Pre-vaccination distribution of human papillomavirus (HPV) genotypes in women with cervical intraepithelial neoplasia grade 3 (CIN 3) lesions in Slovenia

A. Kovanda, U. Juvan, A. Šterbenc, B.J. Kocjan, K. Seme, N. Jančar, E. Vrtačnik Bokal, and M. Poljak

— **A** B S T R A C T

Background: High-risk human papillomaviruses (HPV) are the main etiological factor of cervical cancer. Cervical intraepithelial neoplasia grade 3 (CIN 3) is the latest pre-invasive stage of cervical cancer, with an approximately 20% progression rate to invasive cervical carcinoma.

Objective: To establish the pre-vaccination distribution of HPV genotypes in Slovenian women with CIN 3 lesions, in order to assess the potential benefit of prophylactic HPV vaccination in Slovenia, and to provide baseline data for monitoring the potential replacement of HPV genotypes under selective pressure of HPV vaccines.

Methods and results: A total of 261 cervical swabs collected from women with histologically confirmed CIN 3 lesions were analyzed using four genotyping methods: the Abbott Real*Time* High Risk HPV Assay, the Innogenetics INNO-LiPA HPV Genotyping *Extra* Test, and the in-house PGMY09/11, and CPI/CPIIg polymerase chain reaction (PCR) and sequencing. Of 261 samples, 253 (96.9%) were HPV positive. The most common HPV genotype in CIN 3 lesions in the Slovenian samples was HPV-16 (59.0%), followed by HPV-31 (7.5%), HPV-33 (7.1%), HPV-58 (5.0%), and HPV-51 (4.0%). The presence of more than one HPV genotype was detected in 49/253 (19.4%) samples. Together, HPV-16 and HPV-18 accounted for 67.4% of CIN 3 lesions in this Slovenian population.

Conclusion: The high proportion of CIN 3 lesions caused by HPV-16 and HPV-18 should further support the recent decision to include the prophylactic vaccination against HPV in the national vaccination program in Slovenia.

Introduction

Persistent infection with certain genotypes of human papillomavirus (HPV) is a necessary but not sufficient etiological factor in the development of cervical cancer (1, 2). More than 100 HPV genotypes have been described so far (3). Extensive worldwide studies of HPV genotype distribution in various grades of cervical lesions and cervical cancer have identified 15 high-risk (HR) HPV genotypes: HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-

56, HPV-58, HPV-59, HPV-68, HPV-73, and HPV-82 (3, 4). The latest meta-analysis update (5, 6) showed that HPV-16 and HPV-18 were responsible for more than 70% of cervical cancers worldwide, with the other most common genotypes being HPV-31, HPV-33, HPV-35, HPV-45, HPV-52, and HPV-58, with varying regional prevalence.

Cervical intraepithelial neoplasia grade 3 (CIN 3) is the latest pre-invasive stage of cervical cancer, with an approximately 20% progression rate to invasive cervical carcinoma. The development of CIN 2 and CIN 3 lesions caused by HPV-16 and HPV-18 can be prevented by two prophylactic vaccines composed of L1 virus-like particles (7, 8). In addition to being highly effective against lesions caused by HPV-16 and HPV-18, there is evidence that both vaccines may also offer some additional protection from other high-risk HPV genotypes due to cross-reactivity (8, 9).

Slovenia is a small central European country with a population of approximately 2 million and a cervical cancer rate almost twice that of northern Europe, this rate being higher only in Slovakia, Poland, Lithuania, and the Czech Republic (10, 11). A recent study on a representative population of women with invasive cervical cancer in Slovenia (278 women) showed that the five most common HPV genotypes in cervical cancer lesions were HPV-16 (64.9%), HPV-18 (12.2%), HPV-33 (4.7%), HPV-45 (4.1%), and HPV-31 (3.6%) (12). To the best of our knowledge, the distribution of HPV genotypes in pre-cancerous cervical lesions, including CIN 3, in Slovenia remains unknown. The aim of this study was thus to establish the distribution of HPV genotypes in a population of Slovenian women with histologically confirmed CIN 3 lesions, in order to determine the local HPV genotype specifics and to assess the potential benefit of prophylactic HPV vaccination in Slovenia. In addition, the baseline pre-vaccination distribution of HPV genotypes established in this study will be very helpful for monitoring potential HPV genotype replacement – that is, an increase in the prevalence of non-vaccine-genotype cervical lesions despite a decrease in the prevalence of vaccine-genotype lesions under the selective pressure of the HPV vaccine.

Material and methods

Samples and patients

A total of 261 cervical scrape samples collected from women with histologically confirmed CIN 3 were included in this study. The swabs were collected in 1 ml of Digene Specimen Transport Medium (Qiagen, Gaithersburg, MD) and stored at -20 °C. All samples were consecutive surplus specimens from routine HPV testing between 2005 and 2008 and no samples were col-

lected solely for the purpose of this study. The samples were collected at approximately 40 public clinics or private practices (the number varying by year) from various regions of Slovenia (all major geographical regions were represented each year).

Patient data were retrieved retrospectively from standard medical records. These data included the patient's diagnosis, date of birth, age at diagnosis, and region of residence. The mean age of the patients at the time of diagnosis was 37.8 years (ranging from 20 to 88 years). At the time of diagnosis, 75/261 (28.7%) patients were aged between 20 and 29, 101/261 (38.7%) between 30 and 39, 46/261 (17.6%) between 40 and 49, 21/261 (8.1%) between 50 and 59, 12/261 (4.6%) between 60 and 69 and 6/261 (2.3%) were above the age of 69.

HPV detection and genotyping

DNA was extracted from clinical samples using the Abbott *m*Sample Preparation System on an automated Abbott m2000sp instrument (Abbott Molecular, Des Plaines, Illinois, US), following the manufacturer's instructions. A 1:20 dilution of the original sample was made prior to extraction.

Initial HPV detection and partial HPV genotyping was performed in all samples with the Abbott Real*Time* High Risk HPV assay (Abbott Molecular) on the Abbott m2000rt instrument, following the manufacturer's instructions. The Abbott Real*Time* High Risk HPV assay is a novel real-time PCR assay, which amplifies approximately 150-bp of the HPV L1 gene, and can identify 14 HR or probable HR HPV genotypes: HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, HPV-66, and HPV-68. The assay uses four channels for the detection of fluorescent probes: one for detecting the internal control (human beta-globin), one each for detecting HPV-16 and HPV-18, and one for detecting the remaining 12 HR HPV genotypes.

All samples identified by the Abbott Real*Time* High Risk HPV Assay as containing HR genotypes other than HPV-16 or HPV-18 were additionally genotyped using the commercial INNO-LiPA HPV Genotyping *Extra* CE test (Innogenetics NV, Ghent, Belgium), capable of recognizing 27 alpha-HPV genotypes, as described previously (13). This test detects all 14 HR or probable HR HPV genotypes covered by the Abbott Real*Time* High Risk HPV assay and, in addition, two HR HPV genotypes (HPV-73 and HPV-82) and two probable HR HPV genotypes (HPV-26 and HPV-53).

For samples yielding HR HPV negative results using the Abbott Real*Time* High Risk HPV assay, additional PCR amplification was performed using a HotStart Taq *Plus* DNA Polymerase kit (Qiagen) and consensus PGMY09/11 primers, as described previously, with some modifications (14, 15). The PGMY09/11 primers target approximately 450-bp sized fragments of the L1

Table 1. Relative proportion of HPV genotypes in CIN 3 lesions in Slovenia.

HPV genotype	Epidemiological classification	Number of CIN 3 cases*
HPV-16	high risk	149.2 (59.0%)
HPV-31	high risk	18.9 (7.5%)
HPV-33	high risk	18.0 (7.1%)
HPV-58	high risk	12.6 (5.0%)
HPV-51	high risk	10.0 (4.0%)
HPV-52	high risk	9.5 (3.8%)
HPV-18	high risk	8.4 (3.3%)
HPV-66	probable high risk	7.4 (2.9%)
HPV-45	high risk	5.6 (2.2%)
HPV-35	high risk	4.7 (1.9%)
HPV-39	high risk	2.0 (0.8%)
HPV-67	risk not determined	2.0 (0.8%)
HPV-73	high risk	1.2 (0.5%)
HPV-56	high risk	1.0 (0.4%)
HPV-68	high risk	1.0 (0.4%)
HPV-53	probable high risk	0.5 (0.2%)
HPV-82	high risk	0.5 (0.2%)
HPV-59	high risk	0.5 (0.2%)

*If the presence of more than one HPV genotype was detected in a clinical sample, the relative proportion of each high-risk HPV genotype was calculated. For details, see Material and methods.

gene (14). PCR was carried out in a 25 µl reaction volume containing 5 µl of extracted DNA, 2.5 µl of 10× CoralLoad Buffer, 200 µM (each) of dATP, dCTP, dGTP, and dTTP, 0.75 U of HotStartTaq *Plus* DNA Polymerase, and 3 pmol of each primer of both primer mixes. The thermal cycler program was set to 5 min at 95 °C, followed by 40 cycles consisting of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C. The final extension step was performed at 72 °C for 10 min and the reaction mixtures were then cooled to 4 °C. The resulting PCR products were sequenced directly with the same primers as those used for PCR, as described previously (15).

All samples yielding (HR) HPV-negative results using the Abbott Real*Time* High Risk HPV assay and the PGMY09/11 PCR, were additionally tested using CPI/CPIIg primers (16), targeting approximately 188-bp sized fragments of the HPV E1 gene, as described previously (12). The resulting PCR products were sequenced directly with the same primers as those used for PCR, as described previously (15).

All samples in which the presence of more than one HPV genotype was suspected were additionally tested using the INNO-LiPA HPV Genotyping *Extra* CE test. If the presence of more than one HPV genotype was confirmed, the relative proportion of each HR HPV genotype was calculated. The additional presence of a low-risk HPV genotype (e.g., HPV-6) did not influence the relative proportion of HR genotypes in the same sample.

For the purpose of this calculation, probable HR genotypes (HPV-53 and HPV-66) were considered to be HR genotypes and assigned a score of 1, if found as a single genotype in a clinical sample. In mixed infections containing both HR and probable HR genotypes, the former were assigned a score of 1 and the latter were assigned a score of 0.5.

Results

The 136-bp fragment of human beta-globin, which served as the internal control in the Abbott Real*Time* High Risk HPV assay, was successfully amplified from all 261 samples included in the study. Of the 261 samples, 253 (96.9%) were positive for HPV. Eight samples tested HPV DNA negative using all four methods: Abbott RealTime High Risk HPV assay, INNO-LiPA HPV Genotyping *Extra* CE test and two in-house PCRs. The distribution and relative proportions of HPV genotypes in CIN 3 lesions are summarized in Table 1. As shown in Table 1, the most common HPV genotype in CIN 3 lesions in Slovenia was HPV-16, followed by HPV-31, HPV-33, HPV-58 and HPV-51. HPV-16 and HPV-18 together accounted for 67.4% of CIN 3 lesions in Slovenia.

As summarized in Table 2, a single HPV genotype was detected in 204/253 (80.6%) of HPV positive samples. Of these samples, 196/204 (96.0%) contained one of the established HR genotypes, 6 samples contained a probable HR genotype (HPV-66), and 2 samples contained genotype HPV-67, which is genetically closely related to HPV-16 (3), but is epidemiologically not classified as an HR HPV genotype (4). Both samples containing HPV-67 tested initially HPV negative using the Abbott Real*Time* High Risk HPV assay (as expected) and its presence was determined using PGMY09/11 PCR and sequencing.

As summarized in Table 2, the presence of more than one HPV genotype (multiple infection) was detected in 49/253 (19.4%) samples. The distribution of HPV genotypes in samples with mixed infections classified according to epidemiological classification (4) is shown in Table 2.

Discussion

This study is the first to establish the distribution of HPV genotypes in CIN 3 lesions in a relatively large population of women in Slovenia. The samples included in the study were obtained from women attending roughly 40 different public clinics or private practices across the country. In our opinion, the study population can thus be taken as representative for the population of Slovenian women with CIN 3 lesions.

Table 2. Type-specific prevalence of HPV infection in CIN 3 samples in Slovenia.

HPV Genotype (s)	No. of samples (%)
Single infections total:	**204 (80.6%)**
HPV-16	136 (53.8%)
HPV-18	6 (2.4%)
HPV-31	10 (4.0%)
HPV-33	13 (5.1%)
HPV-35	3 (1.2%)
HPV-39	1 (0.4%)
HPV-45	3 (1.2%)
HPV-51	5 (2.0%)
HPV-52	7 (2.8%)
HPV-56	1 (0.4%)
HPV-58	11 (4.4%)
HPV-66	6 (2.4%)
HPV-67	2 (0.8%)
Multiple infections: two HR or probable HR genotypes total:	**31 (12.2%)**
HPV-16 + HPV-18	3 (1.2%)
HPV-16 + HPV-51	7 (2.8%)
HPV-16 + HPV-31	4 (1.6%)
HPV-16 + HPV-33, HPV-31 + HPV-45, HPV-31 + HPV-66	2 (0.8%) each
HPV-16 + HPV-35, HPV-16 + HPV-45, HPV-16 + HPV-52, HPV-16 + HPV-66, HPV-16 + HPV-68, HPV-31 + HPV-33, HPV-31 + HPV-58, HPV-31 + HPV-68, HPV-31 + HPV-73, HPV-33 + HPV-45, HPV-51 + HPV-52	1 (0.4%) each
Multiple infections: HR and low-risk genotypes total:	**8 (3.2%)**
HPV-31 + HPV-42, HPV-31 + HPV-44(55), HPV-33 + HPV-42, HPV-33 + HPV-44(55), HPV-35 + HPV-81, HPV-39 + HPV-6, HPV-51 + HPV-44, HPV-52 + HPV-69	1 (0.4%) each
Multiple infections: more than 2 genotypes total:	**10 (4.0%)**
HPV-16 + HPV-53 + HPV-58, HPV-16 + HPV-53 + HPV-40, HPV-18 + HPV-31 + HPV-45, HPV-18 + HPV-33 + HPV-82, HPV-16 + HPV-62 + HPV-66 + HPV-73, HPV-16 + HPV-45 + HPV-58 + HPV-66, HPV-16 + HPV-59 + HPV-62 + HPV-6, HPV-33 + HPV-52 + HPV-54 + HPV-84, HPV-16 + HPV-18 + HPV-42 + HPV-54 + HPV-58 + HPV-62 + HPV-70 + HPV-73, HPV-31 + HPV-33 + HPV-35 + HPV-44 (55) + HPV-58 + HPV-62 + HPV-66 + HPV-81 + HPV-82	1 (0.4%) each

In this study, HPV DNA was detected in 96.9% of CIN 3 lesions. In contrast, in the most recent meta-analysis update, the prevalence of HPV in high-grade squamous intraepithelial lesion (HSIL) in European women ranged from 70.7% to 97.8% (5, 6). The prevalence of HPV-16 found in this study (59.0%) was significantly higher ($p = 0.05$) than the mean European prevalence of HPV-16 in HSIL (51.8%; 95% CI 50.1–53.5%) (5, 6), whereas the prevalence of HPV-18 (3.3%) identified in this study was lower than the mean European prevalence in HSIL (6.0%) (5, 6). As shown in Table 3, the distribution of the remaining 13 HR HPV genotypes in CIN 3 lesions in this study differed from the global and European genotype specific distribution in HSIL. This may primarily be due to differences in the HPV detection methods used, as well as the different size and representativeness of the sample popu-

lation. The observed difference may also be inherent to the difference in samples used in the studies. This study used samples with a histologically confirmed CIN 3 diagnosis only, whereas global and European prevalence studies have mainly been carried out on samples with a less-specific HSIL diagnosis.

The distribution of HPV genotypes in Slovenian women with CIN 3 identified in this study differed from the recently reported HPV distribution in Slovenian women with cervical cancer (12). Jančar et al. recently identified the five most common HPV genotypes in Slovenia in cervical cancer samples as (in decreasing order of frequency): HPV-16 (64.9%), HPV-18 (12.2%), HPV-33 (4.7%), HPV-45 (4.1%), and HPV-31 (3.6%) (12). The underrepresentation of HPV-18 and HPV-45 in CIN 3 lesions in comparison to cervical cancer can primarily

Table 3. Comparison of HPV genotype-specific prevalence in HSIL and CIN 3 in different studies.

Diagnosis	HSIL				CIN 3	
Region	Worldwide*		Europe*		Slovenia	
HPV genotype	No. women tested	Prevalence (%)	No. women tested	Prevalence (%)	No. women tested	Prevalence (%)
Any	7,094	84.9	3,464	88.0	261	96.9
HPV-16	6,978	45.4	3,348	51.8	261	59.0
HPV-18	6,978	6.9	3,348	6.0	261	3.3
HPV-31	6,282	8.7	3,263	10.0	261	7.5
HPV-33	6,418	7.3	3,061	8.6	261	7.1
HPV-58	4,181	7.0	1,775	2.9	261	5.0
HPV-52	3,945	5.1	1,782	3.6	261	3.8
HPV-35	4,739	3.8	2,135	3.4	261	1.9
HPV-51	3,509	3.6	1,717	3.0	261	4.0
HPV-45	3,726	2.3	1,932	2.2	261	2.2
HPV-66	2,840	1.9	1,336	1.5	261	2.9
HPV-73	1,464	1.8	623	3.5	261	0.5

*Data from Bosch et al. (6).

be explained by the tighter association of HPV-18 and HPV-45 with cervical adenocarcinoma and adenosquamous carcinoma and their precursors.

The testing algorithm used in this study allowed us to identify HPV-67, a HPV genotype genetically closely related to five HR HPV genotypes: HPV-16, HPV-31, HPV-33, HPV-52, and HPV-56 (3), but still epidemiologically not classified as an HR HPV genotype (4). Due to primer mismatching, HPV-67 is not amplified by the frequently used consensus primer set GP5+/6+ and is not included in any commercially available HPV assay. This is probably the main reason that HPV-67 has been missed by a variety of HPV prevalence studies. Future studies, using appropriate detection methods for HPV-67, are therefore needed in order to determine its true clinical significance.

This study identified 49/253 (19.4%) samples as having more than one HPV genotype. Such a result was expected, given the very sensitive HPV detection methods approach employed in the study. Although some marginal relationship between the number of genotypes present in the sample and the patients' age at diagnosis was observed, we were unable to confirm this statistically due to the relatively small sample size.

HPV-16 and HPV-18, two HR HPV genotypes targeted by both HPV prophylactic vaccines, together accounted for 67.4% of CIN 3 lesions in Slovenia, which is higher than their combined mean prevalence (57.5%) reported in European HSIL studies (5, 6). The relatively high proportion of CIN3 lesions caused by HPV-16 or HPV-18 determined by this study further supports the recent decision to include the prophylactic vaccination against HPV in the national vaccination program in Slovenia.

Acknowledgement

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A U T H O R S ' *Anja Kovanda, BS, PhD student, Institute of Microbiology and*
A D D R E S S E S *Immunology, Faculty of Medicine, Zaloška 4, 1000 Ljubljana, Slovenia*
Urška Juvan, BS, same address
Anja Šterbenc, medical student, same address
Boštjan J. Kocjan, PhD, same address
Katja Seme, Assoc. Prof., same address
Nina Jančar, MD, Department of Obstetrics and Gynecology,
Ljubljana University Medical Center, Šlajmerjeva 3, 1000 Ljubljana,
Slovenia
Eda Vrtačnik-Bokal, MD, PhD, Assoc. Prof., same address
Mario Poljak, MD, PhD, Prof., Institute of Microbiology and
Immunology, Faculty of Medicine, Zaloška 4, 1000 Ljubljana,
Slovenia, corresponding author, E-mail: mario.poljak@mf.uni-lj.si