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Presence of histopathological premalignant lesions and infections caused by high-risk genotypes of human papillomavirus in patients with suspicious cytological and colposcopy results – a prospective study

Prisustvo patohistoloških premalignih lezija i infekcija visokorizičnim genotipovima humanih papilomavirusa kod bolesnica sa sumnjivim citološkim i kolposkopskim nalazima – prospektivna studija

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Abstract

Background/Aim. In patients with premalignant cervical lesions, human papillomavirus (HPV) infection, at any moment, may be spontaneously eliminated, or may persist or transform cervical epithelium from a lower to a higher degree. Due to that, it is necessary to wisely select the patients who are at high risk of cancer development. The aim of the study was to establish the interdependence between a suspicious Papanicolaou (Pap) test and colposcopy with the infection caused by high-risk genotypes of human papillomavirus and the presence of premalignant cervical lesions. **Methods.** This prospective study used cytological, colposcopy, real-time polymerase chain reaction (PCR) of high-risk genotypes of human papillomavirus and histopathological analysis of cervical biopsy specimen. Out of 2,578 female patients sent to cytological analyses in Clinical Center of Montenegro, during 2012, 2013 and 2014, the study included 80 women who had to submit their biopsy specimens due to a suspicious Pap test and atypical colposcopy results. **Results.** In the group of 80 (3.1%; n = 80/2,578) of the selected female patients with suspicious Pap test and colposcopy, 2/3 or 56 (70%) of them had cervicitis, and 1/3 or 24 (30%) had cervical intraepithelial neoplasia. The most common type in cervical intraepithelial neoplasia was HPV16 in 8 female patients, *ie* 61.53% out of the number of infected, or 33.33% out of the total number of premalignant lesions. **Conclusion.** Patients with suspicious Papanicolaou test, colposcopy results and infection which is caused by high-risk HPV infection (HPV 16 in particular) often have premalignant cervical lesions. In these cases, histopathological confirmation of lesions is mandatory, since it serves as a definitive diagnostic procedure.

Key words:
papillomaviridae; uterine diseases; uterine neoplasms; vaginal smears; histology; primary prevention.

Apstrakt

Uvod/Cilj. Kod premalignih lezija grlića materice infekcija visokorizičnim genotipovima humanih papilomavirusa (HPV) može spontano biti eliminisana, opstajati ili dovesti do transformacije epitela grlića nižeg u viši stepen. Zato je važno pravilno odabrati bolesnice koje su u visokom riziku od obolevanja od karcinoma. Cilj studije bio je da se ustanovi međuzavisnost sumnjivog Papanikolau testa i kolposkopskog nalaza sa infekcijom visokorizičnim genotipovima HPV i prisustvom premalignih lezija grlića materice. **Metode.** U ovoj prospektivnoj studiji korištena je citološka, kolposkopska, lančana reakcija polimeraze (PCR) u realnom vremenu visokorizičnih genotipova HPV, kao i patohistološka analiza bioptata grlića materice. Istraživanje je obuhvatilo 2 578 žena u Kliničkom Centru Crne Gore, koje su upućene na citološku analizu tokom 2012, 2013, i 2014. godine. Posmatrano je 80 žena kojima je indikovana biopsija grlića

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materice zbog sumnjivog Papanikolau testa i atipičnog kolposkopskog nalaza. **Rezultati.** U grupi od 80 (3,1%; n = 80/2 578) odabranih bolesnica sa sumnjivim Papanikolau testom i kolposkopijom, njih 2/3 ili 56 (70%) bilo je sa cervicitisom, a 1/3 ili 24 (30%) sa cervikalnom intraepitelnom neoplazijom. Najčešće zastupljeni pojedinačni tip kod bolesnica sa cervikalnom intraepitelnom neoplazijom bio je HPV16 kod 8 bolesnica, tj. 61,53% od broja inficiranih ili 33,33% od ukupnog broja premalignih lezija. **Zaključak.** Kod bolesnica koje imaju sumnjiv Papanikolau test i kolposkopski nalaz i infekciju visokorizičnim genotipovima HPV (posebno HPV16) česta je pojava cervikalnih intraepitelnih neoplazija. U tim situacijama, obavezna je patohistološka provera lezije, kao odlučujući dijagnostički postupak.

Ključne reči:
papilloma virus, humani; materica, bolesti; materica, neoplazme; vaginalni brisevi; histologija; preventivno-medicinska zaštita.

Introduction

Premalignant phase of planocellular cervical cancer is a phase-continuous process of epithelial change, from low degree lesions, classified as low-grade squamous intraepithelial lesion – LGSIL, to lesions of more severe degree, classified as high grade squamous intraepithelial lesion – HGSIL, caused by persistent human papillomavirus (HPV) infection. Carcinogenesis of cervical cancer is a process spanning more years. In this period it is possible to detect infection, virus type, degree of epithelial lesion and apply an appropriate patient treatment. HPV infection of cervical epithelium is a sexually transmitted disease which is highly contagious. At any moment, infection may be spontaneously eliminated, persist or lead to the transformation of cervical epithelium of a lower to a higher degree, due to which it is necessary to wisely select the patients who are at high risk of cancer development [1].

The highest incidence of premalignant intraepithelial cervical lesions is in women aged between 25 and 35. The average life expectancy with diagnosed carcinoma *in situ* is 35 years, and with invasive carcinoma between 48 to 52 years. It has been proven that HPV is the main cause of cervical cancer, but there are a number of contributing factors, such as: smoking, promiscuous behaviour, other sexually transmitted diseases (*Chlamydia trachomatis*, *Herpes simplex* virus type 2, *Cytomegalovirus*), partners' sexual behaviour, socioeconomic status, genetic, hormonal and immunological status of a woman [2].

The majority (80%) of fatal outcomes happens in developing countries which is chiefly due to the lack of preventive programmes for timely detection of premalignant cervical lesions [3]. The preventive programmes include different screening methods: cytology [Papanicolaou (Pap test)], colposcopy, diagnostics of cervical infection by HPV, and pathohistological biopsy specimen. The sensitivity of these methods is different and their combination provides for timely diagnostics of premalignant lesions and cervical cancer prevention [4].

Even though cytological examination of cervical epithelium is primarily used as a primary screening method for the detection of premalignant lesions, today current researches are directed towards finding an optimum screening method to make it possible to assess cervical cancer risk. Some researches show it could be done by combining diagnostic cytopathology and verification of cervical HPV DNA or RNA.

Modern medicine can cure premalignant intraepithelial lesions, therefore, timely diagnostics of intraepithelial lesions is of crucial importance. Use of different diagnostic procedures and their mutual combination increases sensitivity of diagnostics of cervical epithelium changes. With the combination of different diagnostic procedures it is possible to detect 98% of pathological cervical lesions [4].

The aim of this study was to determine the importance of cytological examination (Pap test) in relation to the detection of premalignant cervical lesions, determine the association of HPV DNA diagnostics (high risk) and of premalignant cervical lesions, establish the interdependence between suspicious Pap test and the presence of premalignant cervical lesions, and establish the interdependence between HPV DNA (high risk) and the presence of premalignant cervical lesions, and determine what is the most common type of high-risk HPV DNA in cervical precancerous lesions.

Methods

This prospective study used cytological, colposcopy, HPV DNA (high risk) diagnostics and histopathological analysis of cervical biopsy specimen.

The female patients were made familiar with the proposed diagnostic procedures and gave their consent. Then, they completed a questionnaire containing the questions about age, marital status, menarche, the first intercourse, number of partners, use of contraception and its type, and social status.

This research included 2,578 female patients who had cytological analyses during 2012, 2013 and 2014, and then selected 80 women who had to submit their biopsy specimens due to a suspicious Pap test – atypical squamous cells of undetermined significance (ASCUS; *ie* Pap III A and B) and atypical colposcopy results. All women, apart from Pap test and pathohistological examination, went through HPV DNA diagnostics. The included women were of all ages.

Pap test was performed in such a way that we took a swab prior to bimanual examination and microbiological analysis of the swab, since blood and lubricants must not contaminate the sample. While taking cytological swab, the whole cervix must be visible. If there is excessive secretion, it is necessary to remove it by physiological saline and if there are no signs and symptoms of cervical lesions, a swab should be taken between 10 and 16 days from the beginning of menstrual cycle. A patient is placed in a position typical for gynecological examination. Speculum is cleaned with

physiological saline, lighting should be good, and with an appropriate endocervical brush we collect cells form ectocervix, then from cervical canal with relatively subtle rotations, taking into account that rotations do not cause bleeding, which would contaminate the swab and make further analysis of cytological results more difficult. Swab content should be immediately placed on a Petri dish, within a couple of seconds. It must not be allowed for the swab to dry up in the air, since cytological readings will be compromised. The smear on a petri dish shoud be completely soaked in 95% alcohol and sprayed with polyethylene glycol. A Petri dish should be adequately labelled together with a form which contains: patient's name and family name, last menstruation cycle date, regularity of menstrual cycle, the number of given births, miscarriages and if there were any previous abnormal swabs or treatments. The form should also contain information on using contraceptive pills or intrauterine device [4].

HPV DNA infection diagnostics was performed by real time polymerase chain reaction (RT PCR). In order to take a swab for the diagnostics of HPV infection, a patient assumes the lithotomy position, vaginal wall is spreaded, middle part of the brush with longer bristles (used for cervical swabs specimen, single use only) is put deep into the cervical canal and the rest of the brush with shorter bristles is in contact with the external part of the cervix. The brush is rinsed in a specialised solution – PreservCyt-Solution, which is located in a sampling bottle, the brush is dipped 10 times in the solution and then rotated on the bottom of the bottle. The brush is then removed from the bottle and the lid placed so that the marked line of the lid passes the marked line of the bottle. The bottle is then labelled, the label contains name and family name of the patient, and then all data are inserted into medical documentation of the patient [5].

The swabs taken in such manner are used to detect high-risk HPV genotypes (Abbott High Risk HPV DNK test, Abbott Molecular, USA) in the specimen by PCR method. This analysis is performed on Applied Biosystems 7500 Real-Time PCR System.

Cervix tissue biopsy specimen is taken with a patient in the lithotomy position. External gynaecological organs and the vagina should be cleaned and then vaginal wall spread. Cervix is grasped by a tentaculum and pulled in the direction of vaginal axis which enables cervical tissue biopsy specimen to be taken. Biopsy specimen should be stored in a dish with formalin together with a document form for a specialist of histopathological medicine. The form contains cytological status of the patient and an obligatory information on a possible, previous premalignant lesion of the patient with date and treatment manner. The form was contains patient's personal data: name, family name, age. The cervix tissue biopsy specimen is used for the histopathological diagnostics [6].

Statistical data were analysed using SPSS version 17 software (SPSS Inc., Chicago, IL, USA). Due to small sample size, only descriptive statistics for scores was performed (medians and percentages). The processed data and the results were presented in tabular and graphical forms. Data processing and analysis used the following statistical methods: descriptive statistics, χ^2 test and Fisher's exact test.

Results

Out of 2,578 female patients who had cytological analyses during 2012, 2013 and 2014, this study included 80 (3.1%) who had to submit their biopsy specimens due to an abnormal Pap test and atypical colposcopy results. All the women, apart from Pap test and pathohistological examination, went through HPV DNA diagnostics. The included women were of all ages, the youngest patient was 19, and the oldest one 74 (Figure 1).

In the group of 80 of the selected female patients with abnormal Pap test, 2/3 or 56 (70%) had cervicitis, and 1/3 or 24 (30%) had cervical intraepithelial neoplasia (CIN) (Figure 2).

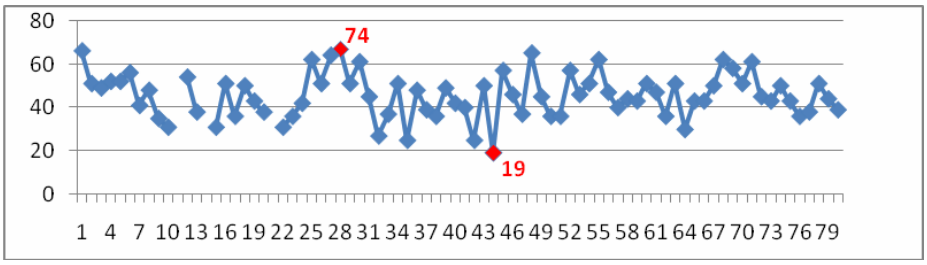

Fig. 1 – Age range of all the patients.

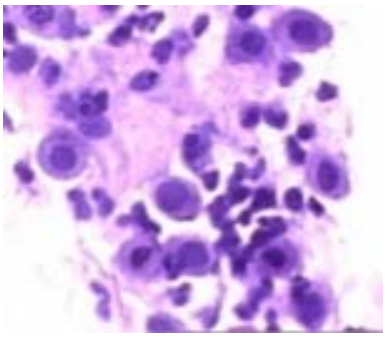

Fig. 2 – Suspicious cytological findings (Papanicolaou, ×400).

CIN I was present in 12 (15%) patients, CIN II in 3 (3.75%), and CIN III in 9 (11.25%) of patients. LGSIL and HGSIL lesions were present in 15%.

Of 80 examined female patients, HPV infection was present in 32 (40%). Combined HPV types were verified in 11 (13.75%) patients, out of the total number of the patients and 39.39% out of the total number of the infected (Table 1, Figure 3).

tal number of infected (16.66%) of all CIN I patients. Others were combined with other genotype.

The group CIN II contained 3 female patients, out of which 1 (33.33%) was with HPV positive genotype. This case involved the combination of HPV31, 33 and 45 genotypes.

The group CIN III contained 9 female patients, out of which 8 (88.88%) were with HPV positive genotype.

Table 1

Frequency of histopathological parameters

Histopatological variables	Frequency (n)	Percentage (%)
Suspicious findings	80/2,578	3.1
CIN I	12	15
CIN II	3	3.7
CIN III	9	11.25
CERVICITIS	56	70
LGSIL	12	15
HGSIL	12	15
CIN	24	30
HPV positive cervicitis	19	33.92
HPV positive CIN	13	54.16

CIN – cervical intraepithelial neoplasia; LGSIL – low-grade squamous intraepithelial lesion; HSIL – high-grade squamous intraepithelial lesion; HPV – human papillomavirus.

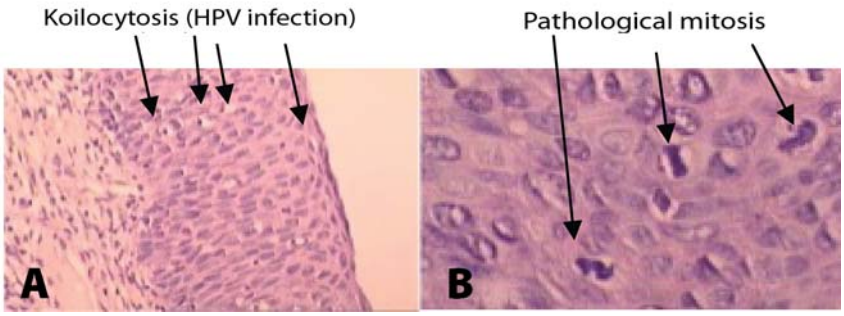

Fig. 3 –Histopathological characteristics of cervical intraepithelial neoplasm in CIN III with A) Koilocytosis (HE ×200); B) Pathological mitosis (HE ×400). CIN – cervical intraepithelial neoplasia; HPV – human papillomavirus.

Out of 56 females with cervicitis, 19 (33.92%) had HPV infection (23.75% of the total number patients). The most commonly individual type present was HPV45 in 8 (42.10%) patients or 14.28% out of the total number of patients with cervicitis. The second individual most common type was HPV31 in 5 (26.31%) patients or 8.92% out of the total number of patients with cervicitis. Common types in cervicitis were found in 4 (21.05%) cases or 7.14% out of the total number of patients with cervicitis.

Out of 24 female patients with cervical intraepithelial neoplasm 13 (54.16%) had HPV infection. The incidence of HPV infection was higher in patients with CIN (54.16%) compared to the patients with cervicitis (33.92%).

The most common type in the CIN was HPV16 in 8 (61.53%) female patients out of the number of infected or 33.33% out of the total CIN number. Combined types in CIN were found in 7 (53.84%) cases out of the number of infected or 29.16% out of the total number of CIN.

The group CIN I contained 12 female patients, out of which 4 (33.33%) were with HPV positive genotype. The most common was HPV16 in two cases (50%) out of the to-

Fisher's test confirmed a statistically significant difference between the presence of HPV infection in patients with CIN III compared to those with cervicitis ($p = 0.002$, $p < 0.01$).

The most common genotype was HPV16 in 6 (75%) of the infected female patients or (66.66%) out of all CIN III patients. The frequency of HPV 16 infection was higher in the infected patients with CIN III (75%) when compared to the patients with cervicitis (10.52%). The second most common genotype was non-existent.

Fisher's test confirmed a statistically significant difference between the presence of HPV16 infection in the patients with CIN III, in comparison to those with cervicitis ($p = 0.026$, $p < 0.05$).

Discussion

Out of all the patients with Pap test for cytological analysis, 3.1% had ASCUS findings. Such percentage is the golden mean of all reports. Namely, Rinku et al. [7] described the presence of ASCUS in 5.3% of Pap tests during the screening procedure. Their data correlate to the results found in

other studies [8]. When it comes to the neighbouring countries of the West Balkan Region, one of the studies conducted in the Republic of Serbia by Ravić [9] found that out of 17,350 women which participated in the screening procedure, 1,038 (5.98%) had suspicious/positive cytological and/or colposcopy result which led then to further histological diagnostics. In one earlier research in the town of Karlovac screening encompassed 2,076 women and, due to suspicious/positive cytological and or colposcopy results in 14.3% of them, biopsy procedure was performed [10]. Additionally, in the 70s and 80s in the municipality of Šabac, 399,203 gynecological exams were performed in order to have organised screening of female genital organs cancer. All the women went through Pap test and colposcopy. Due to suspicions changes indicative of *portio vaginalis uteri* (PVU), biopsy specimens were taken from 2.21% of women [11]. Generally speaking, published data on the percent of pathological-cytological results after the conducted screening procedures is very different and it ranges from 2% to 3% [12–21], 5% to 6% [22–24], 7% to 8% [25–29]. In 2003, France, Luxembourg and Finland reported percentage which is lower than 1.2% [30–32]. Very high percentage, from 10% up to even 25%, was reported from Ibadan, Nigeria, and Taiwan [33, 34]. Interesting data come from France, since different parts of France reported diametrically different data. Namely, they conducted pilot studies on organised screenings. The Isère Department reported only 1.2% of abnormal Pap tests, the Bouches-du-Rhône department reported 4.94%, and the Doubs department as much as 15.11% [35]. Multiple factors influence the number of suspicious/positive Pap tests within screening. One of the most fundamental reason is health of the population. Certainly, this numbers shall be lower in countries which systematically organise cervical cancer screening. One of the most important factors is the quality of cytological laboratory.

The percentage of cases with chronic cervicitis, 56 (70%), is close to the percentage in the study. In the study conducted by Zhang et al. [36], out of 875 female patients with ASCUS, 553 (63.2%) were diagnosed with chronic cervicitis, and Ravić [9] reported that out of 1,038 biopsy specimens, taken on the basis of suspicious and positive Pap and/or colposcopy results, 612 (58.9%) were benign [9]. Massad et al. [37] found lower percentage of benign histological results (45%).

There were 24 (30%) CIN cases, CIN I was present in 12 (15%) of the cases, CIN II in 3 (3.75%), and CIN III in 9 (11.25%). To sum up, LGSIL lesion was present in 15% of the patients and the same percent (15%) was related to HGSIL. Ravić [9] explains that in biopsy specimens, taken on the basis of suspect and positive Pap tests and/or colposcopy results, there were 37.57% of CIN. LGSIL was verified in 268 (25.82%), HGSIL in 122 (11.75%) of the patients. In some studies the percentage of patients diagnosed with LGSIL ranges from 13% to 22%, HGSIL from 26% to 27% [37–39]. Massad et al. [37] stated that there were 33% of cervical dysplasia. Similar distribution and total percentage as in our research can be found in the study of Zhang et al. [36], (33.02%) with cervical intraepithelial neoplasia, out of which 165 cases were with CIN I (18.9%), 45 (5.1%) cases with CIN II, 79 (9.0%) cases with CIN III. Lower percentage is found in the ASCUS-LSIL Triage Study (ALTS) Group. During a two-year monitoring with ASCUS 26% were diagnosed with CIN, out of which LGSIL was present in 15%, and HGSIL in 11% of the cases (6% CIN; 2.5% CIN3) [40]. Higher incidence of CIN I-III (40–66%) than in our research can be found in some other studies [41–43]. Patel et al. [41] in his retrospective study which included 19,215 Pap smears, conducted in the Gujarat Oncology Hospital, found that the presence of CIN III in cytologically detected ASCUS was 38.89% [42]. Similar distribution to found in our study can be found in Rinku et al. [7] with 23% for LGSIL in comparison to 7%–25% in other studies [44]. Rinku et al. [7] found that HGSIL incidence is 11.7% and other authors [45] from 4%–17%.

Out of 80 examined female patients, high risk HPV infection was present in 32 (40%) of them. Higher incidence can be found in a study Jordan et al. [45] (in 41%–50% cases of ASCUS, HPV test was positive). Also, higher percentage can be found in the paper written by Planinić et al. [46]. In women with ASCUS, HPV DNA of high-risk genotypes was detected in (46%) of samples (n = 19/41). Out of 56 female with cervicitis, 19 (33.92%) had HPV infection, or 23.75% of the total number of those with HPV infection. Higher percentage than this can be found in a study of Planinić et al. [46] where HPV DNA was detected in 62 (36.9%) out of 168 of women with cervicitis, and the most frequently detected was DNA of other high-risk genotypes (36/168; 21.4%) and HPV-16 DNA (11/168; 6.5%). Out of 24 female patients with cervical intraepithelial neoplasm, 13 (54.61%) had HPV infection. Much higher percentage can be found in a study of Crum et al. [47] where HPV sequence was detected in 85% of all biopsies and it contained precancerous changes.

In our research most common type in CIN was HPV16 in 8 (61.53%) female patients out of the total number of infected or 33.33% out of the total CIN number. CIN III group contained 9 female patients, out of which 8 (88.88%) were with HPV positive genotype. The most common was HPV16 in 6 (75%) infected female patients or 66.66% of all CIN III patients. In a study of Insinga et al. [48] the most present types of high-risk HPV16 and/or HPV18 were present in 52% of detected CIN2 lesions, 61% for CIN**.** Moscicki et al. [49] presented results, and stated that HPV16 was present in 50% of high-grade CIN [49, 50]. Similar data can be found a paper of in Arbyn and Dillner [51], and they state that HPV 16 and 18 cause half of high-grade cervical squamous intraepithelial lesions and 25% of low-grade cervical squamous intraepithelial lesions. Much higher incidence was reported in a paper of Huang et al. [52], who, in paraffin-embedded biopsy specimen, found HPV-16 in 5 (83.3%) out of 6 cases of CIN I and in 10 (90.9%) out of 11 cases of CIN II/III. Lungu et al. [53] study confirmed that LGSIL changes were exceptionally heterogeneous and out of them any of 40 HPV genotypes can be extracted.

Conclusion

The percentage of suspicious results of Pap tests shown in this study is the golden mean of all reports. We deem it to be the result of quality work performed by the cytological laboratory. Synchronous determination of Pap test and HPV is obligatory,

especially after a suspicious Pap test and abnormal colposcopy. This approach enables classification of women into groups with higher or lower risk of premalignant lesion. One method cannot go without the other. The incidence of HPV infection is higher in patients with cervical intraepithelial neoplasm compared to patients with cervicitis. HPV16 is the most common single cause of cervical intraepithelial neoplasm (especially CIN III). Patients with suspicious Papanicolaou test, colposcopy results and infection caused by high-risk HPV infection (HPV 16 in particular) often have premalignant cervical lesions. In these cases, pathohistological confirmation of the lesion is mandatory, as a definitive diagnostic procedure. This is the only way to make quality diagnostics and provide adequate monitoring and valid treatment to our female patients.

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