

Distribution of human papillomavirus genotypes in women with high-grade cervical intraepithelial lesions and cervical carcinoma and analysis of human papillomavirus-16 genomic variants

Aim To analyze the distribution of high-risk human papillomavirus (HR-HPV) genotypes and the diversity of HPV-16 genomic variants in Croatian women with high-grade squamous intraepithelial lesions (HSIL) and cervical carcinoma.

Methods Tissue biopsy specimens were obtained from 324 women with histopathologically confirmed HSIL or cervical carcinoma, 5 women with low-grade SIL, and 49 women with negative histopathology. HR-HPV DNA was detected with Ampliquality HPV-type nucleic-acid hybridization assay, which identifies 29 different HPV genotypes. HPV-16 genomic variants were analyzed by an in-house sequencing.

Results The most common HPV type in women with HSIL was HPV-16, detected in 127/219 (57.9%) specimens. HPV-16 was also the dominant type in squamous cell cervical carcinoma (46/69 or 66.7%) and in adenocarcinoma (18/36 or 50.0%). Out of 378 patients, 360 had HR-HPV (282 single infections and 79 multiple infections), 3 (0.8%) patients had low-risk HPV, and 15 (4%) tested negative. HPV-16 variants were determined in 130 HPV-16 positive specimens, including 74 HSIL and 46 carcinoma specimens. In HSIL specimens, 41 distinct variants were found, 98.6% belonging to the European branch and 1.4% belonging to the African branch. In cervical carcinoma specimens, 95% isolates grouped in 41 variants belonging to the European branch, one isolate (2.5%) belonged to the North American, and one (2.5%) to the Asian-American branch.

Conclusion HPV-16, mainly belonging to the European branch, was the most frequent HPV genotype in women from Croatia with histologically confirmed HSIL and cervical cancer.

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Cervical cancer is the second leading cause of death in women in low-income countries (1). Persistent infection with particular human papillomavirus (HPV) genotypes is a necessary but not a sufficient requirement for the development of cervical cancer (2). HPV DNA is detected worldwide in nearly all specimens of invasive cervical cancer, including squamous cell carcinomas, adenocarcinomas, and the majority (>95%) of immediate cervical cancer precursors (3). An epidemiological study by Bosch et al (4) has shown that the most common HPV genotypes in HSIL and squamous cell carcinomas were HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-45, HPV-52, and HPV-58, with a combined worldwide relative contribution of 91% and the predominant role of HPV-16, HPV-18, and HPV-45 in cervical adenocarcinoma.

HPV genomic variants are defined as the viruses that vary by 2% or less in specified regions of the genome, and some display different oncogenicity (5). HPV-16 heterogeneity has been extensively investigated (6-12), and HPV-16 genomic variants have been identified to belong to five main branches: European, Asian-American, two African branches, and an Asian branch (13). Two subsequent studies expanded these classifications and reported a new branch: North American 1 (14,15).

Epidemiological studies have shown that non-European HPV-16 variants may promote viral persistence and disease progression (16-19). HPV-16 E6 variants, including the European HPV-16 T350G variant in the E6 gene, were detected up to 20 times more often in patients with high-grade cervical disease compared with controls. A novel HPV-16 variant, identified in Croatia, harboring a 63-bp in-frame duplication in the E1 gene, was presumed to be of reduced oncogenicity (11).

According to the several national or regional studies in women with normal and abnormal cytology, HPV-16 is the most common high-risk genotype in Croatian women (20-27). However, none of these studies involved HPV genotyping in tissue specimens, and the majority were performed in general population with a small number of women with histologically confirmed HSIL or cervical cancer. The genomic diversity of high-risk HPV genotypes in Croatia has not been studied to date. On the other hand, recommended, non-mandatory, free-of-charge, nine-valent HPV vaccine is available in Croatia and is intended for vaccination of both women and men aged 14 to 25 years (28).

The aims of this study were to analyze the distribution of high-risk HPV genotypes (HR-HPV) in women with histo-

logically confirmed HSIL and cervical carcinoma and to analyze the genomic diversity of HPV 16 in HSIL in comparison with invasive cervical cancer.

PATIENTS AND METHODS

Patients and samples

The study enrolled 406 women aged 19-83 years (median 37, standard deviation [SD] 11.31) treated at the Department of Obstetrics and Gynecology, University Hospital Centre Zagreb for abnormal cytology (HSIL or carcinoma) between December 2009 and December 2013. Two tissue specimens (one for histopathological analysis and one for HPV genotyping) were obtained during colposcopy by biopsy of suspected lesions (90 specimens) or during a surgical intervention: large loop excision of the transformation zone, conization, and radical hysterectomy (316 specimens). The procedures were performed by four experienced gynecologists. The specimens were stored in the media provided within the Swab Specimen Collection Kit (Qiagen, Hilden, Germany). The histopathological evaluation was performed by two certified pathologists, who were unaware of the patients' HPV status. The histopathological tissue preparation was performed according to the standard protocol: the tissue was paraffin-embedded, sliced to 3-5 microns, and stained with hematoxylin and eosin.

The selected demographic and clinical data (age, place of residence, cytology results, risk factors for cervical cancer including smoking, parity, age of menarche) were collected with the standardized questionnaire.

The study was approved by the Ethics Committees of Department of Gynecology and the University Hospital of Infectious Diseases in Zagreb. The study was conducted according to principles of the Declaration of Helsinki. All patients signed the informed consent before entering the study.

HPV genotyping

DNA was isolated by using DNA I Blood Cell High Performance II kit on a Magna Pure LC 2.0 instrument (Roche, Pleasanton, CA, USA) and stored at -20 °C until amplification. HPV was detected and genotyped with Ampliquality HPV-type nucleic-acid hybridization assay (AB Analytica, Padua, Italy), which identifies 29 HPV genotypes. The assay is based on the amplification of a target se-

quence of 150 bp within the L1 genomic region by using GP5+/6+ universal primer pair.

Analysis of HPV-16 genomic variants

The target sequence of the L1-LCR-E6 region genome of about 1700 bp HPV-16 was amplified by using g16f-7122, g16f-7122, g16r-7714, g16f-7663, g16r-376, g16f-273, and g16r-913 primers on GenAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) (29,30), and sequenced by using 3500 Dx Genetic Analyzer (Applied Biosystems). The presence of the amplicon was analyzed by gel electrophoresis (0.8% agarose). Nucleotide sequences were analyzed with the Vector NTI program (Thermo Fisher Scientific, Waltham, MA, USA). Nucleotide HPV-16 sequences (E6, E7, and long control region [LCR]) from patients and reference nucleotide sequences were aligned by using MAFFT, v. 6.846 software algorithm (31). A maximum likelihood phylogenetic tree was constructed with the RAxML HPC2, v. 7.6.3 algorithm (32), based on the evolutionary GTRCAT model and with the bootstrap value of 1000.

Statistical analysis

The normality of distribution was tested with the Smirnov-Kolmogorov's test. The differences in age, age of menarche, number of births and abortions, and smoking were compared using the ANOVA test. The Pearson test was used to assess the differences in the distribution of single and multiple infections in the samples according to different pathohistological diagnoses. *P* values lower than 0.05 were considered significant. The analysis was conducted with IBM SPSS, version 25.0 (Armonk, New York, NY, USA).

RESULTS

Histopathologic findings

Seventy patients had a negative result, 5 had cervical intraepithelial neoplasia (CIN) 1 (mild cervical intraepithelial neoplasia – changes limited to the lower third of the epithelium), 15 had CIN 2 (moderate cervical intraepithelial neoplasia – changes involving the lower two-thirds of the epithelium), 206 had CIN 3 (severe cervical intraepithelial neoplasia – changes involving more than two-thirds of the epithelium), 24 had microinvasive carcinoma (MIC), 45 had squamous cell carcinoma, 8 had adenocarcinoma *in situ* (AIS), 20 had adenocarcinoma, 1 had adenosquamous carcinoma *in situ*, and 2 had adenosquamous carcinoma. Five patients had cancer of other origin. One specimen was a mixture of adenocarcinoma and MIC (0.3%) and 4 specimens were a mixture of CIN3 and AIS (1.1%). These specimens were classified according to the more severe clinical diagnosis as invasive lesions. The specimens of cancer of other origin were excluded from the HPV analysis, thus 401 specimens were further tested for the presence of HPV DNA.

Human papillomavirus detection and genotyping

HPV DNA could not be detected in 6 specimens due to the poor quality of the biopsy tissue (negative internal control). In 13 specimens, HPV was not detected (histopathology of those samples was also negative). In 1 specimen with negative histopathology, we detected only low-risk HPV genotype, and in 3 specimens we detected HR HPV of unknown type. Consequently, these 23 specimens were not further analyzed.

TABLE 1. Distribution of human papillomavirus (HPV) types in patients depending on histopathological finding and number of infections

| HPV type | Number (%) of samples; number of mono-infections + number of multiple infections | | | |
|---------------------|--|--|-------------------------------------|----------------------------|
| | negative histopathology (n = 49) | high-grade squamous intraepithelial lesions (n = 219) | squamous cell carcinoma (n = 69) | adenocarcinoma (n = 36) |
| HPV-16 | 21 (42.9); 16 + 5 | 127 (57.9); 99 + 28 | 46 (66.7); 37 + 9 | 18 (50.0); 14 + 4 |
| HPV-31 | 6 (12.2); 4 + 2 | 28 (12.8); 21 + 7 | 10 (14.5); 5 + 5 | |
| HPV-51 | 5 (10.2); 2 + 3 | | | |
| HPV-58 | 4 (8.2); 3 + 1 | 14 (6.4); 11 + 3 | | |
| HPV-33 | | 15 (6.8); 9 + 6 | 4 (5.8); 2 + 2 | |
| HPV-52 | | 13 (5.9); 7 + 6 | | |
| HPV-18 | | 10 (4.6); 3 + 7 | 5 (7.2); 3 + 2 | 15 (41.7); 10 + 5 |
| HPV-45 | | 7 (3.2); 2 + 5 | 5 (7.2); 4 + 1 | 2 (5.6); 1 + 1 |
| HPV-low risk | 2 (4) | 1 (0.5) | | |
| HPV DNA negative | | 8 (3.7) | 3 (4.3) | 4 (11.1) |
| Multiple infections | 12 (25.5) | 43 (19.6) | 14 (20.3) | 7 (19.4) |

HPV genotyping for 324 patients with histologically confirmed HSIL or cervical carcinoma, 5 patients with LSIL, and 49 patients with negative histopathological finding is shown in Table 1.

High-risk HPV types were detected in 360 out of 378 specimens (95.5%). Three (0.8%) specimens had low-risk HPV infection. The histopathology of HR-HPV-positive specimens was as follows: 47 specimens with negative histopathology, 5 LSIL lesions (CIN 1), 210 HSIL lesions (15 CIN 2+, 195 CIN 3), and 98 cervical carcinoma specimens (66 squamous cell subgroup +32 adenocarcinoma subgroup).

A total of 282 out of 360 (78.3%) specimens had a single infection with HR-HPV genotype and 78 (21.7%) specimens had a coinfection with two or more HR-HPV genotypes: 61 specimens with 2 genotypes, 12 specimens with 3 genotypes, and 5 specimens with 4 genotypes. Fifteen out of 378 specimens (4%) tested negative for HPV DNA with the following histopathology results: 8 HSIL, 3 squamous cell carcinoma, and 4 adenocarcinoma.

High-risk human papillomavirus genotype distribution

HPV-16 was the most common genotype in patients with a negative histopathological finding (21/49 or 42.9% spec-

imens); in patients with preinvasive lesions (127/219 or 57.9% specimens); and in patients with squamous cell carcinoma and adenocarcinoma (46/69 or 66.7% and 18/36 or 50%, respectively). The results for other HPV types are shown in Table 1.

There were only 5 patients with LSIL, 3 out of whom had single high-risk genotype infection and 2 had multiple infections. HPV-51 was the most common genotype (2 mono-infections and 1 multiple infection).

Figure 1 shows the distribution of the most common HR-HPV types (including those from multiple infections) in specimens with negative histopathological findings, preinvasive lesions, squamous carcinomas, and adenocarcinomas.

HPV-16 was the most frequently detected genotype in the tested specimens regardless of the histopathological diagnosis. Overall, HPV-16 was detected in 212 of 358 specimens (59.2%); 166 mono-infections and 46 multiple infections. The prevalence of other high-risk HPV genotypes in the specimens was as follows: HPV-31 in 45 (12.6%), HPV-18 in 24 (6.7%), HPV-33 in 22 (6.1%), HPV-52 in 20 (5.6%), and HPV-58 in 18 (5.0%).

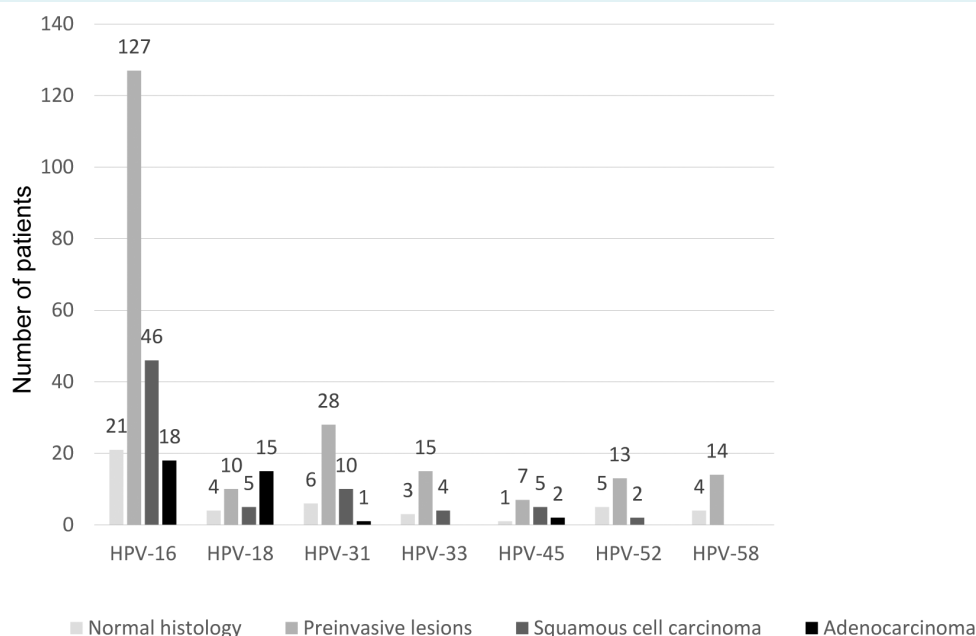


FIGURE 1. Distribution of the most common human papillomavirus (HPV) genotypes (including single and multiple infections) in specimens from women with normal histopathology, preinvasive lesions, squamous cell carcinoma, and adenocarcinoma.

There was no significant difference in the distribution of single and multiple infections in specimens according to different histopathological diagnoses (Pearson χ^2 test, $P=0.899$).

Demographic and clinical findings

The median age was as follows: preinvasive lesions group 35 years, squamous carcinoma group 46 years, adenocarcinoma group 40 years and negative histopathological findings group 35 years (Table 2). The mean parity in the preinvasive lesions group was 1.31 (SD 1.179) children, in invasive lesions group it was 1.89 children (SD 1.146), and in patients with negative histopathological findings it was 1.14 children (SD 1.099). Invasive lesions group, negative histopathological findings group, and preinvasive lesions group significantly differed in the mean age ($F=35.57$, $P\leq 0.001$), number of deliveries ($F=10.60$, $P\leq 0.001$), and smoking ($F=1250$, $P\leq 0.001$), but did not significantly differ in the number of abortions ($F=1.62$, $P=0.19$) and the age of menarche ($F=2.39$, $P=0.09$).

The patients were mostly from Zagreb (165 patients) and Zagreb County (72 patients, a total of 62.7%), but also from other parts of Croatia: Central Croatia 50 (13.2%), Slavonia 44 (11.6%), Dalmatia 34 (9%), and Istria, Primorje-Gorski Kotar County 13 (3.5%).

Human papillomavirus-16 genomic variants in women with high-grade squamous intraepithelial lesions

Due to financial constraints, HPV-16 genomic variants of LCR, E6, and E7 regions were analyzed in 130 randomly selected specimens out of 212 HPV-16-positive specimens.

In total, 35 LCR variants were detected in CIN-3 specimens and one LCR variant was detected in the CIN-2 specimen (Supplementary Figure 1). Five sequences from

CIN-3 specimens were identical to the reference LCR sequence. The analysis of the entire LCR sequence (823 bp, nucleotide [nt] 7155 to 82) showed one deletion and 43 nt substitutions in CIN-3 specimens and 2 nt substitutions in the CIN-2 specimen.

The most distant genetic variant from CIN-3 specimens differed from the reference HPV-16 by 12 nt, whereas the greatest variability of the LCR region in CIN-3 specimens was 1.4%. The most distant genetic variant in the CIN-2 specimen differed from the reference HPV-16 by 2 nt, and the LCR genomic region variability in the CIN-2 specimen was 0.2%. The most common variant detected in CIN-3 specimens was 16-LCR-2, with two mutations (G7193T and G7521A) found in 25/74 (33.8%) isolates. The genomic variant found in the CIN-2 specimen also had 2 mutations (T7230G and A7316G).

Eleven E6 variants were found in CIN-3 specimens (Supplementary Figure 2). The E6 variant in the CIN-2 specimen was identical to the reference sequence. Twenty-five (34.2%) sequences in CIN-3 specimens were identical to the reference E6 sequence. The analysis of the complete E6 sequence (477 bp, nt 83 to 559) showed 18 nt substitutions in CIN-3 specimens. The greatest variability of the E6 region in CIN-3 specimens was 1.67% (8 nt). HPV-16 genomic variant carrying T350G mutation was found in 45/74 (60.8%) CIN-3 specimens.

Four E7 variants were found in CIN-3 specimens (Supplementary Figure 2). The E7 variant in the CIN-2 specimen was identical to the reference sequence. Sixty-six (90.4%) sequences from CIN-3 specimens were identical to the reference E7 sequence. The analysis of the complete E7 sequence (297 bp, nt 562 to 858) showed 5 nt substitutions in CIN-3 specimens. The maximum variability of E7 genomic sequence in CIN-3 specimens was 0.6% (2 nt).

TABLE 2. Distribution of single and multiple human papillomavirus infections (HPV status) according to patients' age and histopathological diagnosis*

| Histopathological analysis | HPV status | | | | Age | | | |
|---|--------------|-------------------|-----------------------------|------------|--------|-----|-----|--------------------|
| | negative (n) | single HPV HR (n) | multiple HPV infections (n) | LR HPV (n) | median | min | max | standard deviation |
| Negative | 0 | 35 | 12 | 2 | 35 | 21 | 69 | 12.41 |
| Low-grade squamous intraepithelial lesions | 0 | 3 | 2 | 0 | 34 | 26 | 50 | 9.78 |
| High-grade squamous intraepithelial lesions | 8 | 167 | 43 | 1 | 35 | 19 | 70 | 9.02 |
| Squamous cell carcinoma | 3 | 52 | 14 | 0 | 46 | 27 | 83 | 11.97 |
| Adenocarcinoma | 4 | 25 | 7 | 0 | 40 | 21 | 69 | 11.86 |

*Abbreviations: HR – high-risk; LR – low-risk.

When analyzing together the LCR, E6 and E7 sequences for each individual isolate, 40 different genomic variants were found in 74 specimens from women with CIN 3 (Table 3). The sequence from the CIN-2 specimen had

a variant in the LCR area, while E6 and E7 were identical to the reference sequence. Overall, 41 genomic variants were found among 74 sequences from CIN-3 and CIN-2 specimens: 73 sequences (98.6%) belonged to the Euro-

TABLE 3. Genomic variants of human papillomavirus virus-16 (HPV-16) in women with cervical intraepithelial neoplasia (CIN) 3, squamous cell carcinoma, and adenocarcinoma; R variants that are identical to the reference sequence of HPV-16*

| Genomic variant (GV) | CIN 3 | | | | | Squamous cell carcinoma | | | | | Adenocarcinoma | | | | | | |
|----------------------|-------------|------------|------------|----|--------|-------------------------|-------------|------------|------------|---|----------------|-----------------|-------------|------------|------------|---|--------|
| | LCR variant | E6 variant | E7 variant | N | Branch | Genomic variant | LCR variant | E6 variant | E7 variant | N | Branch | Genomic variant | LCR variant | E6 variant | E7 variant | N | Branch |
| GV 1 | LCR-R | E6-R | E7-R | 5 | E | GV 1 | LCR-R | E6-R | E7-R | 1 | E | GV 1 | LCR-1 | E6-R | E7-1 | 1 | E |
| GV 2 | LCR-1 | E6-1 | E7-R | 1 | E | GV 2 | LCR-1 | E6-1 | E7-R | 2 | E | GV 2 | LCR-2 | E6-1 | E7-R | 1 | E |
| GV 3 | LCR-2 | E6-2 | E7-R | 20 | E | GV 3 | LCR-1 | E6-2 | E7-R | 1 | E | GV 3 | LCR-3 | E6-2 | E7-R | 1 | E |
| GV 4 | LCR-2 | E6-R | E7-R | 1 | E | GV 4 | LCR-2 | E6-R | E7-R | 1 | E | GV 4 | LCR-4 | E6-3 | E7-R | 1 | E |
| GV 5 | LCR-2 | E6-5 | E7-R | 1 | E | GV 5 | LCR-3 | E6-R | E7-R | 1 | E | GV 5 | LCR-5 | E6-4 | E7-2 | 1 | AA |
| GV 6 | LCR-2 | E6-6 | E7-R | 1 | E | GV 6 | LCR-4 | E6-R | E7-R | 1 | E | GV 6 | LCR-6 | E6-5 | E7-R | 1 | E |
| GV 7 | LCR-2 | E6-7 | E7-R | 1 | E | GV 7 | LCR-5 | E6-R | E7-R | 1 | E | GV 7 | LCR-7 | E6-5 | E7-R | 1 | E |
| GV 8 | LCR-2 | E6-9 | E7-R | 1 | E | GV 8 | LCR-6 | E6-R | E7-R | 1 | E | GV 8 | LCR-8 | E6-5 | E7-R | 1 | E |
| GV 9 | LCR-3 | E6-R | E7-R | 5 | E | GV 9 | LCR-7 | E6-3 | E7-R | 1 | E | GV 9 | LCR-9 | E6-R | E7-1 | 1 | E |
| GV 10 | LCR-4 | E6-2 | E7-R | 1 | E | GV 10 | LCR-7 | E6-4 | E7-R | 1 | E | GV 10 | LCR-10 | E6-R | E7-R | 1 | E |
| GV 11 | LCR-5 | E6-2 | E7-R | 1 | E | GV 11 | LCR-7 | E6-5 | E7-R | 1 | E | GV 11 | LCR-11 | E6-R | E7-R | 1 | E |
| GV 12 | LCR-6 | E6-2 | E7-R | 1 | E | GV 12 | LCR-8 | E6-6 | E7-1 | 1 | NA | | | | | | |
| GV 13 | LCR-7 | E6-2 | E7-R | 1 | E | GV 13 | LCR-9 | E6-R | E7-R | 1 | E | | | | | | |
| GV 14 | LCR-8 | E6-3 | E7-1 | 1 | E | GV 14 | LCR-10 | E6-R | E7-R | 1 | E | | | | | | |
| GV 15 | LCR-9 | E6-2 | E7-R | 1 | E | GV 15 | LCR-11 | E6-R | E7-R | 1 | E | | | | | | |
| GV 16 | LCR-10 | E6-4 | E7-2 | 1 | E | GV 16 | LCR-12 | E6-7 | E7-R | 2 | E | | | | | | |
| GV 17 | LCR-11 | E6-R | E7-R | 1 | E | GV 17 | LCR-13 | E6-R | E7-R | 1 | E | | | | | | |
| GV 18 | LCR-12 | E6-R | E7-R | 1 | E | GV 18 | LCR-12 | E6-8 | E7-R | 1 | E | | | | | | |
| GV 19 | LCR-13 | E6-1 | E7-R | 1 | E | GV 19 | LCR-14 | E6-R | E7-R | 1 | E | | | | | | |
| GV 20 | LCR-14 | E6-R | E7-R | 1 | E | GV 20 | LCR-15 | E6-R | E7-R | 1 | E | | | | | | |
| GV 21 | LCR-15 | E6-R | E7-1 | 3 | E | GV 21 | LCR-16 | E6-R | E7-R | 1 | E | | | | | | |
| GV 22 | LCR-16 | E6-R | E7-R | 1 | E | GV 22 | LCR-17 | E6-9 | E7-R | 4 | E | | | | | | |
| GV 23 | LCR-17 | E6-2 | E7-R | 1 | E | GV 23 | LCR-18 | E6-10 | E7-R | 1 | E | | | | | | |
| GV 24 | LCR-18 | E6-R | E7-R | 1 | E | GV 24 | LCR-19 | E6-9 | E7-R | 1 | E | | | | | | |
| GV 25 | LCR-19 | E6-2 | E7-R | 3 | E | GV 25 | LCR-20 | E6-R | E7-R | 1 | E | | | | | | |
| GV 26 | LCR-20 | E6-2 | E7-R | 1 | E | GV 26 | LCR-21 | E6-R | E7-R | 1 | E | | | | | | |
| GV 27 | LCR-21 | E6-2 | E7-R | 1 | E | GV 27 | LCR-22 | E6-R | E7-R | 1 | E | | | | | | |
| GV 28 | LCR-22 | E6-R | E7-1 | 1 | E | GV 28 | LCR-23 | E6-R | E7-2 | 1 | E | | | | | | |
| GV 29 | LCR-23 | E6-2 | E7-R | 1 | E | GV 29 | LCR-24 | E6-R | E7-2 | 1 | E | | | | | | |
| GV 30 | LCR-24 | E6-2 | E7-R | 1 | E | GV 30 | LCR-25 | E6-R | E7-R | 1 | E | | | | | | |
| GV 31 | LCR-25 | E6-R | E7-R | 1 | E | | | | | | | | | | | | |
| GV 32 | LCR-26 | E6-8 | E7-3 | 1 | Af | | | | | | | | | | | | |
| GV 33 | LCR-27 | E6-R | E7-R | 2 | E | | | | | | | | | | | | |
| GV 34 | LCR-28 | E6-2 | E7-R | 1 | E | | | | | | | | | | | | |
| GV 35 | LCR-29 | E6-2 | E7-R | 2 | E | | | | | | | | | | | | |
| GV 36 | LCR-30 | E6-R | E7-R | 1 | E | | | | | | | | | | | | |
| GV 37 | LCR-31 | E6-2 | E7-R | 1 | E | | | | | | | | | | | | |
| GV 38 | LCR-32 | E6-10 | E7-R | 1 | E | | | | | | | | | | | | |
| GV 39 | LCR-33 | E6-R | E7-R | 1 | E | | | | | | | | | | | | |
| GV 40 | LCR-32 | E6-1 | E7-R | 1 | E | | | | | | | | | | | | |

*Abbreviations: E – European branch; Af – African branch; NA – North American branch; AA – Asian-American branch.

pean branch, while one (1.4%) variant belonged to the African branch.

Human papillomavirus-16 genomic variants in women with squamous cell carcinoma and adenocarcinoma

Twenty-six LCR variants were found in squamous cell carcinoma specimens and 11 LCR variants in adenocarcinoma specimens (Supplementary Figure 3). One isolate in squamous cell carcinoma specimens was identical to the reference LCR sequence. The analysis of the entire LCR region (823 bp, nt 7155 to 82) showed 34 nt substitutions in squamous cell carcinoma specimens and 27 nt substitutions in adenocarcinoma specimens.

In squamous cell cancer, the most genetically distant variant differed from the reference HPV-16 in 15 nt, and the greatest variability of the LCR genomic region specimens was 1.8%. In adenocarcinoma specimens, the most genetically distant variant differed from the reference HPV-16 in 16 nt, and the greatest variability of the LCR genomic region was 1.9%.

The most common variant in squamous cell carcinoma specimens was 16-LCR-17, with two mutations (G7193T and G7521A) found in 4/35 (11%) isolates. In adenocarcinoma specimens, all variants were equally represented (9%), and none of the isolates was identical to the reference sequence.

Eleven E6 variants were found in squamous cell carcinoma and 6 E6 variants in adenocarcinoma specimens (Supplementary Figure 4). Nineteen (54.3%) sequences from the squamous cell carcinoma specimens and 4 (36.3%) sequences from adenocarcinoma specimens were identical to the reference sequence. The analysis of the entire E6 sequence (477 bp, nt 83 to 559) showed 10 nt substitutions in squamous cell carcinoma specimens and 8 nt substitutions in adenocarcinoma specimens. The greatest variability of the E6 region in squamous cell carcinoma specimens was 1.04% (5 nt), and in adenocarcinoma specimens it was 1.25% (6 nt). The genomic variant T350G was found in 15/35 (42.8%) isolates in squamous cell carcinoma specimens and in 6/11 (54.5%) isolates in adenocarcinoma specimens.

Three E7 variants were found in squamous cell carcinoma and 3 E7 variants in adenocarcinoma specimens (Supplementary Figure 4). Thirty-two (91.4%) sequences from squamous cell carcinoma specimens and 8 (72.7%)

sequences from adenocarcinomas were identical to the reference E7 sequence. The analysis of the entire E7 sequence (297 bp, nt 562 to 858) showed 3 nt substitutions in squamous cell carcinoma specimens and 4 nt substitutions in adenocarcinoma specimens. The greatest variability of the E7 region in squamous cell carcinoma specimens was 0.6% (2 nt), and in adenocarcinoma specimens it was 1.0% (3 nt).

When analyzing together the LCR, E6, and E7 sequences for each individual isolate in 35 squamous cell carcinoma specimens, 30 different genomic variants were found (Table 3). When analyzing together the LCR, E6, and E7 sequences in 11 adenocarcinoma specimens, 11 different genomic variants were found (Table 2).

Overall, 44 (95%) isolates in carcinoma specimens grouped into 41 genomic variants belonged to the European branch, one isolate (2.5%) belonged to the North American branch (one specimen of squamous cell carcinoma), and one (2.5%) belonged to the Asian-American branch (one adenocarcinoma specimen).

Human papillomavirus-16 genomic variants in patients with negative histopathological finding

In negative histopathological specimens, 6 LCR variants were found. No sequences were identical to the reference LCR sequence. The analysis of the complete LCR sequence (823 bp, nt 7155 to 82) showed 6 nt substitutions. The greatest variability of the LCR region was 0.36% (3 nt). HPV-16 genomic variant carrying G7193T and G7521A mutations was found in 5/10 (50.0%) specimens.

Two E6 variants were found. Two sequences were identical to the reference E6 sequence. The analysis of the complete E6 sequence (477 bp, nt 83 to 559) showed 1 nt substitution. The greatest variability of the E6 region was 0.21% (1 nt). HPV-16 genomic variant carrying T350G mutation was found in 8/10 (80.0%) specimens.

All E7 variants were identical to the reference sequence. When analyzing together the LCR, E6, and E7 sequences for each individual isolate, 6 different genomic variants were found in 10 specimens.

DISCUSSION

In our study, the most frequent HPV genotype in women from Croatia with histologically confirmed HSIL and cer-

vical cancer was HPV-16. Persistent infection with one or more HPV genotypes with high oncogenic potential has been identified as the most important factor causing cervical neoplasia. At least 12 HR-HPV types have been recognized as carcinogenic: HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, and HPV-59. The prevalence of HR-HPV types varies geographically, with HPV-16 being the most common genotype in premalignant and malignant lesions worldwide.

Cervical cancer is the ninth cause of malignancy in women in Croatia, as at least 300 women develop cervical cancer every year. The prevalence of HR-HPV in the tissue of HSIL and cervical carcinoma has not been systematically studied yet.

Several studies assessed the prevalence of different HPV genotypes, but this was done mostly in the swab specimens, rarely involving histopathological examination. In several cases, HPV DNA was detected in a relatively small number of archival paraffin embedded cervical cancer specimens (20-27). Grce et al in 1997 (21) analyzed 379 scrape specimens from women with previously documented Pap smear cytology classified from 1-4 by in-house PCR method, including consensus primer pair MY09/MY11 and specific primer pairs for HPV6/11, 16, 18, 31, and 33. The study population included 61 specimens with CIN 2, 50 specimens with CIN 3 cytology, and only 18 with CIN 4. However, only 50% of CIN-2 and CIN-3, and 60% of CIN-4 specimens tested positive for HPV. The most commonly detected HPV genotype in HSIL and carcinoma lesions was HPV-16 (18%), followed by HPV-31 in 13% and HPV-18 in only 6% of the cases. Histopathology data were unavailable because of design and methodological limitations, so the study shows only preliminary genotype distribution. Our study, on the contrary, had histopathology evidence for 100% of participants, and HPV was detected in 95% of cases. Fifteen specimens (4%) were HPV-negative (8 HSIL, 3 squamous cell carcinoma, and 4 adenocarcinoma specimens). This is consistent with the previous studies that confirmed the existence of HPV-negative carcinomas (3,33-35), a phenomenon that could be explained by low HPV DNA content, loss of HPV within the tumor, misdiagnosed tumor of another origin, inability of the test to determine certain HPV genotypes, and the existence of cervical cancers independent of HPV.

Milutin-Gasperov et al in 2007 (25) assessed the prevalence of high-risk HPV genotypes among Croatian women by using archival cervical swab DNA specimens from women

with a spectrum of various cytological diagnoses from normal cytology to HSIL (41%), with a very high proportion (25%) of patients with atypical cells of undetermined significance (ASCUS). Overall, HPV DNA was detected in 58.8% of specimens, 64% of which were further genotyped. However, HPV type could not be determined in 35.5% of specimens. In HSIL specimens, the most prevalent type was HPV-16 (23% of cases), followed by HPV-31 (12%), HPV-33 (6.1%), HPV-18 (5%), HPV-52 (2.3%), HPV-58 (1.1%), and HPV-45 (0.9%). Multiple infections were found in 10% of cases. Again, histopathology data were unavailable, and the large number of ASCUS results, which is usually below 5% of cytology findings, indicates that the cytology findings came from different laboratories with different expertise level. In addition, there were no specimens from patients with cervical cancer, so the results were of limited value.

Kaliterna et al in 2007 (22) performed the HPV DNA typing among general population of women from Split-Dalmatia County with unknown cytological diagnosis. Out of 570 tested women, 200 (35%) were HR-HPV positive, with HPV-16 as the most abundant type in 28.5% of the patients, followed by HPV-18 (17.5%), HPV-31 (7.5%), HPV-33 (5.5%), HPV-52 (4%), HPV-59 (2%), and HPV-45 (1%). A large proportion of specimens were of an unresolved genotype (34%).

The same authors in 2013 (23) assessed the HR-HPV prevalence in women from Split-Dalmatia County with reference to cytology results. Out of 1160 tested women, 406 (35%) were HR-HPV positive and 12.2% of all tested samples had an unresolved genotype. Out of those 406 positive results, 148 (36.5%) had HSIL, and a high proportion of women had ASCUS/LSIL – 148 (45.8%). HPV-16 was the most frequent type within each cytological category, with a prevalence of 30.8%. HPV prevalence within the HSIL category was as follows: HPV-16 in 34.5%, HPV-18 in 22.3%, HPV-31 in 6.8%, HPV-33 in 3.4%, HPV-52 and HPV-59 in 2% each, and HPV-45 in 1.4% specimens. There were no histopathological data.

A pilot study by Roksandic-Krizan et al (26) from 2013 conducted in northeast Croatia in 100 women with abnormal cervical cytology analyzed high-risk/intermediate-risk/low-risk HPV prevalence. The authors found multiple infections in 43% specimens, with the most prevalent HR genotypes being HPV-16 (27.6%), HPV-31 (11.8%), HPV-51, and HPV-52 (10.2% each). No data regarding HR-HPV distribution were available within each cytological category, but the authors identified a low number of HSIL cases as the main study limitation.

Marijan et al (24) determined the HR-HPV prevalence in different age groups of women from the Zagreb region. A total of 3440 samples were tested, but the information regarding cervical abnormalities was unavailable for 48% of the specimens. A total of 34% of specimens were identified as ASCUS and only 4.4% samples as HSIL. The HR-HPV prevalence was 34.6%, and it decreased with age. The study limitation was a high proportion of younger patients (44.2% aged 21-30). No data regarding distribution of different HPV genotypes were available.

Grahovac et al (20) explored the HR-HPV prevalence and HPV genotypes among 361 women attending regular gynecological visits. Seventy-two out of 361 women (19.9%) were diagnosed with HSIL, other were without abnormal lesions (56.8%) or had ASCUS/LSIL (23.3%). HR-HPV prevalence increased with the severity of cytological diagnosis, and women with HSIL had HR-HPV prevalence 80.6%. HPV-16 was predominant in all cytological entities. In women with HSIL it was present in 63.8% of patients, while other most frequent types were as follows: HPV-31 (8.6%), HPV-33 (6.9%), and HPV-18 5.2%. There were 10.3% women with indeterminate HPV type. Again, as in other mentioned studies no information regarding histopathology was available.

Our results support the results of Sabol et al (27) that HPV-16 was the most common genotype in HSIL, followed by HPV-31, HPV-58, HPV-33, and HPV-52. However, we cannot directly compare the distribution of HPV genotypes obtained in our study with that obtained by Sabol et al due to a small number of patients with histologically confirmed cancer in their study (N=35), especially with reference to the distribution difference depending on the histological type of cancer.

The present study for the first time explored the genomic heterogeneity of HPV-16, the most common causative agent of HSIL and cervical carcinoma in Croatian patients. It is assumed that some variants carry an increased oncogenic potential. In our study, mainly European genomic variants of HPV-16 were detected both in HSIL and carcinoma specimens.

Similar to our findings, LCR was found to be the most variable segment of the HPV-16 genome in different populations (36,37). At the same time, HPV E7 was highly conserved. The T350G genomic variant of HPV-16 was found in 62.5% of HSIL specimens, in 42.8% of squamous cell carcinoma specimens, and in 63.6% of adenocarcinoma specimens.

Some previous studies (38-41) have indicated a stronger oncogenic potential of non-European genomic variants, while others (42-44) did not support this finding. A German study showed that the oncogenic potential of Asian-American or North American lineages was influenced by polymorphisms in the LCR and possibly other viral genome regions. In the European lineage, this phenomenon appears to be associated with E6 rather than LCR variations (18). In European HPV-16 isolates, polymorphisms in the LCR are more frequent than in the E6 gene (18).

A Slovenian study (45) reported non-European variants only in 5% of the cervical carcinoma specimens, which confirms the predominance of European variants in ethnically homogeneous female populations in Europe.

The present study presents crucial information about the genetic diversity of HPV-16 in southeastern Europe. HPV-16 is the most oncogenic genotype worldwide, and the genomic variants detected in cervical carcinoma specimens represent the most oncogenic variants. The main limitation of our study is a relatively small number of analyzed specimens due to restricted financial resources.

Our results support the previous findings that mostly European HPV-16 variants are discovered in small European populations that are not mixed with different ethnical groups. Nevertheless, further studies are necessary to give more information about the genomic diversity of HPV-16 in cervical carcinoma patients in this part of Europe.

Cervical cancer is the tenth cancer in terms of incidence in Croatian women (46). It is also the third most common cancer among women of fertile age. Therefore, this is a significant public health issue that negatively affects both women's reproductive health and demographic statistics. In 2014, 307 cases of cervical cancer were newly diagnosed, with the highest rate in women aged 45-59 (86.1%) (46). HSIL incidence is the highest in the age-group 30-34, which was the group with the largest share of births in Croatia in 2016 (34%) (47).

Furthermore, as the number of newly discovered patients has not declined since 2010 despite the introduction of the National Early Cervical Cancer Screening Program, there is need for a comprehensive national strategy of cervical cancer prevention to further reduce the incidence of cervical cancer and preinvasive lesions in Croatia. Currently available vaccines, especially the most recent nine-valent vaccine, cover all the most common HPV types detected in

Croatian women with histologically proven lesions (HSIL or cancer), suggesting that this vaccine could be an excellent primary prevention tool in Croatia.

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Declaration of authorship MK, MP, and AV conceived and designed the study; MK, SZL, AP, IG, PP, MC, LH, KFK, MP, and AV acquired the data; MK, SZL, AP, IG, AC, LH, KFK, MP, and AV analyzed and interpreted the data; MK, SZL, AP, IG, LH, KFK, MP, and AV drafted the manuscript; all authors critically revised the manuscript for important intellectual content; all authors gave approval of the version to be submitted; all authors agree to be accountable for all aspects of the work.

Competing interests All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

References

- International Agency for Research on Cancer. GLOBOCAN 2018. Lyon: International Agency for Research on Cancer; 2018. Cancer today. 2018. Available from: <https://gco.iarc.fr/today/>. Accessed: December 6, 2019.
- Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol.* 1999;189:12-9. doi:10.1002/(SICI)1096-9896(199909)189:1<12::AID-PATH431>3.0.CO;2-F Medline:10451482
- de Sanjose S, Quint WG, Alemany L, Geraets DT, Klaustermeier JE, Lloveras B, et al. Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. *Lancet Oncol.* 2010;11:1048-56. doi:10.1016/S1470-2045(10)70230-8 Medline:20952254
- Bosch FX, de Sanjosé S. The epidemiology of human papillomavirus infection and cervical cancer. *Dis Markers.* 2007;23:213-27. doi:10.1155/2007/914823 Medline:17627057
- Bernard HU, Calleja-Macias IE, Dunn ST. Genome variation of human papillomavirus types: phylogenetic and medical implications. *Int J Cancer.* 2006;118:1071-6. doi:10.1002/ijc.21655 Medline:16331617
- de Oliveira CM, Bravo IG, Santiago e Souza NC, Genta ML, Fregnani JH, Tacla M, et al. High-level of viral genomic diversity in cervical cancers: a Brazilian study on human papillomavirus type 16. *Infect Genet Evol.* 2015;34:44-51. doi:10.1016/j.meegid.2015.07.002 Medline:26160543
- Jiang M, Xi LF, Edelstein ZR, Galloway DA, Olsem GJ, Lin WC, et al. Identification of recombinant human papillomavirus type 16 variants. *Virology.* 2009;394:8-11. doi:10.1016/j.virol.2009.08.040 Medline:19758676
- Pande S, Jain N, Prusty BK, Bhambhani S, Gupta S, Sharma R, et al. Human papillomavirus type 16 variant analysis of E6, E7, and L1 genes and long control region in biopsy samples from cervical cancer patients in North India. *J Clin Microbiol.* 2008;46:1060-6. doi:10.1128/JCM.02202-07 Medline:18199779
- Picconi MA, Alonio LV, Sichero L, Mbayed V, Villa LL, Gronda J, et al. Human papillomavirus type-16 variants in Quechua aboriginals from Argentina. *J Med Virol.* 2003;69:546-52. doi:10.1002/jmv.10343 Medline:12601763
- Sabol I, Čretnik M, Hadžisejdić I, Si-Mohamed A, Matovina M, Grahovac B, et al. A new approach for the evaluation of the human papillomavirus type 16 variability with high resolution melting analysis. *J Virol Methods.* 2009;162:142-7. doi:10.1016/j.jviromet.2009.07.029 Medline:19664661
- Sabol I, Matovina M, Gasperov NM, Grce M. Identification of a novel human papillomavirus type 16 E1 gene variant with potentially reduced oncogenicity. *J Med Virol.* 2008;80:2134-40. doi:10.1002/jmv.21304 Medline:19040290
- Tornesello ML, Duraturo ML, Salatiello I, Buonaguro L, Losito S, Botti G, et al. Analysis of human papillomavirus type-16 variants in Italian women with cervical intraepithelial neoplasia and cervical cancer. *J Med Virol.* 2004;74:117-26. doi:10.1002/jmv.20154 Medline:15258977
- Chan SY, Ho L, Ong CK, Chow V, Drescher B, Dürst M, et al. Molecular variants of human papillomavirus type 16 from four continents suggest ancient pandemic spread of the virus and its coevolution with humankind. *J Virol.* 1992;66:2057-66. doi:10.1128/JVI.66.4.2057-2066.1992 Medline:1312620
- Yamada T, Manos MM, Peto J, Greer CE, Munoz N, Bosch FX, et al. Human papillomavirus type 16 sequence variation in cervical cancers: a worldwide perspective. *J Virol.* 1997;71:2463-72. doi:10.1128/JVI.71.3.2463-2472.1997 Medline:9032384
- Yamada T, Wheeler CM, Halpern AL, Stewart AC, Hildesheim A, Jenison SA. Human papillomavirus type 16 variant lineages in United States populations characterized by nucleotide sequence analysis of the E6, L2, and L1 coding segments. *J Virol.* 1995;69:7743-53. doi:10.1128/JVI.69.12.7743-7753.1995 Medline:7494284
- Casas L, Galvan SC, Ordonez RM, Lopez N, Guido M, Berumen J. Asian-american variants of human papillomavirus type 16 have extensive mutations in the E2 gene and are highly amplified in cervical carcinomas. *Int J Cancer.* 1999;83:449-55. doi:10.1002/(SICI)1097-0215(199911)83:4<449::AID-IJC3>3.0.CO;2-0 Medline:10508478
- Hang D, Gao L, Sun M, Liu Y, Ke Y. Functional effects of sequence variations in the E6 and E2 genes of human papillomavirus 16 European and Asian variants. *J Med Virol.* 2014;86:618-26. doi:10.1002/jmv.23792 Medline:24150786
- Kämmer C, Tommasino M, Syrjänen S, Delius H, Hebling U,

- Warthorst U, et al. Variants of the long control region and the E6 oncogene in European human papillomavirus type 16 isolates: implications for cervical disease. *Br J Cancer*. 2002;86:269-73. doi:10.1038/sj.bjc.6600024 Medline:11870518
- 19 Ordonez RM, Espinosa AM, Sanchez-Gonzalez DJ, Armendariz-Borunda J, Berumen J. Enhanced oncogenicity of Asian-American human papillomavirus 16 is associated with impaired E2 repression of E6/E7 oncogene transcription. *J Gen Virol*. 2004;85:1433-44. doi:10.1099/vir.0.19317-0 Medline:15166426
- 20 Grahovac M, Račić I, Hadžisejdić I, Dorić A, Grahovac B. Prevalence of human papillomavirus among Croatian women attending regular gynecological visit. *Coll Antropol*. 2007;31:73-7. Medline:17598508
- 21 Grce M, Husnjak K, Magdić L, Ilijaš M, Zlački M, Lepušić D, et al. Detection and typing of human papillomaviruses by polymerase chain reaction in cervical scrapes of Croatian women with abnormal cytology. *Eur J Epidemiol*. 1997;13:645-51. doi:10.1023/A:1007323405069 Medline:9324210
- 22 Kaliterna V, Andelinović Š, Pejković L, Drmiš Hofman I. Human papillomavirus DNA typing in the cervical specimens among women of Split and Dalmatian County. *Coll Antropol*. 2007;31:79-82. Medline:17598509
- 23 Kaliterna V, Kaliterna M, Pejkovic L, Hofman ID, Andelinovic S. Prevalence and genotyping of the human papillomavirus in the cervical specimens among women of Southern Croatia (Dalmatia County). *Cent Eur J Public Health*. 2013;21:26. doi:10.21101/cejph.a3814 Medline:23741896
- 24 Marijan T, Vraneš J, Mlinarić-Džepina A, Leskovic V, Knežević J, Kvaternik M. Genital human papillomavirus infection in women from the Zagreb region. *Coll Antropol*. 2007;31:83-7. Medline:17600936
- 25 Milutin-Gašperov N, Sabol I, Halec G, Matovina M, Grce M. Retrospective study of the prevalence of high-risk human papillomaviruses among Croatian women. *Coll Antropol*. 2007;31:89-96. Medline:17598510
- 26 Roksandić-Križan I, Bošnjak Z, Perić M, Đurkin I, Zujčić Atalić V, Vuković D. Distribution of genital human papillomavirus (HPV) genotypes in Croatian women with cervical intraepithelial neoplasia (CIN)—a pilot study. *Coll Antropol*. 2013;37:1179-83. Medline:24611331
- 27 Sabol I, Milutin Gašperov N, Matovina M, Božinović K, Grubišić G, Fističić I, et al. Cervical HPV type-specific pre-vaccination prevalence and age distribution in Croatia. *PLoS One*. 2017;12:e0180480-e. doi:10.1371/journal.pone.0180480 Medline:28692681
- 28 Hrvatski zavod za javno zdravstvo. Cijepljenje protiv humanog papiloma virusa (HPV). [Internet] Zagreb: Hrvatski zavod za javno zdravstvo [updated 27.09.2019.] Available from: <https://www.hzjz.hr/aktualnosti/cijepjenje-protiv-humanog-papiloma-virusa-hpv-2018-2019/>. Accessed: December 6, 2019.
- 29 Poljak M, Kocjan B. Poljak M, Kocjan BJ. Commercially available assays for multiplex detection of alpha human papillomaviruses. *Expert Rev Anti Infect Ther*. 2010;8:1139-622010. doi:10.1586/eri.10.104 Medline:20954880
- 30 Platt AR, Woodhall RW, George AL, Jr. Improved DNA sequencing quality and efficiency using an optimized fast cycle sequencing protocol. *Biotechniques*. 2007;43:58. doi:10.2144/000112499 Medline:17695253
- 31 Katoh K, Toh H. Parallelization of the MAFFT multiple sequence alignment program. *Bioinformatics*. 2010;26:1899-900. doi:10.1093/bioinformatics/btq224 Medline:20427515
- 32 Stamatakis A, Hoover P, Rougemont J. A rapid bootstrap algorithm for the RAxML Web servers. *Syst Biol*. 2008;57:758-71. doi:10.1080/10635150802429642 Medline:18853362
- 33 Nicolas I, Marimon L, Barnadas E, Saco A, Rodriguez-Carunchio L, Fuste P, et al. HPV-negative tumors of the uterine cervix. *Mod Pathol*. 2019;32:1189-96. doi:10.1038/s41379-019-0249-1 Medline:30911077
- 34 Tjalma WA, Fiander A, Reich O, Powell N, Nowakowski AM, Kirschner B, et al. Differences in human papillomavirus type distribution in high-grade cervical intraepithelial neoplasia and invasive cervical cancer in Europe. *Int J Cancer*. 2013;132:854-67. doi:10.1002/ijc.27713 Medline:22752992
- 35 Vassilakos P, Tran PL, Sahli R, Low N, Petignat P. HPV-negative CIN3 and cervical cancer in Switzerland: any evidence of impact on screening policies? *Swiss Med Wkly*. 2017;147:w14559. Medline:29185245
- 36 Hubert WG. Variant upstream regulatory region sequences differentially regulate human papillomavirus type 16 DNA replication throughout the viral life cycle. *J Virol*. 2005;79:5914-22. doi:10.1128/JVI.79.10.5914-5922.2005 Medline:15857977
- 37 Kurvinen K, Yliskoski M, Saarikoski S, Syrjänen K, Syrjänen S. Variants of the long control region of human papillomavirus type 16. *Eur J Cancer*. 2000;36:1402-10. doi:10.1016/S0959-8049(00)00121-0 Medline:10899654
- 38 Hildesheim A, Schiffman M, Bromley C, Wacholder S, Herrero R, Rodriguez AC, et al. Human papillomavirus type 16 variants and risk of cervical cancer. *J Natl Cancer Inst*. 2001;93:315-8. doi:10.1093/jnci/93.4.315 Medline:11181779
- 39 Villa LL, Sichero L, Rahal P, Caballero O, Ferenczy A, Rohan T, et al. Molecular variants of human papillomavirus types 16 and 18 preferentially associated with cervical neoplasia. *J Gen Virol*. 2000;81:2959-68. doi:10.1099/0022-1317-81-12-2959 Medline:11086127
- 40 Xi LF, Koutsky LA, Galloway DA, Kuypers J, Hughes JP, Wheeler CM, et al. Genomic variation of human papillomavirus type 16 and risk for high grade cervical intraepithelial neoplasia. *J Natl Cancer Inst*. 1997;89:796-802. doi:10.1093/jnci/89.11.796 Medline:9182978
- 41 Zehbe I, Tachezy R, Mytilineos J, Voglino G, Mikyškova I, Delius H, et al. Human papillomavirus 16 E6 polymorphisms in cervical

- lesions from different European populations and their correlation with human leukocyte antigen class II haplotypes. *Int J Cancer*. 2001;94:711-6. doi:10.1002/ijc.1520 Medline:11745467
- 42 Nindl I, Rindfleisch K, Lotz B, Schneider A, Dürst M. Uniform distribution of HPV 16 E6 and E7 variants in patients with normal histology, cervical intra-epithelial neoplasia and cervical cancer. *Int J Cancer*. 1999;82:203-7. doi:10.1002/(SICI)1097-0215(19990719)82:2<203::AID-IJC9>3.0.CO;2-9 Medline:10389753
- 43 van Duin M, Snijders PJ, Vossen MT, Klaassen E, Voorhorst F, Verheijen RH, et al. Analysis of human papillomavirus type 16 E6 variants in relation to p53 codon 72 polymorphism genotypes in cervical carcinogenesis. *J Gen Virol*. 2000;81:317-25. doi:10.1099/0022-1317-81-2-317 Medline:10644829
- 44 Zehbe I, Wilander E, Delius H, Tommasino M. Human papillomavirus 16 E6 variants are more prevalent in invasive cervical carcinoma than the prototype. *Cancer Res*. 1998;58:829-33. Medline:9485042
- 45 Vrtacnik Bokal E, Kocjan BJ, Poljak M, Bogovac Z, Jancar N. Genomic variants of human papillomavirus genotypes 16, 18, and 33 in women with cervical cancer in Slovenia. *J Obstet Gynaecol Res*. 2010;36:1204-13. doi:10.1111/j.1447-0756.2010.01316.x Medline:21114573
- 46 Hrvatski zavod za javno zdravstvo. Incidencija raka u Hrvatskoj 2015. Šekerija M. ur.[Internet] Zagreb: Hrvatski zavod za javno zdravstvo; 2018; Bilten br. 40. Available from: https://www.hzjz.hr/wp-content/uploads/2018/03/Bilten_2015_rak_final.pdf. Accessed: December 6, 2019.
- 47 Hrvatski zavod za javno zdravstvo. Porodi u zdravstvenim ustanovama u Hrvatskoj 2016. godine. Izvješće za 2016. Rodin U, Draušnik Ž, Cerovečki I. Zagreb: Hrvatski zavod za javno zdravstvo; 2017. Available from: https://www.hzjz.hr/wp-content/uploads/2017/08/Porodi_2016.pdf. Accessed: December 6, 2019.