Redni broj članka: 807

ISSN 1331-2820 (Tisak)
ISSN 1848-7769 (Online)

Risk factors and molecular predispositions for cervical dysplasia among women from east Croatia

Čimbenici rizika i molekularne predispozicije za displaziju vrata maternice u žena iz istočne Hrvatske

Magdalena PERIĆ[1)]
Zinka BOŠNJAK[1)]
Snježana DŽIJAN[2)]
Dinko PAULIĆ[1)]
Ivana ROKSANDIĆ-KRIŽAN[1)]
Ivona DJURKIN KUŠEC[3)]
Maja BOGDAN[1,4)]
Dubravka VUKOVIĆ[1)]
Stjepan RUDAN[5)]

[1)]Microbiology Department, Institute of Public Health Osijek-Baranja County, Osijek
[2)]Department of Transfusion Medicine, University Josip Juraj Strossmayer, University Hospital Centre Osijek, Osijek
[3)]Faculty of Agriculture in Osijek, University Josip Juraj Strossmayer, Osijek
[4)]Faculty of Medicine in Osijek, University Josip Juraj Strossmayer, Osijek
[5)]Faculty of Dental Medicine and Health, University Josip Juraj Strossmayer, Osijek

Key words
Carcinogenesis
Human papillomavirus 16
Human papillomavirus 18
Toll-Like Receptor 9
Uterine Cervical Dysplasia

Ključne riječi
karcinogeneza
humani papilomavirus 16
humani papilomavirus 18
Toll-u 9 slični receptori
displazija vrata maternice

Primljeno: 2017–08–20
Received: 2017–08–20
Prihvaćeno: 2017–09–22
Accepted: 2017–09–22

Original scientific paper

Purpose: The aim of this study was to investigate possible association between high-risk Human papillomavirus (HR HPV) – induced cervical infection, HR HPV-related cervical dysplasia, HR HPV genotypes with two Toll-like receptor (TLR) 9 gene polymorphisms and other risk factors.
Methods: During a three-year period, 100 women positive for cervical HR HPV infection (97 with cervical dysplasia and 3 positive women without dysplasia) were genotyped using the Linear Array HPV Genotyping assay (Roche Diagnostics). Furthermore, two polymorphisms of TLR9 (-1486T/C, rs187084 and 2848C/T rs352140) were determined using real-time Polymerase Chain Reaction; 50 HR HPV negative women of similar ethnicity were included as controls.
Results: This study showed that infections with HPV 16 in women with cervical dysplasia were more frequently found compared with HPV 18 infections (p=0.0539). Comparison between HR HPV positive and negative women showed significant association between age >35 years (p=0.0058), being unmarried women (p=0.0001), nocondom usage (p=0.0304) and active tobacco smoking (p=0.0376) with HR HPV cervical infection. No significant associations between two TLR9 gene polymorphisms, HR HPV infection and cervical dysplasia were found.
Conclusion: Our results indicated that: i) women with cervical dysplasia showed significant higher rate of HR HPV 16 infection compared to HR HPV 18, ii) HR HPV – infection was strongly correlated with social risk factors and iii) TLR9 gene polymorphisms (rs187084; rs352140) did not correlate with HR HPV infection and cervical dysplasia. Further genome-wide association studies could open new frontier in understanding the relationship between polymorphisms at TLR9 and immunological mechanisms in HPV-induced carcinogenesis.

Izvorni znanstveni rad

Cilj: Cilj ovog istraživanja bio je utvrditi povezanost između infekcije vrata maternice visokorizičnim humanim papilomavirusima (engl. *high-risk Human papillomavirus* – HR HPV), displazije vrata maternice uzrokovane visokorizičnim genotipovima HPV-a, visokorizičnih genotipova HPV-a te dva polimorfizma gena za Toll-u sličan receptor (TLR) 9 i drugih rizičnih čimbenika.
Metode: Tijekom trogodišnjeg razdoblja, u 100 žena s cervikalnom infekcijom visokorizičnim genotipovima HPV-a (97 s displazijom vrata maternice i 3 s pozitivnim nalazom HPV-a ali bez displazije vrata maternice) određeni su genotipovi virusa primjenom molekularnog testa Linear Array HPV Genotyping assay (Roche Diagnostics). Također su analizirana i dva polimorfizma gena za TLR9 (-1486T/C, rs187084 i 2848C/T rs352140) koristeći metodu lančane reakcije polimerazom u stvarnom vremenu; te je 50 žena s negativnim HR HPV ili sličnog etniciteta uključeno kao kontrolna skupina.
Rezultati: Istraživanje je pokazalo da su HR HPV infekcije genotipom 16 češće u žena sa displazijom vrata maternice u usporedbi sa ženama s infekcijom genotipa 18 (p=0.0539). Usporedbom žena s pozitivnim i negativnim nalazom na HR HPV, utvrđena je značajna povezanost između dobi >35 godina (p=0.0058), bračnog statusa (slobodne osobe, p=0.0001), nekorištenja kondoma (p=0.0304) i aktivnog pušenja (p=0.0376) i infekcije s HR HPV. Povezanost između dva polimorfizma gena za TLR9, infekcije HR HPV i displazije vrata maternice nije dokazana.
Zaključak: Rezultati ovog istraživanja pokazali su da: i) je u žena sa displazijom vrata maternice značajno veća učestalost infekcije s genotipom 16 u usporedbi s genotipom 18, ii) infekcija s HR HPV povezana je sa socijalnim fak-

torima rizika i iii) polimorfizmi gena za TLR9 (rs187084; rs352140) nisu povezani s HR HPV infekcijom i displazijom vrata maternice. Daljnja genomska istraživanja mogu proširiti spoznaje u razumijevanju odnosa između polimorfizma gena za TLR9 i imunoloških mehanizama u HPV-om induciranoj karcinogenezi.

Introduction

Human papilloma virus (HPV) is one of the most common causes of sexually transmitted diseases worldwide [1]. In Croatia with population of 4.4 million, 355 new cases of cervical cancer and about 100 deaths are registered each year [2]. Recent studies have established the central role of humoral immunity to prevent HPV infection. Duration and elimination of HPV is related to innate immunity [1] although most of infections are asymptomatic and spontaneously resolve in 12 – 18 months [3]. Differently abled innate immunity disrupt the clearance of HPV which cause long-term cervical infection that may progress to malignant lesion [1]. Cells in innate immune system can recognize pattern recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLR) as cellular transmembrane PRRs and PAMP receptors have an important role in innate immunity. By recognizing different PAMPs, TLRs generate immune responses to destroy pathogens. At present members of human TLR family, including TLR2, TLR3, TLR4, TLR7, TLR8 and TLR9 have been identified to recognize viruses [4]. TLRs are present on numerous cell types and have the ability to detect and bind different pathogen-associated molecules. This reaction signals the presence of an invading microbe and starts a direct immune response against it. Ligation of TLRs leads to expression of effector molecules, such as pro-inflammatory and immune-mediating cytokines. Ten TLRs have been identified in man and several have been shown to be important for detection of viruses. TLR3, TLR7, TLR8 and TLR9 are localized intracellularly, where they sense the presence of viral nucleic acid. Also, cell surface-expressed TLR2 and TLR4 interact with viral proteins; however the consequence for the host are less clear [5]. At the beginning of HPV infection, the innate immune system creates a proinflammatory microenvironment by recruiting innate immune cells to eliminate the infected cells. To prevent such outcome, HPV express a wide range of strategies for evading immune-surveillance, generating an anti-inflammatory microenvironment. HPV infects keratinocytes in the cervical epithelium and probably stem cells. The keratinocytes represent immune sentinels of the innate immune defense system. They express several TLRs located on the cell surface (TLR1, TLR2, TLR 4, TLR5, TLR6) or in the endosomes (TLR3, TLR9). Endosomal TLRs have an important role in combating viral infection and in recognition of viral nucleic acids; TLR3 recognizes double-stranded deoxyribonucleic acid (dsDNA), TLR7 and TLR8 single-stranded ribonucleic acid (ssRNA), and TLR9 cytidine-phosphateguanosine (CpG)-rich dsDNA. The activation of these receptors results in production of cytokines and creates a powerful pro-inflammatory environment. For example, activation of TLR9 in keratinocytes results in production of tumor necrosis factor alpha (TNF-α), interleukin-8 (IL8), type I interferon (IFN). The main TLR in relation to dsDNA virus infection is TLR9 [6]. Combinations of PAMPs that trigger TLR3 and TLR4 in synergy with TLR7, TLR8 and TLR9 were shown to trigger vigorous type 1 immunity, showing immunological advantages of engaging multiple TLRs. The viruses are detected by TLRs in dendritic cells (DC) first, by the surface TLR2 interacting with the virions, and second, by the intracellular TLR9 recognizing the viral DNA [7]. HPV activates TLR7 and stimulates dendritic cells to produce Th1-type immune response against HPV infection. Alteration in oncogenes, tumor suppressor genes, or abnormal cell adhesion molecules in the TLR-mediated signaling pathway, can lead to inappropriate activation and carcinogenesis, which is possible complication of HPV infection. In addition to their function of activating immune response, TLRs establish a suitable microenvironment for tumor cell growth, which allows tumor cells to evade immune cells, infiltrate, metastasize, and undergo malignant progression. Moreover, TLRs promote tumor cell proliferation and malignant biological behavior by inducing anti-apoptotic protein expression [4]. The final outcome of infection is different for different hosts and different types of HPV. For example, the HPV oncogenes, E6 and E7 are able to block TLR9-induced cytokine secretion by human keratinocytes [8]. The CpG sequences of E6 and E7 HPV oncogenes, can bind to TLR9 and reduce the viral clearance capability of the host, contributing to persistent infection. TLR9 can also promote tumor cells to secrete a variety of cytokines and cell adhesion molecules, increase tumor cell adhesion and invasion, promote tumor cell growth and inhibit tumor cell apoptosis. Even more, TLR9 can induce immune inhibitor production and restrain the attack of cytotoxic lymphocytes on tumor cells, therefore leading to immune escape [4]. HPV 16 is the most carcinogenic type among high risk (HR) sub-group and interferes with innate immunity by affecting the expression of TLRs [9]. Also, HPV 16 is able to evade immune recognition and this evasion results in its persistence. TLRs whose change in expression is significantly different between women with HPV clearance and those with persistence are TLR2, TLR3, TLR7, TLR8 and TLR9 [5]. Infection of human primary keratinocytes with HPV 16 E6 and E7 inhibits TLR9 transcription and consequently leads to functional loss of TLR9-regulated pathways. However, E6 and E7 from low

risk (LR) HPV subtypes are unable to downregulate the TLR9 promoter. In addition, E6 and E7 from HPV 18, which is known to persist less competently in the host than HPV 16, have reduced efficiency compared with HPV 16 in inhibiting TLR9 transcription. Abolishing innate responses may be crucial step involved in the carcinogenic events mediated by HPVs [9]. Activation of TLR9 pathway by CpG pattern is reduced severely in human keratinocytes expressing HPV 16 E6 and E7 oncoproteins. This event is due to the ability of the viral oncoproteins to down-regulate TLR9 mRNA. TLR9 promoter down-regulation is less significant for HPV 18 compared with HPV 16 and is completely absent in cells expressing E6 and E7 in LR HPVs. In this way, the efficiency of HPV 16 in persistent infection appears to correlate with its ability to downregulate the transcription of TLR9. HPV 16 use TLR9 deregulation as a strategy to avoid viral recognition and has ability to interfere with the first response to infection agents via TLRs. HPV 16 E6 and E7 directly inhibit TLR9-mediated pathways by down-regulating the transcription of the TLR9 gene. The mechanism by which viral CpG-DNA pattern enter the endosomal compartment, where TLR9 is expressed, is presently unclear. It is speculated that, during infection, disassembly of the viral capsid in the endosome results in viral DNA release and this may initiate TLR9 signaling [9]. A key result of TLR activation is release of proinflammatory and immune-mediating cytokines, of which IFN-α2 is considered the most immediate product of TLR activation. It is clear that increase in TLR expression in cervical samples with incident HPV 16 infection is associated with viral clearance and lack of with increased persistence [5]. The ability of TLR9 to inhibit HPV induced carcinogenesis has recently been accentuate by the fact that specific TLR9 polymorphisms are associated with an increased risk of cervical cancer among women [8]. A growing amount of evidence suggests that single nucleotide polymorphisms (SNP) in TLR genes are enough to decrease the ability to respond properly to infection [10]. Based upon those findings and unexplainable nature of different immune responses in HPV infections, we investigate whether TLR9 rs187084 and TLR9 rs352140 polymorphisms correlate with susceptibility to HPV infection.

Materials and Methods

Study population

This cross-sectional study included 100 HR HPV positive women and 50 HR HPV negative women from Osijek-Baranja County tested for sexually transmitted diseases (STDs) between March 2009 and December 2011. The mean age of HR HPV positive women was 28±7.2 years (ages 18 to 56) while of HR HPV negative women was 34±9.3 (ages 18 to 60). All women were referred to our department by their gynecologists and the information about their cervical intraepithelial neoplasia (CIN) was obtained from gynecologist's questionnaires. Informed consent was obtained from all HR HPV analyzed patients. Participants completed a questionnaire that included questions on marital and socio-economic status, education level, sexual and smoking habits. Eight incomplete questionnaires were excluded from the statistical analyses of lifestyle risk factors associated with HPV genital infection. The inclusion criteria in sample formation were adult females (≥18 years), sexually active and exclusion criteria juvenile participant (<18 years), sexually inactive, incomplete informed consent. The study was approved by the Ethics Committee of the Institute of Public Health of Osijek-Baranja County and performed according to ethical principles of the 1975 – 1983 Helsinki declarations.

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.

HPV Genotyping

HR/IR/LR genotyping

The HR HPV positive cervical samples were genotyped by the Linear Array HPV Genotyping Test (Roche Diagnostics, Germany). This test detects 37 HR, inter-mediate risk (IR) and 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 visualization. As internal control, test amplifies beta-globin gene to asses cellular adequacy, extraction and amplification for each processed specimen.

Detection of TLR polymorphisms

Blood was collected using EDTA – vacutainer and whole genomic DNA was extracted and purified using a spincolumn based method according to the manufacturer's instruction (High Pure PCR Template Kit, Roche Diagnostics, Germany). DNA was stored at –20 °C for further analysis.

TLR9 polymorphisms were performed using pre-established LightSNiP assay designed by Tib MolBiol (Tib MolBiol Syntheselabor GmbH, Germany) as followed:

1. TLR9 (rs352140) SNP, allele C/T. PCR was performed using 3 μl DNA, 0.5 μM TLR9 primers (forward 5'– GCATCTCGCAGGCAGTCA – 3', reverse 5' – CACGCCCTGCATGCCAA – 3'), 0.225 μM TLR9 sensor probe (5' – TTCACGGAGCTACCACGACTG – 3'), 0.225 μM TLR9 anchor probe (640 – 5' – GCCCTGGACCTCAGCTACAACAGCC – 3'), 3.0 mM MgCl 2, and Light Cycler FastStart DNA mastermix in a total volume of 20 μl. The PCR was performed using LightCycler 1.5 version at following conditions: initial denaturation step (95 °C for 10 min), 40 cycles consisted of denaturation (95 °C for 10 s), annealing (54 °C for 15 s) and extension (72 °C for 25 s). The melting curve analysis involved 1 cycle at 95 °C for 20 s and 40 °C for 20 s, followed by an increase of temperature to 85 °C at a slope of 0.2 °C/s. Melting temperature values were graphically represented as the negative first derivative of the change in fluorescence with temperature (-dF/dT). The C allele of TLR9 rs352140 had a melting peak of 60.1 °C, while T allele had a melting peak of 68.0 °C.

2. TLR9 (rs187084) SNP, allele T/C. PCR was performed using 5 μl DNA, 0.5 μM TLR9 primers (forward 5'– ACCCCAGATCTGGCACTCCCTGAGC – 3', reverse 5' – GCCCAGAGCTGACCTGCTGGGTGTAC – 3'), 0.225 μM TLR9 sensor probe (5' – GAATGTCAGCTTCTTAAGGGCA – 3'), 0.225 μM TLR9 anchor probe (640 – 5' – TGATCTTTTATCTGCATCCCCAGGATC – 3'), 2.5 mM MgCl 2, and Light Cycler FastStart DNA mastermix in a total volume of 20 μl. The PCR was performed using LightCycler 1.5 version at following conditions: initial denaturation step (95 °C for 10 min), 40 cycles consisted of denaturation (95 °C for 10 s), annealing (60 °C for 10 s) and extension (72 °C for 15 s). The melting curve analysis involved 1 cycle at 95 °C for 20 s and 40 °C for 20 s, followed by an increase of temperature to 85 °C at a slope of 0.2 °C/s. Melting temperature values were graphically represented as the negative first derivative of the change in fluorescence with temperature (-dF/dT). The C allele of TLR9 rs187084 had a melting peak of 62.21 °C, while T allele had a melting peak of 67.1 °C.

Statistical analysis

All participants in this study were classified according to their age into two groups: between 18 and 35 years and above 35 years and HR HPV positive and negative infection. A χ^2 test was used for the analysis of age groups, smoking habits, marital status, number of sexual partners, condom usage, monthly income, chronic illness status and daily dose of drugs and distribution of HR HPV infection. The same statistical test was used to analyze the difference between TLR9 rs187084 and TLR9 rs352140, age groups, smoking habits and Pap smear results (CIN1-3, carcinoma in situ). To test associations between three genetic variants of TLR9 rs187084, three genetic variants of TLR9 rs352140 and distribution of the HR HPV infection χ^2 was used. The χ^2 and FisherFreeman-Halton tests were used to determine the difference between three TLR9 rs187084 genotypes distributions in HR HPV women and HR HPV negative women as well as between three TLR9 rs352140 genotypes distributions in HR HPV positive women and HR HPV negative women.

The nonparametric Mann-Whitney U test was used to compare the distribution of two variables. The magnitude of association between TLR polymorphisms and HR HPV infection was expressed as an odds ratio (OR) with a 95 % confidence interval (95 % CI). The Hardy-Weinberg test of the equilibrium of genotype frequencies was performed using the exact test (HWsim program, 50000 Monte Carlo simulation steps). The linkage disequilibrium Lewontin coefficients D' and r^2 were calculated to reflect allelic linkage (Haploview program http://www.broadinstitute.org). All differences were considered to be significant at $p<0.05$. The Statistica 12.0 software, MedCalc Version 10.2.0.0. and Microsoft Office Excel 2003 (Microsoft) programs were used for statistical analyses.

Results

Distribution of HR HPV infection according to risk factors

Analysis of the HR HPV-related risk factors such as age, marital status, number of sexual partner, monthly income, active tobacco smoking and distribution of HR HPV cervical infection in north-east Croatia present significant differences between HR HPV positive and negative women in four risk factors. Table 1 shows that HR HPV infections were more prevalent in 18 – 35 year old women than in women older than 35 years of age (p=0.0058). Moreover, HR HPV infections were significant higher in unmarried women compared to married women (p=0.0001). The no condom use (p=0.0304) and active tobacco smoking (p=0.0376) were significant associated with HR HPV positive infections compared to HR HPV negative women. On the other hand, no significant association was found between: number of sexual partners, working status, monthly income, other chronically diseases, and daily medicament use with HR HPV infections.

Distribution of two SNPs of TLR9 gene in HR HPV positive women and HR HPV negative women

Comparison of distribution of six genetic variants within two polymorphisms of TLR9 (-1486T/C, rs187084 and 2848C/T rs352140) between 100 HR HPV positive and 50 negative women show no significant associations between investigeted polymorphisms, HR HPV infection and cervical dysplasia (Table 2).

Table 1. Statistical analysis of potential risk factors for HR HPV cervical infection

Tablica 1. Statistička analiza potencijalnih čimbenika rizika za HR HPV infekciju vrata maternice

Risk factor	HPV positive N (%)	HPV negative N (%)	OR [95 % CI]*	p-value
Age (years) 18 – 35 > 35	 84 (84) 16 (16)	 32 (64) 18 (36)	 1.00** 0.34 [0.15; 0.74]	 0.0058
Marital status Unmarried Married	 58 (58) 42 (42)	 12 (24) 38 (76)	 1.00** 0.23 [0.11; 0.49]	 0.0001
No. of sexual partners/year 1 > 1	 87 (87) 13 (13)	 45 (90) 5 (10)	 1.00** 1.34 [0.45; 4.01]	 0.5940
Condom use No Yes	 75 (75) 25 (25)	 45 (90) 5 (10)	 1.00** 3.00 [1.07; 8.39]	 0.0304
Monthly income/month 1.000,00 € > 1.000,00 €	 90 (90) 10 (10)	 46 (92) 4 (8)	 1.00** 1.28 [0.38; 4.30]	 0.6910
Chronic diseases No Yes	 93 (93) 7 (7)	 48 (96) 2 (4)	 1.00** 1.81 [0.36; 9.03]	 0.4657
Drugs therapy No Yes	 92 (92) 8 (8)	 48 (96) 2 (4)	 1.00** 2.09 [0.43; 10.22]	 0.3545
Active smoking (20 cigarettes/day) No Yes	 44 (44) 56 (56)	 31 (62) 19 (38)	 1.00** 2.08 [1.04; 4.16]	 0.0376

* OR, odds ratio; CI, confidence interval; ** reference

Table 2. Distribution of two alleles carriers of TLR9 polymorphisms (rs187084 and rs352140) according to the cervical dysplasia

Tablica 2. Raspodjela dva alela nosača TLR9 polimorfizama (rs187084 and rs352140) prema cervikalnoj displaziji

cervical dysplasia	TLR9 (rs187084)		p-value	χ^2
	CC (%)	CT/TT (%)		
negative	18 (12)	35 (23.3)	0.8365	0.043
positive	36 (24)	61 (40.7)		
total	54 (36)	96 (64)		
	TLR9 (rs352140)			
negative	17 (11.3)	36 (24)	0.9272	0.008
positive	29 (19.3)	68 (45.3)		
total	46 (30.7)	104 (69.3)		

Table 3. HPV genotypes distribution according to TLR9 polymorphism (rs187084)

Tablica 3. Raspodjela HPV genotipova prema TLR9 polimorfizmu (rs187084)

HPV genotype	CC (wt[a]) N = 37	TT (mt[b]) N = 63	p-value	χ^2
mixed	16	27	0.9748	0.001
16	9	12	0.6150	0.253
18	0	2	0.2813	1.161
31	3	3	0.5235	0.407
51	3	2	0.3014	1.068
52	2	3	0.8933	0.018
58	0	3	0.1882	1.732

[a]wt-wild type; [b]mt-mutation type

Table 4. HPV genotypes distribution according to TLR9 polymorphism (rs352140)
Tablica 4. Raspodjela HPV genotipova prema TLR9 polimorfizmu (rs352140)

HPV genotype	CC (wt[a]) N = 30	TT (mt[b]) N = 28	CT (ht[c]) N = 42	p-value	χ^2
mixed	13	11	19	0.9517	0.099
16	7	7	7	0.7573	0.556
18	1	0	1	0.6544	0.848
31	3	2	1	0.4317	1.68
51	0	3	2	0.2064	3.156
52	3	0	2	0.2506	2.768
58	0	1	2	0.5107	1.344

[a]wt-wild type; [b]mt-mutation type; [c]ht-heterozygous type

Table 5. Distribution of HPV 16 and HPV 18 according to cervical intraepithelial neoplasia grades (CIN 1-3)
Tablica 5. Raspodjela HPV genotipova prema TLR9 polimorfizmu (rs352140)

No. of cases (single or mixed infection)	CIN 1	CIN 2	CIN 3	p-value	χ^2
16	13	8	13	0.0539	5.839
18	7	2	0		

Table 6. Allele frequencies of TLR9 polymorphisms
Tablica 6. Učestalosti alela TLR9 polimorfizama

	TLR9 (rs187084)		TLR9 (rs352140)	
Nucleotide	cytosine	thymine	cytosine	thymine
Allele frequency	0.39*	0.61*	0.51*	0.49*

* Hardy-Weinberg equillibrum rs187084 and rs352140

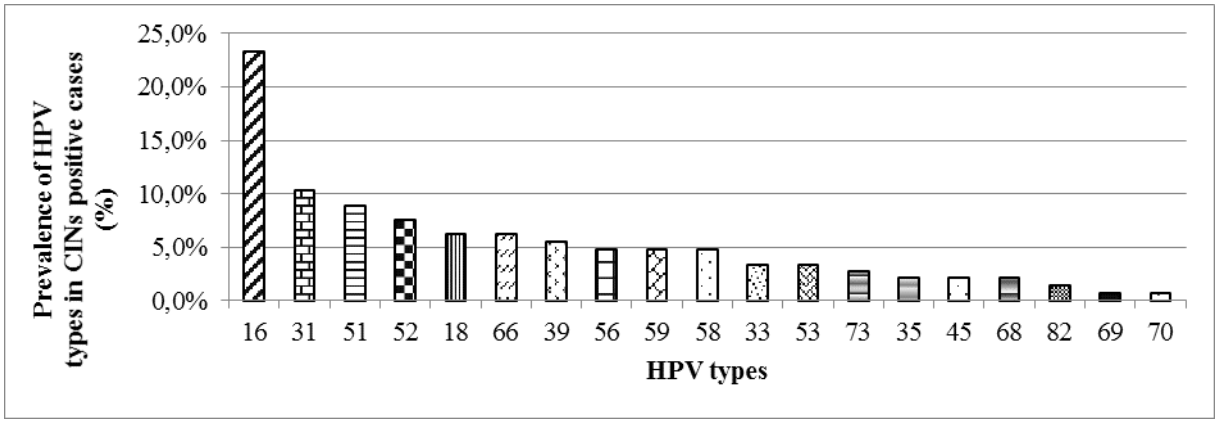

Figure 1. Prevalence of HR HPV genotypes among HR HPV positive women with cervical dysplasia and cervical grades CIN1-3
Slika 1. Prevalencija HR HPV genotipova u HR HPV pozitivnih žena s cervikalnom displazijom i cervikalnim stupnjevima (CIN 1-3)

Distribution of two SNPs of TLR9 gene according to HPV genotypes in HR HPV positive women

Table 3 and 4 show no association between HPV genotypes (HPV 16, 18, 31, 51, 52, 58 and mixed HR HPV infection) and two polymorphisms of TLR9 (-1486T/C, rs187084 and 2848C/T rs352140).

Distribution of cervical dysplasia CIN1-CIN3 according to HPV 16 and HPV 18 cervical infection

Table 5 compares the distribution of three stage of cervical dysplasia (CIN1-CIN3) between number of HPV 16 and HPV 18 infections. Significant difference was found between HPV 16 and HPV 18 cervical infections accord-

ing to cervical dysplasia (p=0.0539). In addition, HPV 16 genotype was more often found in cervical dysplasia compared to HPV 18 genotype.

Prevalence of HR HPV genotypes among HR HPV positive women with cervical dysplasia and cervical grades CIN1-3

Figure 1 shows prevalence of HR HPV genotypes among 97 positive CIN1-3 women. The most prevalent HR HPV was HPV 16 (23.3 %) followed by HPV 31 (10.3 %), HPV 52 (8.9 %), HPV 51 (7.5 %), HPV 18 and 66 (6.2 % each), HPV 39 (5.5 %), HPV 56 and 59 (4.8 % each), HPV 58 (4.1 %), HPV 33 and 53 (3.4 % each), HPV 73 (2.7 %), HPV 35, HPV 45 and 68 (2.1 % each), HPV 82 (1.4 %) and HPV 69 and HPV 70 (0.7 % each).

Distribution of allele frequencies of two SNPs of TLR9 gene

Table 6 shows the distribution of allele frequencies (-1486T/C, rs187084 and 2848C/T rs352140) in our study population. Allele frequencies obtained from our study were strictly similar to those obtained from HapMapCEU study (ss68861046) where C-allele frequency is 0.34 while T-allele frequency is 0.66 for rs187084 while C-allele frequency is 0.52 while T-allele frequency is 0.48 for rs352140.

Discussion

HPV is a viral infection that is passed between people through skin-to-skin contact. It is so common that most sexually active people will get some variety of it at some point, even if they have few sexual partners. It is impossible to know who will develop health problems from HPV, but people with weakened immune systems may be more at risk. However, there are some factors that specifically increase a chance for HR HPV infection. Our study shows that genital HPV infection in women is predominantly acquired in adolescence, and peak prevalence in young adult women (18 – 35). Researchers from all parts of the world suggest largely different prevalence of HPV infection relating age. Worldwide variations in HPV prevalence across age appear to largely reflect differences in sexual behavior across geographical regions [11]. But our analysis showed no significant association with number of sexual partners, probably due to smaller number of participants. The different incidence of HR HPV infection among single and married women implicates that a stable marital relationship protects against HPV infection [12]. The impact of other risk factors, such as avoidance of condom use and active tobacco smoking provides expected results. It is generally thought that, in addition to carcinogenic effects of cigarette smoke on cervical tissue [13], such as genotoxic DNA adducts [14] and the presence of mutagens in cervical mucus [15], the excess risk may be mediated by its effects on immunologic control of HPV infections [16]. Use of male condoms appears to reduce the risk of HPV transmission from men to women, but provides far from absolute protection. Some HPV infections are to be expected despite consistent condom use because the virus can be spread by nonpenetrative genital contact [17]. The rest of socioeconomic factors included in our research, such as working status, monthly income, chronically diseases or daily medicament use, showed no significant association with HPV infection. However, some studies present apparent connection between lower education, higher poverty and potential HPV associated cancers [18].

Cellular and molecular studies over the past years have identified a number of common TLR polymorphisms that modify the cellular immune response and production of cytokines. In addition, human genetic studies suggest that some of these polymorphisms are associated with susceptibility to a spectrum of conditions [19]. TLR9 variants have been reported to have association with many infection and non-infection diseases. To our knowledge, this is the first report on TLR9 polymorphisms and their correlation with HR HPV cervical infection and cervical dysplasia (CIN 1-CIN 3) in HR HPV positive patients from Croatia. Our results have suggested that the investigated two polymorphisms were not associated with HR HPV infection and subsequently stages of cervical dysplasia. This is similar to reports from several other researchers [20, 21] that there are no consistent associations between polymorphism and infection risk, either overall or by type or species. Some studies suggest that this correlation is only marginal, as Pandey et al. showed that the TT genotype of TLR9 (rs352140) displayed borderline significance in increased risk for advanced cervical cancer in a North India population [22]. Contrary to our study, association between TLR9 gene polymorphisms and HR HPV cervical infection was found by the number of others, who claimed that there is a strong connection between certain TLR9 polymorphisms and incidence of cervical dysplasia [23, 24, 25]. These differences in the effect of TLR9 polymorphism on the susceptibility to cervical cancer development between these studies may result from racial heterogeneity, the size of the studied groups, and the action of distinct behavioral and environmental factors [26]. The distribution of two TLR9 gene polymorphisms (-1486T/C, rs187084 and 2848C/T rs352140) in 50 HR HPV negative women and 100 HR HPV positive women from north-east Croatia was comparable to that in European population (the National Center for Biotechnology Information, NCBI). As expected from previous studies, the two SNPs in the TLR9 gene were in linkage disequilibrium. Distribution of those two SNPs of TLR9 gene according to HPV genotypes in HR HPV positive women show no association between HPV genotypes. There is no prevalence of

a single HPV genotype among patients with TLR9 mutation suggesting that it is not the virus responsible for mutation. Similar findings are among other researchers [20], while some present unquestionable connection of certain polymorphism and HPV 16 infection [25]. The fact that some SNPs are more prevalent in younger population (18 – 35 years old) may be due to increased exposure to different HPV types, but the correlation between mutation and a number of sexual partners does not support this hypothesis. The association of certain polymorphism with HPV infection is present only in homozygote variation, indicating a complete mutation is necessary for influence on immune system. What is the cause of TLR9 mutation is presently unknown. We investigate its possible connection with social and environmental factors, but found no association. However, it remains that specific TLR9 polymorphisms could be a genetic risk factor for cervical cancer. Why some SNPs have influence on HPV infection and some not, is inconclusive. There may be some other genetic component that affects baseline TLR expression which results in increased vulnerability to HPV infection of certain population. The cervical vaginal microbiological and immunological milieu is extremely complicated and is affected by a host of environmental and genetic factors which are at the moment beyond our reach [5].

On the other hand, the distribution of cervical dysplasia CIN1-CIN3 according to HPV 16 and HPV 18 cervical infection show significant difference. HPV 16 genotype was more often found in cervical dysplasia compared to HPV 18 genotype. Such outcome is indisputable and equivalent with other studies results [5, 9, 27, 28, 29]. HPV 16 is a member of the human alphapapillomavirus-9 group [30] and has shown to confer a significantly higher risk of developing high-grade CIN lesions or invasive cancers in combination with other HPV types [31]. HPV 16 is the most carcinogenic type among HR sub-group and interferes with innate immunity by affecting the expression of TLRs [9]. This can be explained by much potent influence of HPV 16 protooncogenes comparatively to HPV 18. Also, HPV 16 is able to evade immune recognition and this evasion results in its persistence and indicating by the lack of TLR expression increase in the persistence. Infection of human primary keratinocytes with HPV 16 oncogenes E6 and E7 inhibits TLR9 transcription and leads toward functional loss of TLR9-regulated pathways. In comparison, E6 and E7 from HR HPV 18, which is known to persist less competently in the host than HPV 16, have reduced efficiency in inhibiting TLR9 transcription. The distribution of allele frequencies (-1486T/C, rs187084 and 2848C/T rs352140) in our study population were strictly similar to those obtained from HapMap-CEU study (ss68861046) where C-allele frequency is 0.34 while T-allele frequency is 0.66 for rs187084 while C-allele frequency is 0.52 while T-allele frequency is 0.48 for rs352140. Such allotment suggests that polymorphisms of TLR signaling pathway influence the cellular and molecular mechanisms of different human diseases.

We analyzed distribution between TLR9 polymorphism (rs187084, -1486T/C) in the putative promoter region and synonymous TLR9 polymorphism (rs352140, C2848T) in exon 2 and HPV positive and negative women and their association with HPV cervical infection. No significant difference between both TLR9 polymorphisms (rs187084, rs352140) and cervical HPV infection was observed. Martinez-Campos C et al. reported that genotype TT in the -1486 locus of TLR9 was significantly associated with HPV infection among Mexican women. In present study, genotype TT (63 %) at the same locus is the most prevalent among HPV positive women, which indicates a significant association between analyzed polymorphisms of the TLR9 gene with HPV cervical infection [32].

Meta-analysis of the most intensively studied polymorphisms of TLR9 gene (rs187084, rs352140, and rs5743836) reported that rs187084 polymorphism is associated with increased risk of cervical cancer in Caucasians but not in Asians, whereas rs352140 polymorphism is not associated with any cancer risks neither in Caucasians or Asians [33]. Those findings suggest that diversity between studies could be explained by cancer-types specificity in the positive studied participants and their different carcinogenetic mechanisms, undefined/incomparable features of controls participants and relatively small sample size. In addition, discrepancies in polymorphisms of TLR9 gene between ethnicities indicate a possible influence of genetics and environmental factors (age, sex, smoking, drinking etc.).

After HPV vaccination, microarray studies conducted on HPV infected cells confirmed higher secretion of Th1 cytokines (IFN-γ and interleukin-2) and subsequently, due to above mentioned down regulation of TLR9 receptors, lack of Th1 immune response in HPV positive cervical cells is highly associated with development of HPV cervical infection persistency [34] which contributes with impaired TLR9 signaling and malignant transformation [24].

Due to abolish transcription of TLR9 gene in HR HPV infected cervical keratinocytes and other risk factors such as active smoking, no condom usage and sexual behavior (appeared) suggested that TT genotypes HR HPV positive women from our study may have a greater risk for progression of CIN to cervical carcinoma. However, it should be noted that the present study included low sample size and therefore the results can be considered as preliminary.

It is clear that human variations on genetic level have a strong impact on inflammation and disease susceptibility [19] and host genetic factors of immune related genes may explain the differences in the ability to clear the HPV infection. Additional research has showed that functional variants in xenobiotic metabolism genes have been associ-

ated with cervical cancer risk [35]. Due to such hypothesis, can host genetic factors associated with higher clearance rate of HR HPV serve as a potential biomarker for future HPV related disease [35]? Can it be exploit for therapeutic approach? Is the strong immunologic response in HPV infection desirable or the inflammation could result in DNA damage, which could allow HPV to integrate? Studies have shown that excess TNF-α can result in harmful inflammatory responses, whereas too little can contribute to persistent infection. TNF-α is one of the primary cytokines released after HPV infection and upregulates the expression of antigen-processing and presentation pathway components for class I human leukocyte antigen (HLA) [36]. Has the polymorphism of certain proinflammatory cytokine greater impact on HPV clearance than TLR9 polymorphism? A new association was reported between several TNF polymorphisms and susceptibility to cervical cancer [3]. Accumulative epidemiological evidence suggests that polymorphisms of TLR signaling pathway light up the cellular and molecular mechanisms of human diseases who's gaining a primordial importance [37]. Currently, there is not enough evidence available to understand whether specific infection or diseases are more or less likely to be influenced by TLR polymorphisms. Larger well-designed studies with precise clinical and microbiological phenotyping will be required to validate these observations.

Acknowledgements

This study was supported by the Institute of Public Health of Osijek-Baranja County.

The authors declare that they have no conflict of interests.

Compliance with Ethical Standards

Funding: No funding was received.

Conflict of Interest: The authors declare that they have no conflict of interests.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

References

[1] Fernandes JV, DE Medeiros Fernandes TA, DE Azevedo JC, et al. Link between chronic inflammation and human papillomavirus-induced carcinogenesis. Oncology Letters 2015;9(3):1015–26. doi: 10.3892/ol.2015.2884.

[2] Bošnjak Z, Perić M, Roksandić Križan I, et al. Prevalence and genotype distribution of high-risk human papillomavirus (HR HPV) in male genital samples of Osijek-Baranja County. Coll Antropol 2013;37(4):1203–8.

[3] Bodily J, Laimins LA. Persistence of human papillomavirus infection: Keys to malignant progression. Trends Microbiol 2011;19(1) 33–9. doi: 10.1016/j.tim.2010.10.002.

[4] Hasimu A, Ge L, Li QZ, Zhang RP, Guo X. Expressions of Toll-like receptors 3, 4, 7, and 9 in cervical lesions and their correlation with HPV16 infection in Uighur women. Chin J Cancer 2011;30(5): 344–50. doi: 10.5732/cjc.010.10456.

[5] Daud II, Scott ME, Ma Y, Shiboski S, Farhat S, Moscicki AB. Association between Toll-like Receptors expression and Human Papillomavirus Type 16 Persistence. Int J cancer 2011;128(4): 879–86. doi: 10.1002/ijc.25400.

[6] Amador-Molina A, Hernández-Valencia JF, Lamoyi E, Contreras-Paredes A, Lizano M. Role of Innate Immunity against Human Papillomavirus (HPV) Infections and Effect of Adjuvants in Promoting Specific Immune Response. Viruses 2013;5(11):2624–42. doi: 10.3390/v5112624.

[7] Sato A, Linehan MM, Iwasaki A. Dual recognition of HSV by TLR2 and TLR9 in dendritic cells. PNAS 2006;103:17343–48. doi: 10.1073/pnas.0605102103.

[8] Hasan U. Human papillomavirus (HPV) deregulation of Toll-like receptor 9. Oncoimmunology 2014;3(1):e27257. doi: 10.4161/onci.27257.

[9] Hasan U, Bates E, Takeshita F, et al. TLR9 Expression and Function Is Abolished by the Cervical Cancer-Associated Human PapillomavirusType 16. J Immunol 2007;178(5):3186–97. doi: https://doi.org/10.4049/jimmunol.178.5.3186.

[10] Perić M, Bošnjak Z, Šarkanj B, et al. Polymorphisms of Toll-like receptors 2 and 4 in chronically infected hepatitis C patients from northeast Croatia. Arch Virol 2015;160(1):297–304. doi: 10.1007/s00705-014-2283-0.

[11] Smith JS, Melendy A, Rana RK, Pimenta JM. Age-specific prevalence of infection with human papillomavirus in females: a global review. Adolesc Health 2008;43(4):S5-25, S25.e1-41. doi: 10.1016/j.jadohealth.2008.07.009.

[12] Kero KM, Rautava J, Syrjänen K, Kortekangas-Savolainen O, Grenman S, Syrjänen S. Stable marital relationship protects men from oral and genital HPV infections. Eur J Clin Microbiol Infect Dis 20104;33(7):1211–21. doi: 10.1007/s10096-014-2061-7.

[13] Xi LF, Koutsky AL, Castle PE, et al. Relationship between cigarette smoking and human papillomavirus type 16 and 18 DNA load. Cancer Epidemiol Biomarkers Prev 2009;18(12): 3490–6. doi: 10.1158/1055-9965.EPI-090763.

[14] Phillips DH, She MN. DNA adducts in cervical tissue of smokers and non-smokers. Mutat Res 1994;313:277–84. doi: 10.1002/ijc.2910450417.

[15] Holly EA, Petrakis NL, Friend NF, Sarles DL, Lee RE, Flander LB. Mutagenic mucus in the cervix of smokers. J Natl Cancer Inst 1986;76:983–6.

[16] Szarewski A, Maddox P, Royston P, et al. The effect of stopping smoking on cervical Langerhans' cells and lymphocytes. Bjog 2001;108:295–303. doi: 10.1111/j.1471-0528.2001.00074.x.

[17] Winer RL, Hughes JP, Feng Q, et al. Condom use and the risk of genital human papillomavirus infection in young women. N Eng J Med 2006;354(25):2645–54. doi: 10.1056/NEJMoa053284.

[18] Benard VB, Johnson CJ, Thompson TD, et al. Examining the association between socioeconomic status and potential human papillomavirus-associated cancers. Cancer 2008;113(10):2910–8. doi: 10.1002/cncr.23742.

[19] Misch EA, Hawn TR. Toll-like receptor polymorphisms and susceptibility to human disease. Clin Sci (Lond) 2008;114(5):347–60. doi: 10.1042/CS20070214.

[20] Oliveira LB, Louvanto K, Ramanakumar AV, Franco EL, Villa LL. Polymorphism in the promoter region of the Toll-like receptor 9 gene and cervical human papillomavirus infection. J Gen Virol 2013;94(8):1858–64. doi: 10.1099/vir.0.052811-0.

[21] Wang JL, Yang YZ, Dong WW, et al. Application of human papillomavirus in screening for cervical cancer and precancerous lesions. Asian Pac J Cancer Prev 2013;14(5):2979–82.

[22] Pandey S, Mittal B, Srivastava M, et al. Evaluation of Toll-like receptors 3 (c.1377C/T) and 9 (G2848A) gene polymorphisms in cervical cancer susceptibility. Mol Biol Rep 2011;38:4715. doi: 10.1007/s11033-010-0607-z.

[23] Lai ZZ, Ni-Zhang, Pan XL, Song L. Toll-like receptor 9 (TLR9) gene polymorphisms associated with increased susceptibility of human papillomavirus-16 infection in patients with cervical cancer. J Int Med Res 2013;41(4):1027–36. doi: 10.1177/0300060513483398.

[24] Roszak A, Lianeri M, Sowińska A, Jagodziński PP. Involvement of toll-like receptor 9 polymorphism in cervical cancer development. Molecular Biology Reports 2012;39(8):8425–30. doi: 10.1007/s11033-0121695-8.

[25] Deshpande A, Nolan JP, White PS, et al. TNF-alpha promoter polymorphisms and susceptibility to human papillomavirus 16-associated cervical cancer. J Infect Dis 2005;191:969–76. doi: 10.1086/427826.

[26] El-Omar EM, Ng MT, Hold GL. Polymorphisms in Toll-like receptor genes and risk of cancer. Oncogene 2008;27:244–52. doi: 10.1038/sj.onc.1210912.

[27] Jaisamrarn U, Castellsagué X, Garland SM, et al. Natural history of progression of HPV infection to cervical lesion or clearance: analysis of the control arm of the large, randomised PATRICIA study. PLoS One 2013;8(11):e79260. doi: 10.1371/journal.pone.0079260.

[28] Pista A, Oliveira A, Verdasca N, Ribeiro F. Single and multiple human papillomavirus infections in cervical abnormalities in Portuguese women. Clin Microbiol Infect 2011;17(6):941–6. doi: 10.1111/j.1469-0691.2010.03387.x.

[29] Melchers WJG, Claas HCJ, Quint WGV. Use of the polymerase chain reaction to study the relationship between human papillomavirus infections and cervical cancer. Eur J Clin Microbiol Infect Dis 1991;10(9): 714–27. doi: 10.1007/BF01972496.

[30] Burk RD, Harari A, Chen Z. Human papillomavirus genome variants. Virology 2013;445:232–43. doi: 10.1016/j.virol.2013.07.018.

[31] Trottier H, Mahmud S, Costa MC, et al. Human papillomavirus infections with multiple types and risk of cervical neoplasia. Cancer Epidemiol Biomarkers Prev 2006;15:1274–80. doi: 10.1158/1055-9965.EPI-06-0129.

[32] Martinez-Campos C, Burguete-García AI, Madrid-Marina V. Role of TLR9 in Oncogenic Virus-Produced Cancer. Viral Immunol 2017;30(2):98–105. doi:10.1089/vim.2016.0103.

[33] Wan GX, Cao YW, Li WQ, Li YC, Zhang WJ, Li F. Associations between TLR9 Polymorphisms and Cancer Risk: Evidence from an Updated Meta-analysis of 25,685 subjects. PLoS One 2014; 15(19):8279–85. doi:10.1371/journal.pone.0071785.

[34] Scott M, Nakagawa M, Moscicki AB. Cell-Mediated Immune Response to Human Papillomavirus Infection. Clin Diagn LabImmunol 2001;8(2):209–20. doi:10.1128/CDLI.8.2.209-220. 2001.

[35] Sudenga SL. Host Genetic Factors Associated with Cervical Human Papillomavirus Clearance. Dissertation. University of Alabama, Birmingham; 2013.

[36] Qidwai T, Khan F. Tumour necrosis factor gene polymorphism and disease prevalence. Scand J Immunol 2011;74(6):522–47. doi: 10.1111/j.1365-3083.2011.02602.x.

[37] Zidi S, Sghaier I, Gazouani E, Mezlini A, Yacoubi-Loueslati B. Evaluation of Toll-Like Receptors 2/3/4/9 Gene Polymorphisms in Cervical Cancer Evolution. Pathol Oncol Res 2016;22(2):323–30. doi: 10.1007/s12253-015-0009-6.