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A variant of human papillomavirus (HPV) type 66 is common among HPV-infected women from the Republic of Macedonia.

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ABSTRACT

Epidemiological and molecular studies have implicated the human papilloma virus (HPV) as the main risk factor for the development of cervical intraepithelial neoplasia (CIN) and cervical cancer. Currently, 45 HPV types are known to infect the genital tract, of which at least 10 are associated with cancer. For many new and rare HPV types the oncogenic potential is still unknown, due to the limited number of reports concerning their association with particular dysplastic and neoplastic lesions.

Here, we present the prevalence, association with cytological and histological cervical lesions and sequence variations of a variant of HPV 66 among Macedonian woman. Fifteen women or ~7.9% of the 190 HPV positive women in whom the HPV type was determined, were infected with the HPV 66 variant. Thus, this variant is the third most common type, after HPV 16 (~28.4%), and HPV 31 (12.1%), among Macedonian women. The DNA sequence analysis of the L1 region showed less than 2% difference from the published HPV 66 sequence, thus confirming that this virus is a variant of HPV type 66.

The results from the cytological and/or histological analyses showed that the HPV 66 was present in two women with normal cervical findings, one with non-specific chronic cervical inflammation, seven with low-grade squamous intraepithelial lesions (SIL) and five with high-grade SIL.

In conclusion, a variant of HPV 66 is a relatively common intermediate risk HPV type among Macedonian women with cervical abnormalities.

Key words: human papilloma virus (HPV), HPV 66 variant, cervical lesions, squamous intraepithelial lesions (SIL)

INTRODUCTION

Epidemiological and molecular studies have implicated the HPV as the main risk factor for the development of CIN and cervical cancer [1,2]. Persistent infection with high-risk HPV types and high viral load confer an increased risk for persistent or progressing CIN [3,4]. HPV testing, microbicidal agents that kill HPV and vaccines to protect against HPV are new strategies to detect and prevent cervical cancer. HPV testing has been suggested as a supplement to cytology (Papanicolau smears) in cervical cancer screening programs [5-7]. However, the management of the patients depending on HPV type identification requires characterization of the oncogenic potential of every individual HPV type.

At present there are 45 different HPV types known to infect the genital tract [8]. Papilloma viruses are defined by genomic sequence similarities [9]. An HPV genome is defined as a new type if it differs by more than 10% in its nucleotide sequence compared with other known types in the L1 open reading frame. HPVs that differ by 0 to 2% in their nucleotide sequence compared with the reference sequence of known HPV types are referred to as variants, and those that differ by 2 to 10% are referred to as subtypes. Based on their presence in normal, premalignant or malignant lesions, the genital HPV types have been classified into three groups: high-risk, intermediate-risk and low-risk [10]. HPV types 16 and 18, as well as some less prevalent HPV types, such as HPVs 45 and 56, belong to the group of high-risk HPV types due to their frequent detection in genital cancer. HPVs 31, 33, 35, 52, 58 and some others, are usually referred to as HPVs with an intermediate risk for tumor induction since they are more frequently detected in high-grade SIL than in cancers. HPVs 6 and 11, that are responsible for the great majority of condylomatous lesions and are very rarely found in genital cancers, and some rare HPV types, such as 42, 43 and 44, are considered as low-risk HPV types. For many new and rare HPV types, the oncogenic potential is still unknown due to the limited number of reports concerning their association with particular dysplastic or neoplastic lesions.

HPV testing in the Republic of Macedonia was initiated in 1998 with the aim of determining the distribution of HPV types among Macedonian women with different cervical abnormalities, and to provide additional information for accurate decision-making in the treatment of cervical lesions.

Here we present the prevalence of a variant of the HPV type 66 in the Republic of Macedonia, the sequence variations of this HPV type among our patients and the association of HPV 66 with different cytological/ histological cervical lesions.

MATERIALS AND METHODS

Materials. Since 1998, a total of 800 women, attending the Out-Patient Clinic of the Department of Gynecology and Obstetrics, Skopje, Republic of Macedonia, for routine gynecological check-ups, were tested for the presence of HPV. Informed consent was obtained from all patients. Clinical samples were taken by scraping the endo- and exo-cervix with a cytology brush. The specimens were smeared onto a slide, fixed with

absolute ethanol, air-dried and sent to the laboratory for HPV testing. Fifteen women who showed the presence of a variant of HPV 66 were included in this study.

Methods. For routine HPV testing,DNA was isolated using the proteinase K digestion-phenol/chloroform extraction-ethanol precipitation method [11]; a negative control was included for every DNA isolation. HPV was detected by polymerase chain reaction (PCR) analysis using the MY09/MY11 consensus primers located in the L1 region of the virus [12]. The DNA quality of each specimen was ascertained by coamplification of b-globin gene fragment using the GH20/PC04 primers. AmpliTaq Gold polymerase (Applied BioSystems, Foster City, CA, USA) was used for all PC reactions. Positive and negative controls were included for every amplification. Aliquots of the PCR were run on a 1.5% agarose gel and analyzed under UV light following ethidium bromide staining. To confirm the specificity and to increase sensitivity, the PCR fragments were transferred to a nylon membrane and hybridized to a DIG-ddUTP labeled HPV group specific oligonucleotide probes (GP5+/GP6+). The detection was performed with Anti-DIG/AP conjugate and CDP* Star (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire, UK) chemilluminiscence autoradiography. Samples with high viral load were typed by restriction fragment length polymorphism (RFLP) analysis of PCR amplified MY09/MY11 fragments using *Bam*HI, *Dde*I, *Hae*III, *Hin*fI, *Pst*I, *Rsa*I and *Sau*3 AI restriction enzymes [13], while samples with low viral load were typed by dot-blot hybridization with nine type- specific oligonucleotide probes (HPV types 6, 11, 16, 18, 31, 33, 39, 58 and 66). The nucleotide sequence of the MY09/ MY11 PCR fragment of the HPV 66 variant was determined by fluorescent cycle sequencing on ABI PRISMO 310 (Applied BioSystems).

RESULTS AND DISCUSSION

From a total of 800 analyzed samples, 290 tested positive for the presence of the HPV infection. The type of the virus was determined in 190 samples (Table 1), while in 90 samples (29.6%) the HPV type remained unknown due to insufficient material and/or low viral load with uncommon HPV types. The most frequent type was HPV 16 (28.4%), followed by HPV 31 (12.1%), HPV 66var (7.9%), HPV 6 (7.4%), HPV 58 (6.8%), HPV 33 (5.2%) and HPV 18 (4.7%). Thirteen other HPV types (53, CP8304, 11, MM7, LVX160, 56, 61, 39, 54, 62, MM8, MM9 and LVX100) were detected with a frequency of 14.2%, while double and triple HPV infections were detected with a frequency of 13.3%.

The HPV 66 variant was found to be the third most common HPV type among Macedonian women. HPV 66 in our group of patients differs from the published HPV 66 type in one restriction enzyme site (*Rsa*I) and was named the HPV 66var. The 450 bp MY09/ MY11 fragment of the HPV 66 type contains no *Rsa*I restriction enzyme site, while the HPV 66var contains one *Rsa*I site. The *Rsa*I digestion of the 450 bp fragment of HPV 66var generates two fragments of ~380 and ~70 bp (Fig. 1). The nucleotide sequence of the MY09/MY11 DNA fragment of HPV 66var in comparison with the published HPV 66 sequence (GeneBank Acc No U31794) is shown in Figure 2. The DNA sequence analysis of this region showed only eight nucleotide differences between

this variant and the published HPV 66 MY09/MY11 sequence, that is <2%, thus confirming that this virus is a variant of HPV type 66. One of these nucleotide changes creates an *Rsa*I restriction enzyme site at position 72 bp from the 5' end of the MY09/ 11 fragment.

Cytological/histological findings in Macedonian women infected with a variant of HPV type 66 are shown in Table 2. Cervical cytology was performed in all 15 women, and biopsy specimens were taken from eight women. The results from the cytological and/or histological analyses showed that the HPV 66 was present in two women with normal cervical findings, one with nonspecific chronic cervical inflammation, seven with low-grade SIL and five with high-grade SIL.

In conclusion, a variant of HPV 66 is a relatively common type among Macedonian women with cervical abnormalities. It can be considered as an intermediate risk HPV type since it has been detected both in women with low- and high-grade SIL. The fact that some uncommon HPV types are relatively frequent in certain populations, such as HPV 66 among Macedonians, should be taken into consideration when designing an HPV detection protocol.

Table 1. HPV types detected among Macedonian women with cervical abnormalities

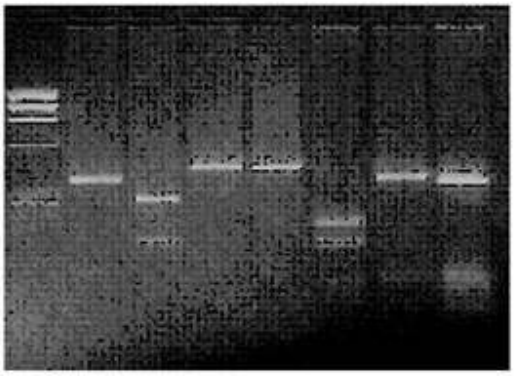
HPV Type	**n**	**%**
16	54	28.4
31	23	12.1
66	15	7.9
6	14	7.4
58	13	6.8
33	10	5.2
18	9	4.7
Other types*	27	14.2
>2 HPV types	25	13.3
Total	**190**	**100.0**

* HPVs 53, CP8304, 11, MM7, LVX160, 56, 61, 39, 54, 62, MM8, MM9 and LVX100.

Table 2. Cytologica/histological findings in women infected with a variant of the HPV type 66

Cytological/Histological Findings	**n**	**%**
Normal cervical findings	2	13.3
Non-specific chronic inflammation	1	6.7
Low-grade SIL	7	46.7
High-grade SIL	5	33.3
Total	**15**	**100.0**

Figure 1. RFLP pattern of the MY09/MY11 PCR fragment of the HPV 66var. L: ladder (DNA molecular weight marker IX; Boehringer Mannheim, Mannheim, Germany); B: *Bam*HI (366 + 83 bp); D: *Dde*I (291 + 158 bp); H: *Hae*III (449 bp); Hi: *Hin*fI (449 bp); P: *Pst*I (207 + 150 +66 +26 bp); R: *Rsa*I (~380 +~70 bp); S: *Sau*AI (366 + 63 + 20 bp).



HPV66	gcacagggcc ataataatgg catatgctgg ggtaatcagg tatttgttac tgttgtggat
HPV66var	gcacagggcc ataataatgg catatgctgg ggtaatcagg tatttgttac tgttgtggat
HPV66	actaccagaa g**c**accaacat gactattaat gcagctaaaa gcacattaac taaatatgat
HPV66var	actaccagaa g**tac**caacat gactattaat gcagctaaaa gcacattaac taaatatgat
***Rsa*I**	
HPV66	gc**c**cgtgaaa tcaatcaata ccttcgccat gtggaggaat atgaactaca gtttgtgttt
HPV66var	gc**a**cgtgaaa tcaatcaata ccttcgccat gtggaggaat atgaactaca gtttgtgttt
HPV66	caactttgta aaataacctt aactgcagaa gttatggcat atttgcataa tatgaataat
HPV66var	caactttgta aaataacctt aactgcagaa gttatggcat atttgcataa tatgaataat
HPV66	actttattag acgattggaa **t**attgg**c**tt**a** tccccaccag ttgcaactag cttagaggat
HPV66var	actttattag acgattggaa **c**attgg**a**tt**g** tccccaccag ttgcaactag cttagaggat
HPV66	aaatataggt atattaaaag cacagctatt acatgtca**g**a gggaacagcc ccctgcagaa
HPV66var	aaatataggt atattaaaag cacagctatt acatgtca**a**a gggaacagcc ccctgcagaa
HPV66	aagcaggatc ccctggctaa atataagttt tggga**a**gtta atttacagga cagcttttct
HPV66var	aagcaggatc ccctggctaa atataagttt tggga**g**gtta atttacagga cagcttttct
HPV66	gcagacctgg atcagtttcc tttgggtag
HPV66var	gcagacct**a**g atcagtttcc tttgggtag

Figure 2. Nucleotide sequence of the MY09/MY11 DNA fragment of the HPV 66var compared to the published HPV 66 sequence.

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