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Human Papiloma Viruses Detection in Adolescent Women with Abnormaln Cytology in Croatia

Utvrdjivanje humanih papiloma virusa sa sa abnormlnim citološkim nalazom kod adolescentkinja u Hrvatskoj

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Summary The association between certain human papillomaviruses (HPV) and cervical intraepithelial neoplasia (CIN) is well documented, but still unknown among Croatian adolescent women. Women between the age of 15 and 20 with cytomorphologically abnormal smears (CIN I-IV) were tested for the presence of HPV. Consensus and specific primers were used in the polymerase chain reaction (PCR to detect the most common types: 6, 11, 16, 18, 31 and 33. Beside low-risk HPV 6/11 (25.8%) the most frequently observed types were high-risk HPV types 16 (20.2%) and 31 (17.8%). Globally, the HPV positivity rate declines with age. The presence of HPV DNA significantly increased from 35.5 to 61.1% along with the severity of the cervical intraepithelial neoplasia (CIN I-IV). HPV type 6/11 was strong associated with CIN I (33.8%), HPV type 31 with CIN II (22.9%) and HPV type 16 with CIN III (50%).

Key words: Cervical intraepithelial neoplasia, Human papilloma viruses, Adolescents

Sažetak Povezanost određenih humanih papiloma virusa (HPV) i cervikalne intraepitelne neoplazije (CIN) dobro je dokumentirana, no još uvijek nije poznata među hrvatskim adolescentkinjama. Žene u dobi od 15 do 20 godina s abnormalnim citomorfološkim brisom (CIN I-IV) testirane su na prisustvo HPV-a. Konsenzus i posebne početnice korišteni su u lančanoj reakciji polimeraze (PCR za otkrivanje najčešćih tipova: 6, 11, 16, 18, 31 i 33. Osim niskorizičnog HPV-a 6/11 (25,8%), najčešće utvrđeni bili su visokorizični tipovi HPV-a, tip 16 (20,2%) i 31 (17,8%). Uopšteno, stopa pozitivnosti na HPV značajno opada s godinama. Prisutnost HPV DNK znatno se povećala s 35,5 na 61,1% uz ozbiljnost cervikalne intraepitelne neoplazije (CIN I-IV). HPV tip 6/11 snažno je povezan s CIN I (33,8%), HPV tip 31 s CIN II (22,9%) i HPV tip 16 s CIN III (50%).

Ključne reči: Cervikalna intraepitelna neoplazija, Humani papiloma virusi, Adolescenti

Introduction

Increasing evidence from both laboratory (1) and epidemiological (2) studies indicate that sexually transmitted HPV infections may be the leading couse of cervical cancer world-wide (3). This study presents the major risk factor in the development of cervical neoplasia into cervical cancer and the presence of HPV infection in the female genital tract. These findings are of particular concern in Croatia where cervical cancer is the third most common form of cancer among women, surpassed only by breast carcinoma and stomach carcinoma.

Approximately 34 HPV types are assotiated with various lesions in the lower genital tract of women (4,5). They range from histologically benign flat and exophytic condyloma to several stages of cervical intraepithelial neoplasm (CIN), and also include invasive carcinoma (6). The presence of these HPV types is most often first detected by an abnormal Pap smear, however, a normal Pap smear does not prove the absence of HPV infections.

According to the HPV phylogenetic tree (7), the so-called low-risk HPVs, namely types 6,11, and rarely 42, 43 and 44, are most commonly associated with benign or condylomatous lesions on the anogenital tract.

On the orher hand, the high-risk HPVs, namely types 16 and, to a lesser extent, types 18, 45, 56, 31, 33, 35, 51, 52, 58 (7), are primarly associated with CIN (8). The presence of HPV DNA is confirmed for research purposes through different methods of hybridization, Southern blot hybridization being a gold standard. However, the most reliable and commonly used method of specific DNA detection is the polymerase chain reaction (PCR), which has replaced Southern blot hybridization (9). Through the use of the PCR method, this study investigates the presence of different types of HPV in women with cytological indications of HPV infections and pre-malignant lesions.

Material and methods

Population study and data collection. The patient population was made up of consenting women between the age of 15 to 20 with known abnormal cytological cervices.

The enrollment procedure consisted of a personal interview and physical examination which included a pelvic examination with specimen collection. Cotton or brush swabs were used to obtain the endocervical samples, and scraping specimens were collected in 5 ml sterile phosphate-buffer saline. All of the participiants had previously undergone cytological analysis (Pap smear) prior to being sampled for HPV.

DNA preparation. The cervical cell samples were pelleted by low speed centrifugation (3000 g for 10 min), resuspended in 500 mcl lysis buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 7.9, 0.5% SDS), and treated with Rnase (100 mcg/ml) during 1 hour and Proteinase K (100 mcg/ml) overnight at 37°C. DNA was isolated by a phenol extraction followed by one chloroform/isoamyl alcohol (24:1) extraction and ethanol precipitation. The DNA precipitate was then finally resuspended in 50-100 mcl tridistillated sterile water. DNA concetration and quality was determined both spectrophotometrically and by agarose gel electrophoresis (10).

Detection of HPV-DNA. The cervical DNA was tested for the presence of HPV DNA using the general primer PCR (polymerase chain reaction) based method. The amplification reaction included modified consensus primer pair MY09/MY11 (11) by introducing inosine in the most degenerated sites (12). According to internal control the quality of the target DNA and the absence of PCR inhibition, ß-globin-specific primers (13) were used in the multiplex reaction with a HPV consensus-primer-mediated PCR. Type-specific primers for low-risk HPV 6/11 (14), and high-risk HPV 16, 18, 31 and 33 (15) were also used either in the single (HPV18) or multiplex (HPV6/11 with 16, and HPV31 with 33) PCR reactions.

The aforementioned primer pairs were synthesized according to the directions of the manufacturer of the Cyclone Plus DNA synthetiser (MilliGen/Biosearch Division of Millipore). Recombinant plasmid HPV DNA types 6, 11, 16, 18 (kindly provided by Prof H. zur Hausen), 33, 34, 39, 42 (kindly provided by Prof G. Orth) as well as DNA from human cervical carcinoma cell lines containing integrated HPV 16 genome (CaSki, 600 copies per cell; kindly provided by Prof G. Orth) and HPV 18 (HeLa; 20-50 copies per cell) cells were used to evaluate the specificity of the primers. DNA from cell lines without HPV genome (A431; kindly provided by Prof G. Orth) and with no DNA in the PCR reaction was used as negative control.

Results and discussion

All the cervical specimens were examined by PCR using the consensus primer pairs and the specific primer pair for HPV 6/11, 16, 18, 31 and 33. Specimens with negative ß-globin amplification were not taken into consideration for this study. Only 8 DNA samples were ß-globin negative, undetectable spectrophotometrically and on 1% agarose gel subjected to electrophoresis. The remaining 379 DNA samples were ß-globin positive and HPV positive or negative in the multiplex PCR. After that PCR, all 379 samples were subjected to HPV specific primer mediated PCR, i.e.with HPV 18 primer pairs alone, HPV 6/11 and 16 primer pairs in a second reaction and HPV 31 and 33 primer pairs in a third reaction. We tested about 20 samples in a multiplex PCR containing all specific HPV (6711, 16, 18, 31 and 33) primers and discovered that such a system was less sensitive than the previously elaborated specific primers directed PCR. Furthermore, several non- specific PCR products were detected (not shown).

Our study consisted of 78 positive and 216 negative samples with both consensus and specific primer mediated PCR. There were also 62 positive samples with only specific primer mediated PCR. Finally, there were 23 positive samples with consensus primers that were negative with specific primer mediated PCR. These samples were classified as being of uncertain HPV risk type. Our findings indicate the necessity of simultaneously performing consensus and specific primer mediated PCR. The PCR method has proven itself to be a convenient, reliable, rapid confirmation technique for the screening and typing of HPV in cervical scrapes, and thus an excellent tool in the prevention of cervical cancer.

The results of HPV detection are summarised in Table 1. Of the 379 specimens, 163 (43.0%) contained one or more HPV types. As previously mentioned, HPV infection (determined by slot-blot hybridization with digoxigenin labelled HPV type 6, 11, 16 and 18 DNA probes) is prevalent among Croatian women and has reached epidemic proportions (17). Cases involving HPV infection have increased from 4.6% in 1990 to 38.2% in 1993 and, according to the findings of this study, the figure has increased and continues to grow.

Table 1. Distribution of cervical HPV infections in Croatia according to patient age and cytology

Tabela 1. Distribucija cervikalnih HPV infekcija prema uyrastu i citolo[kom nalayu kod adolescentkinja u Hrvatskoj

Patient age	Number of analysed specimens	HPV-positive specimens[a]	Percentage
< 20	41	28	68.3
21-30	208	86	41.3
31-40	63	21	33.3
41-50	48	16	33.3
≥51	19	12	63.1
Cytology			
CIN I	183	65	35.5
CIN II	128	61	47.6
CIN III	50	26	52.0
CIN IV	18	11	61.1
Total	**379**	**163**	**43.0**

[a] In some specimens more than one HPV type were detected. Six specimens showed double,
low-risk/high-risk HPV and 8 specimens showed double high-risk/high-risk HPV infections.
In two specimens three different low-risk/high-risk HPV types were detected.

The results (Table 2) of this study demonstrate that among Croatian adolescent women there is a high prevalence of conventional HPV types (140/163; 87,5%), as well as a significant distribution of HPV types of uncertain-risk (14,1%). These incertain-risk HPV types (HPV X) will be further characterised in the near future by restriction fragment length polymorphism of consensus PCR products. Low-risk HPVs (types 6 and 11), moderate-risk HPVs (types 31 and 33) and high-risk HPVs (types 16 and 18) appeared equally in 25.8, 24.5 and 25.8% of the cases, respectively. Multiple infections were found in only 9.8% of the cases. Among moderate and high-risk HPVs, types 16 (20.-2%) and 31 (17.8%) were far more common than HPV types 18 (5.5%) and 33 (6.7%). The geographical distribution of different HPV types is well established. Among HPVs associated with high grade CIN and cervical cancer, HPV 16 is the most prevalent in western countries, HPV 18 in Africa and HPV 52 and 58 in Asia (4). Our results show that HPV 16 and HPV 31 seem to be equally distributed among Croatian adolescent women. Additional research is necessary to establish an eventual preferential geographical distribution of HPV 31 in Croatia.

Table 2. HPV distribution according to risk type, patient age and citology

Tabela 2. Distribucija HPV prema tipu, uzrastu pacijentkinja i citoloskom nalazu

Patient age	HPV positive specimens	HPV risk type								
		Uncertain	Low	Moderate			High			Multiple
		X[a]	6/11	31	33	Sum	16	18	Sum	Different types
		N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	
<16	28	3 (10.7)	11 (39.3)	5 17.8)	2 (7.1)	7 25.0)	4 (14.3)	2 (7.1)	6 (21.4)	1 (3.6)
16-17	86	12 (13.9)	17 (19.8)	17 (19.8)	6 (7.0)	23 (26.7)	19 (22.1)	3 (3.5)	22 (25.6)	12 (13.9)
17-18	21	2 (9.5)	7 (33.3)	5 (23.8)	1 (4.9)	6 (28.6)	3 (14.4)	1 (4.8)	4 (19.0)	2 (9.5)
18-19	16	4 (25.0)	4 (25.0)	1 (6.2)	2 (12.5)	3 (18.7)	5 (31.2)	0 (0)	5 (31.2)	0 (0)
=20	12	2 (16.7)	3 (25.0)	1 (8.3)	0 (0)	1 (8.3)	2 (16.7)	3 (23.0)	5 (41.7)	1 (8.3)
Cytology										
CIN I	65	12 (18.5)	22 (33.8)	11 (16.9)	2 (3.1)	13 (20.0)	8 (12.3)	1 (1.5)	9 (13.8)	9 (13.8)
CIN II	61	6 (9.8)	15 (24.6)	14 (22.9)	5 (8.2)	19 (31.3)	10 (16.4)	6 (9.8)	16 (26.2)	5 (8.2)
CIN III	26	3 (11.5)	3 (11.5)	3 (11.5)	2 (7.7)	5 (19.2)	13 (50.0)	1 (3.8)	14 (53.8)	1 (3.8)
CIN IV	11	2 (18.2)	2 (18.2)	1 (9.1)	2 (18.2)	3 (27.3)	2 (18.2)	1 (9.1)	3 (27.3)	1 (9.1)
Total	**163**	**23 14.1)**	**42 (25.8)**	**29 (17.8)**	**11 (6.7)**	**40 (24.5)**	**33 (20.2)**	**9 (5.5)**	**42 (25.8)**	**16 (9.8)**

[a] X = undetermined HPV type based in positivity of consensus primers directed PCR and negativity of specifis primers directed PCR

As previously shown (18, 19), the frequency of HPV infections decreased with patient age (Table 1), with the exception in our study of women beyond 51 years of age. However, this may be due to a statistical error brought about by the low number (19 cases) af analysed adolescent women who exhibited mostly CIN IV cytology. The same age dependency of HPV detection was previously observed among women with negative cytology (20). This may be explained by acquisition of immunity with age.

Among teenagers (age group < 16), low risk HPVs (types 6 and 11) were the most prevalent, 39.3%, while, moderate-risk HPVs (types 31 and 33) and high-risk HPvs (types 16 and 18) were almost equally prevalent, i.e. 25.0 and 21.4% respectively (Table 2). In the 16 to 17 age group, HPV infections were mostly represented by moderate-risk HPVs (26.7%) and high-risk HPVs (25.6%), but also by low-risk HPVs (19.8%) and uncertain-risk HPVs (13.9%). Surprisingly, coinfection by different HPV risk types is the highest in this age group (13.9%). In the 17 to 18 age group, the prevalence of low-risk HPVs (33.3%) was greater than moderate-risk HPVs (28.6%) and high-risk HPVs (19.0%). In the 19-20 age group, high-risk HPVs were most common (31.2%) while the presence of low-risk HPVs (25.0%) and moderate-risk HPVs (18.7%) was less frequent. In this age group there was no coinfection, but the group did clain the highest prevalence (25.0%) of uncertain-risk HPV types as compared to the other agr groups. This may be explained by a more complicated infection by this HPV type, as compared to the conventional types (6/11, 16, 18, 31 and 33). HPV 6/11 is the most easily spread, as indicated by the highest prevalence of HPV 6/11 primo-infection among teenagers. Among women over 20 years of age, high-risk HPV types (41.7%) were the most common, followed by low-risk HPV types (25.0%), uncertain-risk HPV types (16.7%) and moderate-risk HPV types (8.3%). This is an unexpectedly high prevelence of HPVs, but as we have already mentioned this could be due to the low number (19 cases) of analysed women who exhibited mostly high grade cervical neoplasia.

The presence of different HPV types significantly (the Pearson Chi-square test value being $X^2 = 8.11$, $p \leq 0.043$) increased from 35.5 to 61.1% along with the severity of cervical intraepithelial neoplasia (from CIN I to IV) (Table 1). This common result is not surprising, and reflects data found in previous reports (7, 21). Therefore, high-risk HPV type 16 is increasing from CIN I to III, other HPV types 18, 31 and 33 are increasing only from CIN I to II, while low-risk HPV types 6 and 11 are decreasing from CIN I to III (Table 2). Uncertain-risk HPV types were not consistent with the cytologic findings, which suggests that they represent different risk HPV types that have not yet been determined. HPV types 6/11 were strongly associated with CIN I (33.8%) and CIN II (24.6%), HPV type 31 also with CIN II (22.9%), and HPV type 16 with CIN III (50%) (Table 2). According to a previous report, the progression to high-grade CIN is strongly associated with high- and moderate-risk HPV types while no or a few pregression is associated with the presence of low-risk HPV types or the absence of HPV (22), which further stresses the importance of HPV DNA typing.

Multiple infections were found in 16 cases, mostly among women between 16 to 17 years of age with CIN I. This finding is most probably due to greater sexual activity with different partners at that age, whereby HPV infection can be easily spread.

Conclusion

The results of this study indicate a high prevalence of conventional high-risk HPV types among Croatian women, in addition to a significant distribution of HPV types of still uncertain risk. High-risk HPV types 16 and 31 were the most Common types were found in the cases studied. There was also a correlation between the distribution of HPV infections and the severity of cervical intraepithelial neoplasia, i.e. the frequency of low-risk and uncertain-risk HPvs decreased while the frequency high-risk HPVs (especially HPV 16) increased. The youngest women (< 16), exhibited the highest rate of HPV infections, and are thereby exposed to oncogenic viruses very early in life. HPV infection with high-risk HPV types is the major risk factor for cervical neoplasia, nevertheless, infection with multiple types of HPV or with an HPV other than the conventional high-risk types may also increase the risk of developing high-grade cervical neoplasia.

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