Coll. Antropol. **31** (2007) Suppl. 2: 73–77
Short communication

Prevalence of Human Papillomavirus among Croatian Women Attending Regular Gynecological Visit

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A B S T R A C T

Human papillomavirus (HPV) infection has been identified as major risk factor for cervical intraepithelial neoplasia (CIN) and invasive cervical cancer. About 40 HPV viral types are commonly found in the genital tract. Most HPV infections resolve spontaneously, while persistent infection with oncogenic types, namely HPV 16 and 18 is necessary for CIN to occur and progress to cancer. Cervical screening is presently based on the Pap smear that is designed to diagnose precancerous lesions and cervical cancer. The aim of this study was to investigate the prevalence of HPV DNA and to determine HPV types distribution among 361 women attending regular gynecological visit. There were 205 women (29±8 years old) without determined abnormal cervical lesions and 156 women (34±15 years old) with abnormal Pap smear; low grade squamous intraepitehelial lesions (LSIL, n=69), high grade squamous intraepithelial lesions (HSIL, n=72) and atypical squamous cells of undetermined significance (ASCUS, n=15). HPV DNA detection and genotyping was performed by Hybrid Capture 2 assay and additionally by consensus and type-specific primers directed PCR. The overall prevalence of high-risk HPV (hrHPV) in women with abnormal Pap smears was 67.9% (106/156), of which in ASCUS 33.4% (5/15), LSIL 62.3% (43/69) and HSIL 80.6% (58/72). In HPV positive specimens, HPV 16 was found as predominant type in 60.4% cases, followed by HPV 31 (8.5%), HPV 33 (6.6%) and HPV 18 (3.7%). In the group of women without obvious cervical changes the overall hrHPV prevalence was 35.6% with HPV 16 found in 43.8% cases, followed by HPV 31 (17.8%), HPV 33 (9.5%) and HPV 18 (6.8%). In both study groups, women with and without cervical lesions, the prevalence of HPV of indeterminate type was 14.2% and 13.7%, respectively. Our results indicate that cervical intraepithelial lesions are largely associated with HPV type 16, followed by HPV types 31, 33, 18 and HPV of indeterminate type. Although there is a significant difference in hrHPV DNA prevalence among two groups, no significant differences between particular hrHPV types distribution were observed.

Key words: *Cervical intraepithelial lesions, Human papillomavirus(HPV) detection, hybrid capture, HPV prevalence, polymerase chain reaction*

Introduction

Human papillomavirus (HPV) infection has been identified as major risk factor for cervical intraepithelial neoplasia (CIN) and invasive cervical cancer[1,2,]. Epidemiological studies indicate a strong association of high-risk human papillomavirus (hrHPV) genotypes with cervical carcinoma and malignant transformation of cervical epithelial cells[1,2,3].

There are about 40 HPV viral types that are commonly found in the genital tract[4]. The most prevalent hrHPV 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%)[1]. Most HPV infections resolve spontaneously over 6 to 18 months, while viral persistence is necessary for CIN lesions to progress[5]. According to the Croatian National

Received for publication January 31, 2007

Cancer Registry[6], the incidence of cervical cancer in 2004 was 14.4 new cases *per* 100,000 women. There are only a few reports describing HPV genotype distribution in abnormal cervical Pap smears in Croatia[7,8]. Initial screening is presently based on the Pap smear and cytological examination. Cytology-based cervical cancer screening using Pap smears and new technologies such as liquid-based cytology have made significant impact on reducing cervical cancer rates worldwide[10]. Early detection and appropriate treatment of cervical lesions provide the best approach to prevent cervical cancer[11–13]. Treatment of pre-invasive lesions identified in screening programmes is very effective, but care should be taken to avoid unnecessary over-treatment. HPV testing and prophylactic vaccination will change substantially the use of cervical cytology, probably in favour to HPV testing as the primary test in secondary prevention[10].

Current studies evaluate the implementation of HPV testing in screening algorithms for the women with an increased risk for development of cervical cancer and those which could undergo unnecessary re-testing[14,15].

The aim of this study was to investigate the prevalence of HPV DNA and HPV types distribution among 361 women attending regular gynecological visit. According to cytological findings they were separated in the two study groups: women with normal and women with abnormal Pap smears.

Materials and Methods

This study was performed among 361 women attending regular gynecological examination in Zagreb and Rijeka County between 2004 and 2006. Informed consent was sign by all women who participated in the study. Cervical lesions were classified according to »Zagreb 2002« Uniform Classification of Uterine Cervix Cytological Findings in Croatia[9] adapted from the Bethesda system into normal epithelium, low-grade intraepithelial lesions (LSIL or CIN1) indicating a low risk of malignant transformation which may resolve spontaneously, high-grade intraepithelial lesions (HSIL, CIN2 or CIN3-carcinoma *in situ*) having a potential to progress to invasive cervical cancer, and finally borderline Pap smears, classified as atypical squamous cells of undetermined significance (ASCUS). Cervical smears were obtained from 156 women (34±15 years old) with LSIL (n=69) and HSIL (n=72) and ASCUS (n=15). All women from this group were diagnosed by cytological examination followed by subsequent HPV detection and genotyping. A second group of women consisted of 205 women (29±8 years old) undergoing regular preventive gynecological examinations. Women in this group had normal cytological smears and were also tested for the presence of HPV DNA, followed by subsequent genotyping of HPV DNA positive cases. All cervical smears were taken with cervical brush and collected to specimen transport medium (Digene Diagnostic, USA) and stored at +4 °C until testing. Samples were divided into aliquots for hybridization in the liquid phase and polymerase chain reaction (PCR) tests.

Hybrid capture HPV assay

The Hybrid Capture assay (HC2, Digene Diagnostic, USA) is based on signal amplification by chemiluminescent detection[16]. Specimens containing the target DNA hybridize with a specific HPV RNA probe cocktail. The RNA:DNA hybrids are captured onto the surface of a tube coated with antibodies specific for hybrids and detected with a chemiluminescent substrate. The intensity of the light emitted denotes the amount of target DNA in the specimen. RNA probe mix for the detection of hrHPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) was used according to the manufacturer's instructions.

DNA extraction and PCR analysis

DNA was isolated by NucleoSpin®Tissue kit (Macharey-Nagel, Duren, Germany) according to the manufacturer's instructions. Successful DNA extraction was confirmed by the presence of β-globin gene sequence[17]. Detection of HPV DNA was performed by PCR using E6 and E7 consensus primers pU-1M/pU-2R, which are specific for HPV 16, 18, 31, 33, 35, 52 and 58 types[18] (Human Papillomavirus Typing Set, Takara Biomedicals, Japan). HPV typing of HPV 16, 18 and 33 was performed by PCR with primers chosen within the E6 region (Human Papillomavirus Detection Set, Takara Biomedicals, Japan)[19], while HPV 31 was amplified with primers chosen within the E7 region of the HPV genome[3].

PCR products of 228 to 268 bp for generic amplification, and 100 and 140 bp for type-specific amplification were resolved by electrophoresis on 1.5 to 2% agarose gel.

Statistical analysis

HPV prevalence was expressed as percentage of HPV positives against all cases tested for HPV. The prevalence of individual hrHPV genotypes was determined as single infection. Multiple hrHPV infections were defined as two hrHPV genotypes. The distribution of non-continuous cytological variables *versus* HPV status was analysed with the Chi-square test (χ^2). P values of <0.05 were used as the cut-off for statistical significance.

Results and Discussion

High-risk HPV DNA was detected in 179 and 154 out of 361 cervical smears by HC2 and PCR, respectively (Table 1). It was demonstrated that correlation between HC2 and PCR was between 85.8% (91/106) to 86.3% (63/73) This data were additionally confirmed by type-specific PCR amplification which failed to detect any HPV type in 13.7% and 14.2% of cases in the group with CIN and the group with normal cervical findings, respectively, and was referred as HPV of indeterminate types (Table 1).

In 25 women, the positive HPV-DNA results obtained by HC2 test were not confirmed by type-specific PCR amplification for HPV types 16, 18, 31 and 33, suggesting a presence of another HPV type which could be detected by

TABLE 1
HIGH-RISK HUMAN PAPILLOMAVIRUS PREVALENCE AND TYPES DISTRIBUTION IN WOMEN WITH (N=156) AND WITHOUT (N=205) CERVICAL ABNORMALITIES

	n	hrHPV negative		hrHPV positive		HPV 16		HPV 18		HPV 31		HPV 33		HPV indeterminate*		Multiple infection**	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
ASCUS	15	10	66.7	5	33.3	3	60.0	0	-	1	20.0	0	-	0	-	1	20.0
LSIL	69	26	37.7	43	62.3	24	55.8	1	2.3	3	6.9	3	6.9	9	20.9	3	6.9
HSIL	72	14	19.4	58	80.6	37	63.8	3	5.2	5	8.6	4	6.9	6	10.3	3	5.2
Women with abnormal Pap smears (34±15 years)	156	50	32.1	106	67.9	64	60.4	4	3.7	9	8.5	7	6.6	15	14.2	7	6.6
Women with normal Pap smears (29±8 years)	205	132	64.4	73	35.6	32	43.8	5	6.8	13	17.8	7	9.5	10	13.7	6	8.2

hrHPV – high-risk human papillomavirus, *HPV indeterminate: HPV types other than 16, 18, 31, 33, **Multiple HPV infections: positive for HPV 16/18, 16/31 and 18/31

the probes of broader HPV type spectrum in the HC2 assay[16].

Comparison of the HC2 and PCR with hrHPV primers demonstrated concordant results in as many as 86.0% (154/179) of HPV-DNA positive samples. Therefore, PCR method with E7 consensus primers pU-1M/pU-2R which achieved sensitivity comparable to HC2 is also a good method for screening assessments. Development of methods for simple, rapid and accurate detection of HPV DNA has a central role in many strategies designed to reduce the risk of cervical cancer[20,21].

High-risk HPV infection was present in 73 out of 205 (35.6%) women with normal Pap smear (control group) and in 106 out of 156 (67.9%) women with abnormal Pap smear. The hrHPV prevalence is significantly different between these two groups (χ^2=37.060, p≤0.001) (Table 1). Statistically significant difference in hrHPV prevalence was detected in women with LSIL – 62.3% (43/69) and those with HSIL – 80.6% (58/72) compared to women with ASCUS – 33.3% (5/15) (χ^2=14.511, p≤0.001 for trend).

Out of all HPV positive specimens HPV 16 was the predominant type in all cytological entities detectable in 60.0% of ASCUS, 55.8% of LSIL and 63.8% of HSIL as well as in 43.8% women with normal Pap smears (Table 1). In most studies HPV 16 was found to be the most prevalent HPV genotype in cervical cancer, precursors lesions and cytologically normal Pap smears[5,22]. The association of HPV types and histological type of the cancer is well established; HPV16 being the most frequently found genotype in squamous cell carcinoma (SCC), while HPV18 in adenocarcinoma[23]. Our previous study demonstrated the high overall prevalence of HPV-DNA in cervical neoplasia (>90%)[24]. In CIN3 and SCC, HPV 16 was the most common hrHPV type, identified in 65% and 52% of cases, respectively[24]. Grce et al. have demonstrated that among Croatian non-pregnant women increase in hrHPV prevalence is associated with CIN grade changes from 1 to 3 (35.1%, 64.6% and 81.0%, respectively) with HPV 16 being predominant type in all cervical lesions[7].

HPV 18 was detected in 3.7% (4/106) of women with intraepithelial lesions and in 6.8% (5/73) specimens of women with normal smears. HPV 31 and 33 are detected in 8.5% (9/106) and 6.6% (7/106) cases of CIN, respectively, and in 17.8% (13/73) and 9.5% (7/73) of normal smears. The high prevalence of HPV 31 (17.8%) was found in women with normal Pap smears, although the most relevant studies report average prevalence between 5 and 8%[1,3,7,8]. Hadžisejdić et al.[24] have demonstrated the unexpected high prevalence of HPV 31 of 10% and 26% in CIN3 and SCC, but in almost half of these cases as part of multiple infection.

HPV of indeterminate type was found in 14.2% (15/106) and 13.7% (10/73) of cases from abnormal and normal Pap smear groups, respectively. In LSIL cytological group, the prevalence of indeterminate HPV type 20.9% was the highest one, although with no statistical significance. The possible explanation for this finding could be the fact that HPV of indeterminate type could be low- as well as high-risk HPV types; there is always a possibility of cross-hybridization of HC2 RNA probes and in case of PCR with generic primers amplification of a large number of different HPV types.

Low prevalence of multiple infections in LSIL and HSIL as well as in normal Pap smears was found in 6.9%, 5.2% and 8.2% cases, respectively. These data suggest that single hrHPV infection in LSIL and HSIL (>90%) are significant for establishing and maintaining proliferative growth of epithelial cells[25].

Conclusion

Our results indicate that cervical intraepithelial lesions are largely associated with HPV types 16, 31 and 33 followed by HPV 18 and HPV of indeterminate type. A significant difference between hrHPV prevalence among the group of women attending regular examination with normal cytology and the group of women with abnormal Pap smear was observed. However, no significant differences between particular hrHPV types distribution were

observed between the two groups of women. The significance of higher prevalence of HPV 31 type in women with normal Pap smears as well as indeterminate HPV type in low-grade intraepithelial lesions should be considered in future epidemiological studies of hrHPV prevalence in borderline cervical intraepithelial lesions.

Acknowledgment

The Ministry of Science, Education and Sports supported this study (grant number 0196006). We would like to thank Tanja Kovačević and Kornelija Balić for excellent technical assistance.

REFERENCES

1. MUNOZ N, BOSCH FX, DE SANJOSE S, HERRERO R, CASTELLSAGUE X, SHAH KV, SNIJDERS PJF, MEIJER CJLM, N Engl J Med, 348 (2003) 518. — 2. INTERNATIONAL AGENCY FOR RESEARCH ON CANCER (IARC). IARC Handbook of Cancer Prevention. Cervical Cancer Screening, Vol.10, (IARC Press, Lyon, France, 2005) 2. — 3.WALBOOMERS JMM, JACOBS MV, MANOS MM, BOSCH FX, KUMMER JA, SHAH KV, SNIJDERS PJF, PETO J, MEIJER CJLM, MUNOZ N, J Pathol, 189 (1999) 12. — 4. FAUQUET CM, MAYO MA, In: FAUQUET CM, MAYO MA, MANILOFF J, DESSELBERGER U, BALL LA, (Eds) Virus Taxonomy (Elsevier Academic Press, London, New York, 2005). — 5. BOSCH FX, MANOS MM, MUNOZ N, SHERMAN M, JANSEN AM, PETO J, SCHIFFMAN MH, MORENO V, KURMAN R, SHAH KV, J Natl Cancer Inst, 87 (1995) 796. — 6. CROATIAN NATIONAL CANCER REGISTRY, Cancer Incidence in Croatia 2003. Bulletin No 28. (Croatian National Institute of Public Health, Zagreb, 2004) — 7. GRCE M, HUSNJAK K, MATOVINA M, MILUTIN N, MAGDIC L, HUANJAK O, PAVELIC K, J Clin Microbiol 42(2004) 1341. — 8. GRCE M, HUSNJAK K, BOZIKOV J, MAGDIC L, ZLACKI M, LUKAC J, FISTONIC I, SIKANIC-DUGIC N, PAVELIC K, Anticancer Res 21 (2001) 579. — 9. OVANIN-RAKIĆ A, PAJTLER M, STANKOVIĆ T, AUDY-JURKOVIĆ S. Gynecol Perinatol 12 (2003) 148. [In Croat.]. — 10. KITCHENER KC, CASTLE PE, COX JT, Vaccine 24 S3 (2006) 63. — 11. van den GRAFF Y, MOLIJN AC, DOORNEWAARD H, QUINT WGV, van DOORN LJ, van den TWEEL J, Am J Epidemiol, 156 (2002) 158. — 12. WALLIN KL, WIKLUND F, ANGSTROM T, BERGMAN F, STENDAHL U, WADELL G, HALLMANS G, DILLNER J, N Engl J Med, 341(1999)1633. — 13. van DOORN LJ, KLETER B, QUINT WG, Expert Rev Mol Diagn 1,(2001)394. — 14. CUZICK J, MAYRAND MH, RONCO G, SNIJDERS P, WARDLE J, Vaccine 24S (2006) S3/90. — 15. ARBYN M, SASIENI P, MEIJER CJLM, CLAVEL C, KOLIOPOULOS G, DILLNER J, Vaccine 24S3 (2006) S3/78. — 16. CLAVEL C, MASURE M, BORY JP, PUTAUD I, MANGEONJEAN C, LORENZATO M, GABRIEL R, QUEREUX C, BIREMBAUT P, Br J Cancer 80 (1999) 1306. — 17. SAIKI RK, SCHARF S, FALOONA F, MULLIS KB, HORN GT, ERLICH HA, ARNHEIM N, Science 230 (1985) 1350. — 18. FUJINAGA Y, SHIMADA M, OKAZAWA K, FUKUSHIMA M, KATO I, FUJINAGA K, J Gen Virol 72 (1991)1039. — 19. SHIMADA M, FUKUSHIMA M, MUKAI H, KATO I, NISHIKAWA A, FUJINAGA K, Jpn J Cancer Res, 81 (1990) 1. — 20. GRAVITT PE, PEYTON CI, ALESSI TQ, WHEELER CM, COUTLEE F, HILDESHEIM A, SCHIFFMAN MH, SCOTT DR, APPLE RJ, J Clin Microbiol 38 (2000) 357. — 21. SODERLUND-STRAND A, RYMARK P, ANDERSSON P, DILLNER J, DILLNER L, J Clin Microbiol 43 (2005) 3260. — 22. MUNOZ N, J Clin Virol 19 (2000) 1. — 23. ANDERSSON S, SAFARI H, MINTS M, LEWENSOHN-FUCHS, GYLLENSTEN U, JOHANSSON B, Br J Cancer 92 (2005) 2195. — 24. HADŽISEJDIĆ I, ŠIMAT M, BOSAK A, KRAŠEVIĆ M, GRAHOVAC B, Coll Antropol, 30 (2006) 879. – 25. ZIELINSKI GD, SNIJDERS PJ, ROZENDAAL NF, DALMEIJER EK, RISSE FJ, VOORHORST NM, JIWA HC, VAN DER LINDEN FA, DE SCHIPPER A, RUNSINK AP, MEIJER CJ, J Pathol 201 (2003) 535.

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PREVALENCIJA HPV U CERVIKALNIM BRISEVIMA UZETIM PRIGODOM REDOVITOG GINEKOLOŠKOG PREGLEDA

SAŽETAK

Infekcija humanim papilomavirusom (HPV) je jedan od glavnih rizičnih čimbenika za nastanak cervikalne intraepitelijske neoplazije (CIN) i invazivnog karcinoma vrata maternice. Oko 40 HPV tipova je utvrđeno u urogenitalnoj regiji žena. Iako u većini slučajeva infekcija prolazi spontano, trajna infekcija s HPV tipovima visokog rizika, posebice HPV-16 i HPV-18 je neophodna za nastanak CIN-a i invazivnog karcinoma. Probiranje na prekancerozne lezije i karcinom vrata maternice danas se temelji na citološkom pregledu Papa-razmaza. Cilj i svrha ove studije je bila da se ispita prevalencija HPV-DNK i odredi raspodjela HPV tipova visokog rizika u skupini žena (n=361), koje su došle na redovni ginekološki pregled i pristale da sudjeluju u studiji. U skupini je bilo 205 žena životne dobi 29±8 godina s urednim Papa-nalazom i 156 žena u dobi 34±8 godina s promjenama u Papa-nalazu; s niskim stupnjem intraepitelijske lezije (LSIL, engl. *Low grade Squamous Intraepithelial Lesions*) 68 žena, s visokim stupnjem intraepitelijske lezije (HSIL, engl. *High grade Squamous Intraepithelial Lesions*) 72 žene i s atipičnim nalazom pločastih stanica neodređenog značaja (ASCUS, engl. *Atypical Squamous Cells of Undetermined Significance*) 15 žena. Određivanje HPV-DNK i tipizacija provedeni su metodama Hybrid Capture 2 (HC2) i lančanom reakcijom polimeraze (PCR, engl. Polymerose Chain Reaction) temeljenim na koncenzus i tip-specifičnim početnim oligonukleotidima. Ukupna prevalencija HPV-DNA visokog rizika u žena s abnormalnim Papa-razmazom bila je 67,9% (106/156) i to s ASCUS-om 33,4% (5/15), LSIL-om 62,3%

(43/69) i HSIL-om 80,6% (58/72). Od svih HPV-pozitivnih uzoraka u toj skupini, HPV-16 ja nađen u 60,4% žena, HPV--31 u 8,5%, HPV-33 u 6,6% i HPV-18 u 3,7% žena. U skupini žena bez promjena na vratu maternice, ukupna prevalencija HPV-DNK visokog rizika je utvrđena u 35,6% žena, s udjelom HPV-16 od 43,8%, HPV-31 s 17,8%, HPV-33 s 9,5% i HPV-18 s 6,8%. U obje skupine, prevalencija neodređenog HPV-tipa bila je 14,2% i 13,7%. Naši rezultati su pokazali da su intraepitelijska oštećenja vrata maternice udružene s HPV-tipovima visokog rizika i to 16, 31, 33 i 18 u više od 85% slučajeva, dok preostali dio predstavljaju netipizirani HPV-tipovi.