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Prevalence of Human Papillomavirus Genotypes in Cervical Cancer and Precursor Lesions

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A B S T R A C T

There are no data obtained in biopsy material on the prevalence of human papillomavirus (HPV) and HPV genotypes in Croatian women with cervical carcinoma and precursor lesions. Therefore, the prevalence of HPV and HPV genotypes was investigated in archival material of cervical carcinoma and precursor lesions kept at Department of Pathology, School of Medicine, University of Rijeka. DNA was isolated from formalin fixed, paraffin embedded tissue, histologically classified as cervical intraepithelial neoplasia (CIN) III (n=43), squamous cell carcinoma (SCC) (n=54) and adenocarcinoma (ADC) (n=40). HPV testing was performed by polimerase chain reaction (PCR) using generic and genotype specific primers. The prevalence of HPV DNA was 93.02%, 92.59%, and 92.5% in CIN III, SCC and ADC, respectively. In CIN III and SCC, HPV-16 was the most common high-risk genotype, identified in 65% and 52%, followed by HPV-18 in 22.5% and 28% of cases, respectively. HPV-18 showed a statistically significant prevalence in ADC (67.6%) as compared with SCC (χ^2=9.924; p≤ 0.01). Study results revealed a high prevalence of HPV-DNA in examined cervical lesions (>90%). HPV-16 predominated in SCC and HPV-18 in ADC. Single infection was more frequently present than multiple infections in all three histological groups.

Key words: *cervical intraepithelial neoplasia, cervical carcinoma, HPV genotype prevalence*

Introduction

Approximately 510,000 cases of cervical cancer are reported each year, with nearly 80% in developing countries[1,2]. Epidemiological studies indicate a strong association of high-risk human papillomavirus (HPV) genotypes with cervical carcinoma and malignant transformation of cervical epithelial cells[3]. Recent studies have shown that more than 90% of cervical cancers contain HPV DNA, and some are even suggesting that there is no cervical cancer without HPV infection[4–7]. More than 50 HPV genotypes are known to infect female genital tract, and a subset of ~12 of these are known to have a strong oncogenic potential[8]. Therefore, HPV genotypes have been classified according to their association with cervical cancer and precursor lesions into high-risk (oncogenic) and low-risk HPV genotypes[9]. The most prevalent high-risk HPV genotypes worldwide, which infect uterine cervix, are HPV-16 (~53%), followed by HPV-18 (~15%), HPV-45 (~9%), HPV-31 (~6%) and HPV-33 (~3%)[10].

According to the Croatian National Cancer Registry[11], the incidence of cervical cancer in 2002 was 15.9 new cases *per* 100,000 women, yet there are only a few reports describing HPV genotype distribution in abnormal cervical PAP smears in Croatia[12–14]. There are no data obtained in biopsy material on the prevalence of HPV and HPV genotypes in Croatian women with cervical carcinoma and precursor lesions. Therefore we embarked upon this retrospective analysis to assess the distribution of HPV genotypes in the archival material kept at Department of Pathology, School of Medicine, University of Rijeka. Archival (cervical intraepithelial neoplasia) CIN III and cervical carcinomas were analyzed by polymerase chain reaction (PCR) for the presence of HPV DNA and distribution of HPV genotypes.

Materials and Methods

A total of 137 formalin fixed, paraffin embedded samples were chosen on the basis of availability of tissue in the pathology archive. There were 43 CIN III, 54 squamous cell carcinoma (SCC) and 40 cervical adenocar-

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cinoma (ADC) (including 15 adenocarcinoma *in situ* (AIS) specimens obtained from patients treated at Department of Gynecology and Obstetrics, Rijeka University Hospital Center, during the 1995–2005 period. The samples were histopathologically examined by an expert pathologist at Department of Pathology, School of Medicine, University of Rijeka. The mean age of patients with CIN III diagnosis was 31.5, range 24–50 years. In SCC group there were 21 patients with microinvasive carcinoma (MICA). The mean age of patients with MICA and invasive SCC was 40 (range 30–48) and 46 (range 34–67) years, respectively. The mean age of patients with AIS and invasive ADC was 39 (range 26–53) and 45 (range 43–67) years, respectively.

DNA extraction

Total DNA was isolated from formalin fixed, paraffin embedded samples. Great care was taken on sample sectioning to avoid any contamination between the samples. Depending on the amount of biopsy material embedded in paraffin, 4–10 sections (5 μm thick) were placed in a microcentrifuge tube. The sections were deparaffinized by adding 1 mL of xylene and heating at 55 °C for 30 minutes, followed by centrifugation and subsequent removal of the supernatant. Upon dewaxing with three washes of xylene, 1 mL of 100% ethanol was added to remove residual xylene. The tissues were dried at 37 °C for 30 minutes and DNA was isolated using NucleoSpin®Tissue kit (Macharey-Nagel, Duren, Germany) according to the manufacturer's instructions.

PCR analysis

To assess the quality of extracted DNA, β-globin PCRs were performed using four primer combinations spanning 110, 250, 345 and 408 bp (Takara Biomedicals, Japan). Primers targeting highly conserved regions within the L1 and E6/E7 open reading frame (ORF) were used to detect HPV DNA. These included the $GP5^{+}/GP6^{+15}$ and SPF 10 primers (INNO-LiPA Genotyping, Innogenetics N.V., Ghent, Belgium) of the L1 ORF and primers from Human Papillomavirus Typing Set (Takara Biomedicals, Japan), which amplify sequences within E6 and E7 ORF. The HPV types in positive samples were further characterized by using hybridization assay for identification of HPV genotypes (INNO-LiPA Genotyping, Innogenetics N.V., Ghent, Belgium) and type specific PCR amplifying sequences of HPV-16, 18, and 33 within E6 and E7 ORF (Human Papillomavirus Detection Set, Takara Biomedicals, Japan).

Statistical analysis

HPV prevalence was expressed as percentage of all cases tested for HPV in different histological groups (accounted only once). When determining the prevalence of high- and low-risk HPV genotypes, women were counted more than once if they harbored multiple infections with a mixture of both. The prevalence of individual HPV genotypes was determined as they appeared as either single or multiple infections. Multiple high-risk HPV infection was defined as two or more high-risk HPV genotypes with or without additional low-risk HPV genotypes. The χ^2 test was used to assess statistical significance of differences in the prevalence and distribution of HPV genotypes, and to examine the relationship between multiple and single HPV infection with different histological types of cervical cancer. Statistical significance was established at the $p<0.05$ level.

Results

Of 137 study specimens, 127 (91.97%) were positive for HPV DNA. HPV prevalence according to histological groups is presented in Table 1 and overall high-risk HPV prevalence in Table 2. The prevalence of HPV infection in CIN III, SCC and ADC was 93.02% (40/43), 92.59% (50/54) and 92.5% (37/40), respectively. In total, 10 differ-

TABLE 1
HPV DISTRIBUTION ACCORDING TO HISTOLOGICAL GROUPS

	Histological group		
HPV	CIN III	SCC	ADC
HPV neg.	3 (6.9)	4 (7.4)	3 (7.5)
6/11	–	–	1 (2.5)
16	17 (39.5)	22 (40.7)	3 (7.5)
18	3 (6.9)	6 (11.1)	20 (50)
31	2 (4.6)	3 (5.5)	1 (2.5)
33	–	–	1 (2.5)
45	–	1 (1.8)	–
52	–	1 (1.8)	–
6/11+16	4 (9.3)	–	–
16+18	4 (9.3)	2 (3.7)	1 (2.5)
16+31	–	–	1 (2.5)
18+31	–	3 (5.5)	–
18+33	2 (4.6)	–	–
31+33	–	4 (7.4)	–
31+52	–	1 (1.8)	–
31+70	1 (2.3)	–	–
45+68	1 (2.3)	–	–
6/11+16+18	–	2 (3.7)	4 (10)
6/11+18+31	–	1 (1.8)	–
16+31+33	–	–	1 (2.5)
16+31+52	1 (2.3)	–	–
31+52+74	–	1 (1.8)	–
x	5 (11.6)	3 (5.5)	4 (10)
Total	43	54	40
		137	
HPV pos. (%)	93.02	92.59	92.5

Values are expressed as number of samples and percentage in parentheses, CIN – cervical intraepithelial neoplasia, SCC – squamous cell carcinoma, ADC –adenocarcinoma, HPV neg. – all samples without positive HPV DNA test result, HPV pos. – all samples with positive HPV DNA test result.

TABLE 2
OVERALL PREVALENCE OF HPV GENOTYPES IN HPV DNA POSITIVE SAMPLES.

Sample type (N)	HPV pos (N)	Overall distribution of HPV types										
		6/11	16	18	31	33	45	52	68	70	74	x
CIN III (43)	40	4 (10)	26 (65)	9 (22.5)	4 (10)	2 (5)	1 (2.5)	1 (2.5)	1 (2.5)	1 (2.5)	0 (0)	5 (12.5)
SCC (54)	50	3 (6)	26 (52)	14 (28)	13 (26)	4 (8)	1 (2)	3 (6)	0 (0)	0 (0)	1 (2)	3 (6)
ADC (40)	37	5 (13.5)	10 (27)	25 (67.6)	3 (8.1)	2 (5.4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (10.8)
Total (137)	127	12 (9)	62 (49)	48(38)	20 (16)	8 (6)	2 (2)	4 (3)	1 (1)	1 (1)	1 (1)	12 (9)

Because of the existence of multiple HPV infections, women were counted more than once where appropriate, values are number of samples and percentage in parentheses. CIN – cervical intraepithelial neoplasia, SCC – squamous cell carcinoma, ADC –adenocarcinoma, HPV pos. – all samples with positive HPV DNA test result.

ent oncogenic HPV genotypes were identified: 9 in CIN III, 8 in SCC and 5 in ADC. High-risk HPV genotypes that were most prevalent in all three groups accounted for 87.5% (35/40) of all HPV positive cases of CIN III, 90.0% (47/50) of SCC and 86.4% (32/37) of ADC. Low-risk HPVs were detected as part of mixed low/high-risk HPV infections, except for one ADC case. A great diversity of high-risk HPV genotypes was detected in the group of squamous cell lesions, whereas only 4 different HPV genotypes and HPV-X were identified in the ADC group (Table 1). Overall, HPV-16 was the predominant genotype detected in 26/40 (65%) and 26/50 (52.0%) CIN III and SCC HPV positive cases, respectively. The ADC group showed a predominance of HPV-18 genotype, recorded in 25/37 (67.5%) of all HPV ADC positive cases (Table 2). The observed predominance of HPV-16 in CIN III (χ^2=13.923, p<0.001) and SCC (χ^2=5.717, p<0.025), as well as HPV-18 in ADC (χ^2=11.428, p<0.001) was statistically significant (Table 3).

In CIN III group, 77.5% (31/40) of the samples were positive for a single high-risk HPV genotype and 22.5% (9/40) for multiple high-risk HPV genotypes (Figure 1). The overall prevalence of high-risk HPV genotypes found in CIN III in descending order was as follows: HPV-16 (65%), HPV-18 (22.5%), HPV-X (12.5%), HPV-31 (10%), HPV-33 (4.6%), followed by HPV-45, HPV-52, HPV-68 and HPV-70 (Table 2). Single infection was present in 72% and multiple infections in 28% of SCC cases (Figure 1). Considering overall prevalence of high-risk HPV genotypes in the SCC samples, the predominant genotypes were HPV-16 (52%), HPV-18 (28%), HPV-31 (26%) and HPV-33 (8%), followed by HPV-52, HPV-X, HPV-45 and HPV-74. In the ADC group, 81% (29/36) of the samples were positive for a single high-risk and 19% (7/36) for multiple high-risk HPV genotypes (Figure 1). HPV-18 as the predominant high-risk HPV genotype (67.6%) in the ADC group was followed by HPV-16 (27%), HPV-X (10.8%), HPV-31 (8.1%) and HPV-33 (5.4%) (Table 2). The difference in the predominant HPV-16 genotype distribution in CIN III and SCC *versus* HPV-18 in ADC (χ^2=16.121, p<0.001) was statistically significant, and so was the difference yielded by comparison between SCC and ADC (χ^2=9.924, p<0.01) samples.

Multiple high-risk HPV infections were found in all three groups without a statistically significant association with the histological diagnosis (Figure 1, Table 1).

TABLE 3
DISTRIBUTION DIFFERENCE OF HIGH-RISK HPV GENOTYPES 16 AND 18 IN CERVICAL CANCER AND CIN III.

Diagnosis	n	Pos.	Neg.	HPV-16 +ve (n)	HPV-18 +ve (n)	χ^2-test	p
CIN III	43	40	3	26	9	13.923	0.001
SCC	54	50	4	26	14	5.717	0.025
ADC	40	36	3	10	25	11.428	0.001

CIN – cervical intraepithelial neoplasia, SCC – squamous cell carcinoma, ADC – adenocarcinoma

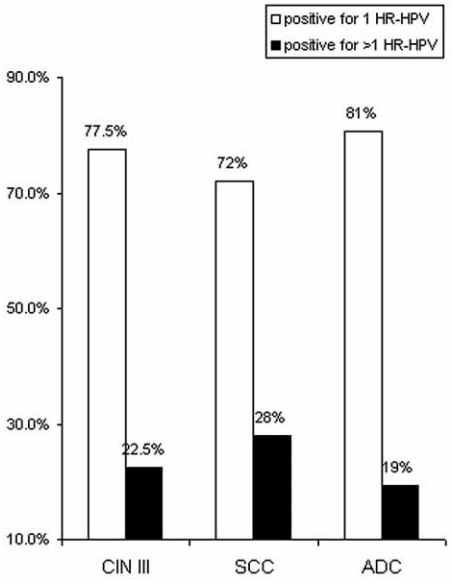

Fig. 1. Prevalence of single and multiple high-risk HPV infections associated with histological groups of cervical carcinoma. HR--HPV – high-risk HPV, CIN – cervical intraepithelial neoplasia, SCC – squamous cell carcinoma, ADC – adenocarcinoma.

Discussion

Epidemiological and molecular biology studies have shown that infection with high-risk HPV is the most important etiologic agent in the pathogenesis of cervical SCC [10,16]. However, the pathogenic role of HPV in cervical adenocarcinoma remains unclear[17–19]. Cross-sectional studies indicate that HPV infection is most frequently found among sexually active young women and that its prevalence decreases with age. In most studies HPV-16 was found to be the most prevalent HPV genotype in cytologically normal women, women with CIN, and women with cervical cancer[7,16]. Yet, recent studies have shown the distribution of HPV genotypes detected in cervical cancer to vary depending on the histological type of the cancer. Whereas HPV-16 is the most frequent genotype in SCC, HPV-18 is a predominant genotype in ADC and AIS[20–23]. Some studies indicate that both HPV-16 and HPV-18 play a prominent role in the development of ADC of the cervix[18,19]. The overall frequency of HPV DNA associated with cervical SCC has been reported to be 91%–100% [7,10]; yet, the HPV DNA detected in adenocarcinomas varies significantly from 32% to 100%, depending on the detection method used [18]. The overall prevalence of HPV DNA detected in our study in all three histological groups was high (>90%). We found that HPV-16 was the most prevalent genotype in CIN III and SCC, whereas HPV-18 was most frequent in ADC. Our results are consistent with previous reports, which also indicate a high occurrence of HPV-16 in CIN III and SCC[22,24]. We demonstrated HPV-18 to be the most common genotype in ADC, which is consistent with several studies conducted in Europe[20,23,25]. In our study, the HPV genotypes detected in ADC were HPV-18, HPV-16, HPV-X, HPV-31 and HPV-33. The only difference between our study and the meta-analysis reported by Clifford *et al*.[23] was that we did not detect HPV-45 genotype in ADC, which could have been due to some specificity of the HPV genotypes found in this area or a smaller sample size. The difference in the prevalence of high-risk HPV-16 and HPV-18 genotypes recorded in SCC and ADC was statistically significant. In most cancer registries, about 75% of all cervical cancer cases are reported as SCC, whereas ADC and adenosquamous cell carcinoma account for 10%–15% of cervical cancer cases, with increasing incidence rates during the last decade, particularly in younger women[26]. The influence of multiple HPV infection on the development of cervical carcinoma is still controversial. It has been shown that multiple high-risk HPV infections were less frequent in high grade than in low grade cervical neoplasia[27]. Multiple infections were significantly less common in ADC, which suggests that the ultimately invasive growth of glandular epithelial cells is triggered by the action of a single HPV genotype rather than by the potentially synergistic action of multiple HPVs[18,28]. Our study revealed a single high-risk HPV genotype to be more frequent in CIN III, SCC and ADC samples.

Taking into account oncogenic potential of HPV-16 and HPV-18, these data deserve special attention in further development of screening programs for cervical cancer in Croatia, as well as future vaccination programs, to lessen the burden of this neoplasm.

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PREVALENCIJA GENOTIPOVA HUMANOG PAPILOMA VIRUSA U KARCINOMU CERVIKSA I PREKURSORSKIM LEZIJAMA

S A Ž E T A K

Ispitali smo prevalenciju humanog papilomavirusa (HPV) i distribuciju HPV genotipova u arhivskom materijalu karcinoma cerviksa i prekanceroznih lezija, prikupljenom u periodu 1995–2005 godina u Zavodu za patologiju, Medicinskog fakulteta Sveučilišta u Rijeci. Iz tkiva uklopljenog u parafin, histološki klasificiranog kao cervikalna intraepitelna neoplazija (CIN) III (n=43), pločasti karcinom cerviksa (SCC) (n=54) i adenokarcinoma cerviksa (ADC) (n=40) izolirana je DNA, a HPV analiza je izvršena pomoću polimeraza lančane reakcije (PCR) koristeći generičke i genotip specifične PCR primere. Prevalencija HPV-DNA utvrđena je u 93.02% slučajeva CIN-a III, 92.59% SCC-a i 92.5% ADC-a. Od visoko rizičnih genotipova HPV-16 je bio najčešći u 65% CIN-ova III i 52% SCC-a, dok je HPV-18 utvrđen u 22.5% CIN-ova III i 28% SCC-a. Pronađena je statistički značajna prevalencija HPV-18 genotipa u ADC-u, 67.6%, u usporedbi sa 28% u SCC-u, (χ^2=9.924; p≤ 0.01). U manje od 15 % slučajeva utvrđene su miješane infekcije s HPV genotipovima 31, 33, 45, 52, 68, 70 i neutvrđenim genotipom X. U sva tri histološka tipa infekcija s jednim HPV-genotipom bila je dominantna u odnosu na miješanu infekciju.