual inspection of the cells and determines the localization of infection. This method allows the analysis of samples fixed in paraffin (Schlecht et al., 2011). Information on histological details in combination with HPV diagnosis can exclude unnecessary invasive treatments, especially in young women. The requirement for in situ hybridization to be positive is the presence of 5 000 viral genome copies per milliliters (Coutlee et al., 1997).

The polymerase chain reaction (PCR) has become a useful method in molecular research, and at the beginning of 2012 it was introduced to the Institute of Public Health of Vojvodina. Real-time PCR revealed that the proportion of HPV among women from the South Bačka District during a one-year period was 59.8%. Many authors have shown interest in comparing the results obtained by PCR and hybridization (Biedermann et al., 2004; Kelesitis, 2011). In situ hybridization and rPCR, respectively, revealed that 19.8% and 32.7% women with normal cytology and 43.1% and 61% women with abnormal cytology had HPV infections of high-risk types. A higher percentage of multiple infections in the tested groups was detected by the rPCR method. A significantly higher percentage of HPV-positive cases of high risk types and multiple infections were found among patients tested by PCR. This was due to the ability of PCR to detect more HPV genotypes and its greater sensitivity. These findings support the results of most comparative studies, which show the greater sensitivity of PCR as compared to in situ hybridization (Biedermann et al., 2004). There are studies that show equal sensitivity of these two methods in the case of severe intraepithelial changes such as occur in CIN2/3 and carcinoma (Kelesitis 2011). The results of our study reveal the higher sensitivity of PCR in detecting high-risk HPV types. This is due to the availability of appropriate primers and fluorescent probes for their detection. However, the method of in situ hybridization has proven to be better at detecting low-risk HPV in benign lesions, such as genital warts, which have a large number of viral particles and are highly infectious. They are indicators of sexual promiscuity (Mayeaux, 2008).

It was observed that among women from the South Bačka District, high-risk types of HPV were prevalent compared to low-risk types. Genital HPV infection is the most common in the population of young women under 30 years of age. According to our results, the peak of HPV infection was in the early twenties, and after that its prevalence decreases, as was also shown by others (Bruni et al., 2011). Similar to other populations of women from the South Bačka District, HPV type 16 was the most prevalent, followed by HPV types 31, 51, 18. The previously described distribution of HPV genotypes was characteristic for women up to the age of 40. In older age groups, beside HPV 16, genotypes HPV 51 and 58 were also present at a high percentage. These data are similar to the results provided by the multicenter study for the region of Eastern Europe (WHO/ICO, 2010). Data on the presence of high and low oncogenic risk HPV genotypes in different age groups is important for the development of prevention programs for malignant tumors, as well as for vaccination programs.

CONCLUSION

Screening by the Papanicolaou method is the test of choice for the detection of cervical cancer in many countries. Application of molecular HPV diagnostics is valuable because it increases the sensitivity of the screening test. The application of both tests to detect cervical cancer is a great improvement in the prevention of malignancy.

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