Distribution of Genital Human Papillomavirus (HPV) Genotypes in Croatian Women with Cervical Intraepithelial Neoplasia (CIN) – A Pilot Study

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A B S T R A C T

Genital infection with high-risk human papillomavirus (HR HPV) associates with increased risk of developing precancerous lesions, such as cervical intraepithelial neoplasia (CIN). The objective of this pilot study conducted in north-east Croatia was to determine the prevalence of HPV genital infection in women with abnormal cervical cytology and to determine its association with their age and HPV genotype(s). From March 2009 to December 2011, cervical swabs from 100 women were analysed for HR HPV infection (AMPLICOR HPV Test, Roche Diagnostics) and genotyped for high risk (HR), intermediate (IR) and low risk (LR) HPVs (Linear Array HPV Genotyping Test, Roche Diagnostics). The most prevalent HR genotypes in women with CIN were HPV 16 (27.6%), HPV 31 (11.8%), HPV 51 and HPV 52 (10.2% each). The most prevalent IR genotypes were HPV 66 (30%) and HPV 62 (23.3%). The most prevalent LR genotype was HPV 6 (20.3%). Women between 21 and 25 years of age showed the highest rate of HPV infection (44.2%). Moreover, women younger than 35 years showed a significant association ($p<0.01$) and positive correlation ($r=0.67$; $p<0.05$) between HR HPV infection and CIN stages 1 and 2. Multiple HPV infections were found in almost half of the women. This is the first study that analysed the prevalence of genital infection with HR/IR/LR HPVs in women with CIN from north-east Croatia. Despite the preliminary nature of this pilot study, the lower prevalence of some HR HPVs (HPV18) and the higher prevalence of other HR HPVs (HPVs 51, 52 and 31) may imply the necessity for the development of more targeted anti-HPV vaccines or other strategies for more efficient protection against oncogenic HPV infection in women from our region.

***Key words**: Human Papillomavirus, HPV genotypes, genotype distribution, cervical intraepithelial neoplasia, HR HPV, Linear Array HPV Genotyping Test (Roche Diagnostics), multiple HPV infections, genital tract infections, Osijek-Baranja County, age-related distribution*

Introduction

Genital infection with human papilloma virus (HPV) is the most common sexually transmitted disease in women. Due to its infection persistency and oncogenic potential, infections with high risk HPV genotypes play a crucial rule in the development of cervical lesions, accounting for 43% to 53% of CIN-positive women in the Western world. Although 70–90% of HPV infected women clear their infections within one to two years through natural immune process[1,2], cervical cancer represents the second most common female cancer worldwide. Elfgren et al. reported that 370,000 new cases and 190,000 deaths occur yearly in less developed countries[1].

Since the last 30 years, cervical screenings by cytological Papanicolaou (Pap) test and quadrivalent vaccinations against the most oncogenic HPVs have decreased the incidence of cervical cancer and its related cancers such as vulvar and colorectal cancer. Early treatment of HPV infection helps to prevent cervical cancer and decreases mortality[3]. Cross sectional studies across the world showed that the prevalence of HPV infection varies by age, country and race[4,5]. In Croatia, with its overall incidence of 13.6 per 100,000, cervical carcinoma is the second most common cancer in highly sexually active 25–40 year-old women[5]. Due to its skin-to-skin, vaginal,

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oral and sexual transmission and often asymptomatic nature, HPV infection spreads easily among young population[1].

According to phylogenetic differences and relative risk for HPV-related progression to cervical dysplasia, HPV genotypes are classified into 3 subtypes:

HR HPV (high-risk HPV): 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 82, 73;

IR HPV (intermediate-risk HPV): 26, 40, 53, 54, 55, 61, 62, 64, 66, 67, 70, 83;

LR HPV (low risk HPV): 6, 11, 42, 43, 44, 81, 84, CP6108.

The aims of this pilot study were to determine the distribution of HR/IR/LR HPV genotypes, their association with age and CIN stages, and the age-related prevalence of single and multiple HPV infections in women from Osijek-Baranja County diagnosed with CIN.

Material and Methods

Study population

From March 2009 to December 2011, cervical swabs from 100 symptomatic women were provided by gynecologist offices from the Osijek-Baranja County. Collection of cervical specimens was performed according to the AMPLICOR HPV test instructions (Roche Diagnostics, Germany). All women were referred to our department by their general practitioners or gynaecologists and the information about their CIN was obtained from gynecologist's questionnaires. All study participants gave informed consent and the Ethics Committee of the Institute of Public Health of the Osijek-Baranja County approved the study.

HR HPV detection

DNA was isolated using the High Pure PCR Template Kit and analyzed for the presence of HR HPV by the AMPLICOR HPV test (Roche Diagnostics, Germany) according to the manufacturer's instructions. DNA from positive swab specimens was stored at –20 °C until subsequent HPV genotyping.

HR/IR/LR genotyping

The HR HPV positive cervical samples were genotyped by the Linear Array HPV Genotyping Test (Roche Diagnostics). This test detects 37 high risk (HR), intermediate risk (IR) and low risk (LR) HPV genotypes (HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, IS39 and CP6108). It is based on the Polymerase Chain Reaction (PCR)-based amplification of polymorphic L1 region, hybridization to the HPV probes and colorimetric visualisation. As internal control, test amplifies beta-globin gene to asses cellular adequacy, extraction and amplification for each processed specimen.

Statistical analysis

Women were classified according to their age (under 20, 21–25, 26–30, 31–35, 36–40 and above 40 years) and Pap smear status (CIN1-3). Prevalence of HR/IR/LR HPV genital infection and association with CIN stages were analyzed by chi-square and Spearman's correlation rank tests (Figures 1, 2 and 3). The prevalence of multiple and single HPV infections according to age was analyzed by Fisher exact test (Figure 4). Statistica 8.0 software was used for all calculations (StatSoft).

Results

Prevalence of HR, IR and LR HPV genotypes

HPV 16 was the most prevalent HR HPV genotype (27.6%), followed by HPVs 31 (11.8%), 51 and 52 (10.2% each), 18 (7.1%), 39 (6.3%), 56 and 59 (5.5% each), 58 (4.7%), 33 (3.9%), 45 and 68 (2.4% each), 35 (1.6%), and 82 (0.8%). HPV 66 was the most prevalent IR HPV genotype (30%), followed by HPVs 62 (23.3%), 53 and 61 (16.7% each), and 54, 55, 70, and 83 (3.3 % each). HPV 6 was the most prevalent LR HPV genotype (20.3%), followed by HPVs 42 and CP 6108 (25% each), and 81 and 84 (20% each) (results not shown).

HPV prevalence according to CIN and age

HR HPV infection significantly associated with CIN stages 1 and 2 ($p<0.01$) as shown by the χ^2-test (Figure 1). Moreover, there was a positive correlation between HR HPV cervical infections and CIN 1 and 2 (Spearman's correlation coefficient $r=0.67$; $p<0.05$). On the other hand, no significant association was detected between: i) HR HPV infection and CIN 3; ii) IR HPV genital infection and CIN stages 2 and 3; iii) LR HPV genital infection and any CIN stage (Figures 2 and 3). Using the

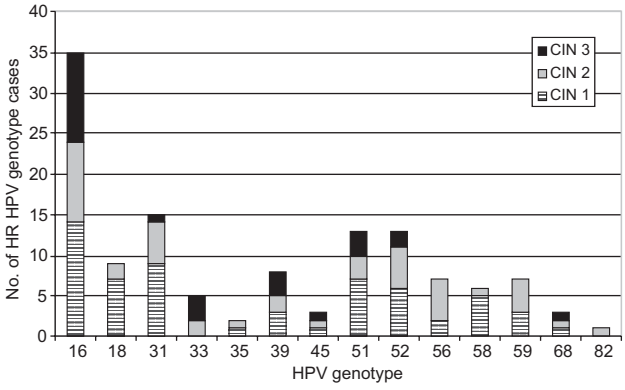

Fig. 1. Distribution of HR HPV genotypes according to CIN.

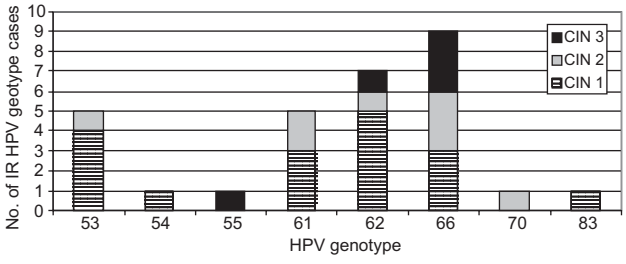

Fig. 2. Distribution of IR HPV according to CIN.

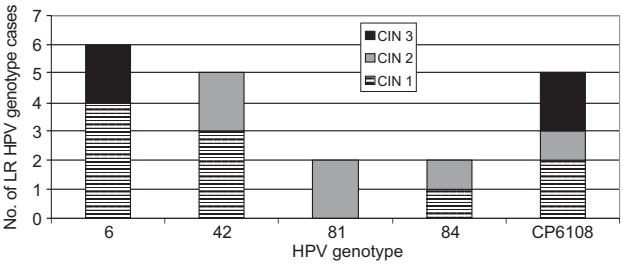

Fig. 3. Distribution of LR HPV according to CIN.

Spearman's rank test, no correlation was detected between genital infections with IR/LR HPVs and CIN.

CIN in women younger than 35 years showed a significant association with HR HPV infection ($p<0.01$). CIN in women older than 36 did not significantly associated with HR HPV. Moreover, women younger than 30 years had a significantly higher prevalence of IR HPV infection ($p<0.01$).

Prevalence of single and multiple HPV infections according to age

Out of 100 women diagnosed with CIN, 57% of them were infected with a single HPV genotype and 43% of them with multiple HPV genotypes. Figure 4 shows that single HPV infections were mostly prevalent in 26–30 and 21–25 year old women (15.2% and 14.1%, respectively), followed by women between 30 and 35 years and older than 40 (8.1% each), women younger than 20 (6.1%), and 36–40 years old women (5.1%).

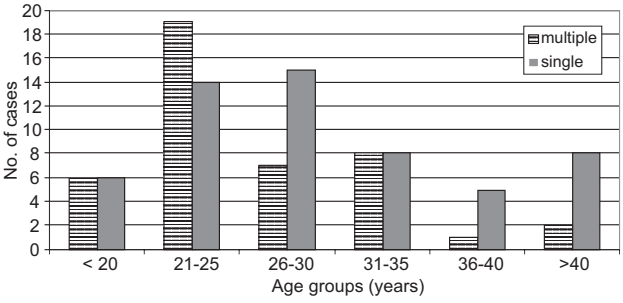

Fig. 4. Multiple and single HPV infections according to age.

Multiple HPV infections were most prevalent in 21–25 year old women (19.2%) but less common in other age categories. There, 8.1% of 31–35 year old women, 7.1% of 26–30 year old women, 6.1% of women younger than 20, 2% of women older than 40, and 1% of 36–40 year old women were infected with two or more HPV types (Figure 4).

Discussion

HR HPV

In this pilot study of 100 women with CIN from north-east Croatia, the two most common high risk HPV genotypes were HPVs 16 and 31 (28% and 12%, respectively). Both genotypes significantly associated with CIN1 and CIN2 ($p<0.01$ each) but not with CIN3. The latter may be due to the low number of study participants with severe dysplasia (CIN3). Similar prevalence of HPV 16 and 31 was reported in two studies from Italy[6] and Spain[3]. The prevalence of HPV 31 in our study population (12%) was however higher than in women with CIN from Brazil[7], Canada[8,9], and the USA[10] (10%, 3% and 7%, respectively).

Contrary to the world-wide epidemiological studies[11] and reports from Brazil[7], Iran[12], and the USA[10] (46%, 12% and 13%, respectively), the prevalence of HPV 18 in our study population was low (7.1%) but similar to some European countries[12–26]. Although this difference in the prevalence of HPV 18 may be caused not only by different sensitivities of PCR-based tests used for HPV genotyping, but also due to different stages of cervical dysplasia in tested study populations. Indeed, a clinical study from northern Croatia (Istra) detected a higher prevalence of HPV 18 in women with invasive adenocarcinoma and adenocarcinoma in situ[14].

The prevalence of high risk HPV 52 in our study (10.2%) was higher than in women with CIN from the nearby regions such as Split-Dalmatia County[13] and neighbouring Slovenia[15] (1.4% and 5%, respectively). The prevalence of high risk HPV51 in our study (10.2 %) was similar to that in women with CIN1 from Canada (13%)[16] and the USA (12%)[17], higher than in Spain[3] and Brazil[7] (7% and 3%, respectively) but lower than in women from Italy (37%)[4].

At this point, it remains unclear whether the afore-mentioned discrepancies in the prevalence of certain HR HPVs reflect the »true« region- or race-specific differences and not just a consequence of different sensitivities of HPV detection methodologies. We find nevertheless interesting that the prevalence of oncogenic HPV 18 in women with CIN from our County was lower than expected. On the other hand, some oncogenic HPVs (HPVs 31, 51 and 52) in our study population were more frequent than in reports from other regions/countries. This may suggest a need for a more specific anti-HPV vaccine in order to achieve some protection against high grade cervical disease although currently available quadrivalent vaccine shows cross protective effect on precancerous lesions and carcinoma in situ caused by oncogenic HPV types other than vaccine strains[22].

IR HPV

The most prevalent intermediate risk HPV in our region was HPV 66 (30%), similarly to reports from Tanzania[21] (16%), Spain[3] (9%), and Canada[23] (5%). However, this genotype was not found in Italy[6] and Slovenia[15] and was very low in women from the USA[10] (1.1%). Whether this difference is due to different hybridization specificities and/or cross-reactivity to other genotypes remains to be elucidated. Since HPV 66 is often detected in symptomatic women with multiple HPV infections but is rarely found in women with cancer[24,25] and single HPV66 infection, it may not be a strong contributing factor in

the development of cervical cancer[23]. Other IR genotypes that were observed in women from our study were HPVs 62, 53, and 61 (23%, 17%, 17%, respectively). Interestingly, HPVs 62 and 61 were not detected in women from Spain[3]. Finally, we found no significant association between IR HPV infection and CIN grades 2 and 3.

LR HPV

With a slight predominance of HPV6, the prevalence of the most common LR HPVs (6, 42, CP1208, 81, 84) in our study population was comparable (30%, 25%, 25%, 20%, 20%). A similar predominance of HPV6 was reported in women with CIN from Brazil (24%)[7]. On the contrary, a study from Tanzania[21] reported a strong predominance of HPV 81 and 84 genotypes (28% each). As expected, we detected no significant association between LR HPV infection and CIN.

Age-specific distribution of HR HPV in women with CIN

A recent Croatian study reported that 55–60% of women younger than 30 with cervical dysplasia show a significantly higher prevalence of HR HPV infection[26,27]. We also observed a significant association between CIN and HR HPV infection(s) in women younger than 35. This may be due to more frequent and/or less protected (no condom) sexual activity of younger women, intercourse with uncircumcised men and history of herpes simplex virus or Chlamydia infections[28,29]. For example, Ljubojević et al. reported that the usage of oral contraceptives associated with less frequent usage of condoms. Similar tendency was reported for women with more than 4 lifetime sexual partners and those whose partners had more than 16 female partners[26].

No association between HR HPV infection and CIN in our study participants older than 36 may be explained by their decreased sexual activity, lower number of sexual partners and possible clearance of HR HPV infection(s). HPV status and the number of lifetime sexual partners, as well as choosing male partners who have previously had less than 16 other female sexual partners were strong predictors of CIN regression[26].

Age-specific distribution of single and multiple HPV infections in women with CIN

Similarly to the recent study of women from Osijek-Baranya County[30], we also observed multiple HPV infections in our study participants. Five percent of cervical swabs were infected with more than 5 different HPVs, whereas 46% of swabs showed concurrent infections with several HPV genotypes. The rate of multiple infections was however lower than that in women with CIN from the USA (66%)[2,31]. As expected, we also found that the highest rate of multiple HPV infections occurred in women younger than 30. When compared to our study population, Danish women with CIN2/3 in the same age category had a higher rate of multiple infection (30 *vs.* 61%)[25].

There are several limitations to this study: i) low number of cases with high grade cervical dysplasia (CIN2, 3) and CIS; ii) unknown medical history of previous genital infections; iii) disproportion between younger and older study participants. Nevertheless, the results presented here are useful in designing future prospective case-control studies and better prevention methods against acquiring HR HPV infection.

Conclusion

This pilot study of 100 women with CIN from north-east Croatia showed that cervical HR HPV infections associated with CIN. The highest prevalence of multiple HPV infection was detected in young women (21–25 year old). HPV genotypes distribution (HPV16, HPV18, and 31) was very similar to distribution obtained by others. Contrary, HR HPV52 prevalence in women with CINs was significantly higher.

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RASPODJELA SPOLNIH LJUDSKIH PAPILOMAVIRUS GENOTIPOVA KOD ŽENA SA INTRAEPITENOM NEOPLAZIJOM GRLIĆA MATERNICE (CIN)

S A Ž E T A K

Spolna HPV infekcija, posebno s onkogenim visokorizičnim ljudskim papiloma virusima (HR HPV prema engl. High Risk Human Papillomavirus), se dovodi u svezu s povećanim rizikom razvoja prekanceroznih promjena kao što su cervikalne intraepitelne neoplazije (CIN). Cilj ove pilot studije je utvrditi prevalenciju genitalnih HPV infekcija u žena s abnormalnim cervikalnim citološkim nalazom i njihovu povezanost sa životnom dobi pacijentica i genotypom HPVa. Svi uzorci testirani su na nazočnost HR HPV uporabom AMPLICOR HPV testa (Roche Diagnostics) i genotipizirani uporabom Linear Array HPV Genotyping testa (Roche Diagnostics). Najučestaliji HR HPv genotipovi u žena s CIN su HPV 16 (27,6%), HPV 31 (11,8%), HPV 51 and 52 (10,2% svaki). U grupi srednjerizičnih HPV-a (IR HPV) najučestaliji su HPV 66 (30%) i HPV 62 (23,3%) dok je u grupi niskorizičnih HPV-a (LR HPV) najučestaliji HPV 6 (20,3%). Najveću prevalenciju HPV infekcija imale su žene između 21 i 25 godina (44,2%). Nadalje, žene mlađe od 35 godina imale su značajnu povezanost ($p<0,01$) i pozitivnu korelaciju ($r=0,67$; $p<0,5$) između infekcija HR HPV i CIN 1 i 2. Multiple infekcije dokazane su u skoro polovine ispitanica. Ovo je prvo istraživanje prevalencije genitalnih infekcija sa HR/IR/LR HPV genotipovima kod žena sa abnormalnim citološkim nalazom u Osječko-baranjskoj županiji. Usprkos preliminarnoj prirodi ovog pilot-istraživanja, niža prevalencija nekih HR HPV genotipova (HPV18) koji su uključeni u dostupno četverovalentno cjepivo kao i viša prevalencija nekih drugih HR HPV (HPV51, 52 i 31) protiv kojih cjepivo pruža djelomičnu unakrsnu zaštitu upućuju na potrebu za njegovom širom primjenom ali i za razvijanjem djelotvornijih zaštitnih mjera protiv onkogenih HPV infekcija žena u našoj regiji.