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High-risk human papilloma virus genotypes in cervical carcinoma of Serbian women: Distribution and association with pathohistological findings

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ABSTRACT

A significant role of high-risk Human papilloma viruses (HR HPV) in the development of cervical carcinoma is well known. HR HPV 16 and 18 account for approximately 70% of all cases of cervical cancer worldwide. The incidence of cervical cancer in Serbia, is one of the highest in Europe. The aim of our study was to investigate the distribution of HR HPV types in cervical carcinoma of Serbian women, as well as association between the HPV types and pathohistological findings. The study included 80 archival cervical cancer tissues from the same number of patients. The presence of HPV DNA was determined using MY09/MY11 primers for L1 gene and GP1/GP2 primers for E1 gene. HPV was detected in 78.75% tissues. HR HPV genotypes found in the decreasing order of frequency were: HPV16 (80.39%), HPV33 (7.84%), HPV58 (5.88%), HPV18 (1.96%), HPV45 (1.96%) and HPV53 (1.96%). The examined tissues were 91.25% squamous cell carcinomas and 8.75% adenocarcinoma. The high frequency of HPV 16 was observed in both types of carcinoma (80.8% and 75%, respectively) while the prevalence of HPV18 was low. These results may contribute to the implementation of cervical carcinoma prevention program in Serbia, including the selection of the most appropriate vaccine and immunization program.
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1. Introduction

Human papilloma viruses (HPV) are species specific viruses, classified into five papillomavirus genera (*Alpha*-, *Beta*-, *Gamma*-, *Mu*- and *Nupapillomavirus*) [1,2]. According to the differences in the viral genome, HPV type is defined if the nucleotide sequence of L1 gene differs by more than 10% when compared with the closest known HPV type, differences between 2% and 10% define a subtype and less than 2% a variant [3].

A significant role of HPV in the development of cervical carcinoma is very well document [3,4,5]. The majority of HPV infections with both high-risk and low-risk types are asymptomatic. Due to the host immune response these infections resolve spontaneously in most cases. However, in rare instances, HPV infection persists. If persistent infection with high-risk types is not detected and treated, it may progress towards premalignant squamous intraepithelial lesions. The process of malignant progression usually takes at least ten years. Since high-risk HPV types are detected in all cervical cancers, it is generally considered that the persistent infection is one of the prerequisites for the development of cancer [4].

More than 200 types of HPV have been discovered and are generally classified as high-risk (HR) or low-risk (LR) types on the basis of their oncogenicity and association with cervical carcinoma. According to the International Agency for Research on Cancer (IARC), 12 different HR HPV types are classified as carcinogenic to humans: types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59. These types belong to a several phylogenetically related species 5, 6, 7, 9 and 11 of the *Alphapapillomavirus* genus [6,7].

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Among these high-risk types, HPV 16 and 18 account for approximately 70% of all cases of cervical cancer worldwide [8].

Cervical cancer is the fourth most common cancer in women, with an estimated 527,624 new cases and 265,653 deaths in 2012, as stated by WHO [9]. It is also one of the leading cancer mortality causes among the female population in Serbia. According to the WHO estimates, the incidence of cervical cancer in Serbia, is one of the highest in Europe with age standardised rate of 23.8 per 100.000 [10]. From 2003 to 2009, the incidence rates were 21.6–27.1 per 100.000 according to the Cancer Registry of Serbia [11]. More recent data for 2013 shows that the age standardised rate of 20.3 per 100.000 [12].

The data on HPV genotype distribution in cervical cancer in Serbia are scarce. So, the aim of this study was to investigate the distribution of HPV types in cervical carcinoma tissues of Serbian women, and to correlate the distribution of HPV types with pathohistological findings of cervical carcinomas. It is expected that the results obtained will contribute to the implementation of a cervical cancer prevention program in Serbia and help in the selection of the most appropriate available HPV vaccines.

2. Methods

The protocol of the study was reviewed and approved by the Ethical Committee, School of Medicine, University of Belgrade, decision number 29/III-19.

2.1. Tissue samples

During 2010–2012, a total of 80 formalin-fixed, paraffin embedded (FFPE) tissue samples of cervical carcinoma from the same number of patients were collected by the University Clinic of Gynecology and Obstetrics "Narodni Front", Belgrade, and Clinical Hospital Center "Zemun", Belgrade. These specimens were analysed in 2014 and 2015. General information about the age of each patient was obtained from medical records. Pathohistological type of cervical carcinoma was determined according to World Health Organization (WHO) criteria, and staging was determined according to the protocol of International Federation of Gynecology and Obstetrics (FIGO) [13,14].

2.2. HPV detection

From each paraffin tissue block, five to seven 5 μm serial sections were cut and collected for DNA extraction. In order to avoid cross-contamination, microtome blade was carefully cleaned with xylene after cutting each tissue block. Prior DNA extraction, deparaffinisation of samples with xylene and 96% ethanol was done. DNA extraction from FFPE samples was performed using QIAamp DNA Mini Kit (QIAGEN Inc., USA) according to the manufacturer's protocol.

The amplification of HPV DNA L1 and E1 gene was done with two sets of primers, MY09/MY11 and GP1/GP2, respectively [15,16]. PCRs were performed in a 25 μL volume reaction mix containing QiagenTaq PCR Master Mix-250U (QIAGEN Inc., USA), μmol of each primer and 5 μL of extracted DNA. PCR protocol for the detection of L1 gene comprised of initial denaturation at 95 °C for 5 min, followed by 40 cycles of 30 s at 94 °C, 30 s at 58 °C, 1 min at 72 °C and a final elongation of 20 min at 72 °C [15]. The protocol for the detection of E1 gene comprised of initial denaturation at 85 °C for 5 min, followed by 40 cycles of 1 min at 94 °C, 1 min at 50 °C, 90 s at 72 °C and a final elongation of 10 min at 72 °C [16]. The presence of specific 450 bp band for L1 gene and 450 bp band for E1 gene detected by agarose gel electrophoresis with ethidium bromide staining was considered as a positive result.

2.3. HPV genotyping

The identification of HPV genotypes was performed by the direct DNA sequencing method. HPV-positive samples were purified by QIAGEN MinElute PCR Purification Kit (QIAGEN Inc., USA) according to the manufacturer's protocol. The purified PCR products were subsequently sequenced with Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems, USA) using PCR primers as sequencing primers. Sequencing reactions were analyzed on the ABI Prism 310 Genetic Analyzer. The obtained nucleotide sequences were analysed using Sequence Analysis 5.1 software. HPV genotypes were determined by comparison with HPV reference strains and documented virus sequences available in the GeneBank database using BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/). The nucleotide sequence was assigned to an HPV type if it corresponded in more than 95% in 350–400 bp with a known HPV genotype.

2.4. Statistical analysis

Descriptive statistics and Fisher's exact test were used for statistical analysis. Analysis was performed in SPSS v.23 (SPSS Inc., USA) software. Differences with a p-value of <0.05 were considered to be significant.

3. Results

The presence of HPV DNA was detected in 63 (78.75%) cervical carcinoma tissue samples by PCR method. HPV genotypes were identified in 51 samples, while in 12 samples genotypes were not identified due to the appearance of numerous ambiguous or overlapping peaks in the DNA sequencing tracings. Genotyping revealed the presence of 6 HR HPV types: 16, 33, 58, 45, 18 and 53. The most prevalent type was HPV 16, found in 41 cervical carcinoma tissues (80.39%). Types 33 and 52 were identified in 7.84% and 5.88% samples, respectively. All other types were detected at frequencies ≤2% (Table 1).

FIGO staging of cervical carcinoma tissues with identified HR HPV types showed that most of the samples were classified as Stage I (36 out of 51). Statistically significant association between the distribution of HR HPV types and stage of the disease was not found (Table 2). Women were stratified by age (≤40 and >40years) and the majority of women whose samples were examined in this study were in the group >40 years (78.75%). Although, more HR HPV types were identified in the group >40years, the statistical significance was not found (Table 2).

The histology of the 80 FFPE cervical carcinoma tissues identified 73 (91.25%) tumors as squamous cell carcinoma (SCC) and 7 (8.75%) as adenocarcinoma (ADC). HR HPV types were identified in 47 SCC of and 4 ADC samples. HPV 16 was identified in 80.8% of SCC tissues and 75% of ADC tissues. Other HR HPV types were identified in higher frequency in the SCC tissues, however statistical significant association between the distribution of HR HPV types in SCC and ADC samples was not found (Table 2).

Table 1
HPV genotypes distribution in cervical carcinoma tissues.

HPV type	Phylogenetic group	n	%
16	Alpha-papillomavirus 9	41	80.39
33	Alpha-papillomavirus 9	4	7.84
58	Alpha-papillomavirus 9	3	5.88
45	Alpha-papillomavirus 7	1	1.96
18	Alpha-papillomavirus 7	1	1.96
53	Alpha-papillomavirus 6	1	1.96
Total		51	100.00

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