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Liquid-Based Cytology – New Possibilities in the Diagnosis of Cervical Lesions

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A B S T R A C T

Liquid-based cytology (LBC) enables the use of supplementary methods in the diagnosis and prognosis of cervical lesions. The aim of this study was to analyze the correlation between $p16^{INK4a}$ immunoexpression in ThinPrep cervical cytologic samples and human papillomavirus (HPV) detection by polymerase chain reaction (PCR) from the same sample. LBC-ThinPrep (Cytyc, USA) cervical cytology samples, prepared and stained by Papanicolaou method, were analyzed using modified Bethesda cytologic classification named »Zagreb 2002«. A second ThinPrep slide, prepared from the same sample, was immunostained for $p16^{INK4a}$ using CINtec $p16^{INK4a}$ Cytology Kit (DakoCytomation, Denmark). Increased expression of the high-risk (HR) HPV E6 and E7 oncogenes results in a highly specific increase in p16 protein expression and overexpression of $p16^{INK4a}$ acts as a potential biomarker for cervical cancer progression from premalignant lesions. Brown nuclear and/or cytoplasmic staining of abnormal cells was considered a positive result. Residual material was used for 13 HR HPV-DNA detection by the PCR based AMPLICOR HPV test (Roche Molecular Systems). A total of 120 ThinPrep Pap tests with the following cytologic diagnoses: 17 within normal limits, 17 atypical squamous cell (ASC) (7 ASC of undetermined significance /ASCUS/ and 10 ASC of high-grade squamous intraepithelial lesions cannot be excluded /ASC-H/), 26 low-grade squamous intraepithelial lesions (LSIL) corresponding cervical intraepithelial neoplasia (CIN) I, 57 high-grade SIL (HSIL) i.e. 24 CIN II and 33 CIN III and 3 squamous cell carcinoma (SCC) were included in the study. All CIN III (n=33) and SCC (n=3) specimens expressed $p16^{INK4a}$ immunoreactivity, whereas the HR HPV test was positive in 97% (32/33) of CIN III and 100% (3/3) of SCC specimens. The $p16^{INK4a}$ biomarker was positive in 87.5% (21/24) of CIN II and 69% (18/26) of CIN I, while the HR HPV was positive in 75% (18/24) of CIN II and 50% (13/26) of CIN I. In ASCUS cytology, $p16^{INK4a}$ and HR HPV showed the same rate of positivity (28.5%; 2/7). Expression of $p16^{INK4a}$ was detected in all cytologic (10/10) ASC-H lesions, in contrast to HR HPV detected in only 20% (2/10) of ASC-H cases. These data suggest the $p16^{INK4a}$ evaluation in ThinPrep cervical samples to be significantly associated with HR HPV testing by PCR in the same sample for the diagnosis of HSIL lesions and cervical carcinomas. A prospective study with longer follow up may clarify the predictive values in the management of LSIL and ASC diagnosis.

***Key words**: cervical intraepithelial neoplasia, cervical cancer, liquid-based cytology, human papillomavirus, $p16^{INK4a}$*

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Introduction

The Papanicolaou (Pap) test has been the most successful cancer screening test in the history of modern medicine. Since its introduction in the 1940s, the conventional Pap test has dramatically decreased the morbidity and mortality of cervical cancer by identifying and classifying cellular and morphological changes associated with progression to cancer, but the disease has not been eradicated[1,2]. Among other factors, particularly sampling errors, the inadequate efficiency of the screening program results also from inaccurate cytology findings, largely because the evaluation of the Pap test relies on individual subjective diagnostic skills and experience[3,4]. This indicates the need for adjunct methods, among them liqid-based cytology (LBC)[5,6] and specific biomarkers ($p16^{INK4a}$, L1)[7–11]. LBC has been developed in the last few decades as an alternative to conventional cytology reporting to increase the sensitivity of cervical cytology and the proportion of slides that are satisfactory for assessment[5,6,12–15]. LBC slides can be read more quickly than conventional cytology slides[15] and the liquid sample can be used for human papillomavirus (HPV) DNA testing and for other molecular tests[16–19]. Epidemiological studies have clearly established that persistent infection with high-risk human papillomavirus (HR HPV) is the primary risk factor for the development of cervical cancer and its precursor lesions[20–23]. However, HR HPV cannot accurately discriminate patients whose squamous intraepithelial lesions (SIL) will persist or progress to invasive carcinoma from those whose lesions will regress spontaneously. It has been proposed that $p16^{INK4a}$ is a useful biomarker for the identification of cervical intraepithelial lesions (CIN) because it is a measure of persistent HPV infection rather than viral presence only[7–9,26–28]. $p16^{INK4a}$ as a specific inhibitor of the cycline-dependent kinase (cdk4 and cdk6) plays a crucial role in the regulation of the cell cycle (the G1 checkpoint) by retinoblastoma protein phosphorylation[24,25]. Increased expression of the HR HPV E6 and E7 oncogenes through binding to the retinoblastoma protein and release of transcription factor E2F results in a highly specific increase in $p16^{INK4a}$ protein expression in dysplastic and malignant cells of squamous and columnar epithelium of the cervix, which is detectable by a specific monoclonal antibody[26–28].

The aim of the present study was to analyze the correlation between $p16^{INK4a}$ immunoexpression in ThinPrep cervical cytologic samples and HPV detection by polymerase chain reaction (PCR) in the same sample.

Materials and Methods

Specimen preparation and processing

During the one-year study period (January to December 2008), a total of 120 cervical cytology specimens were received at the University Department of Gynecology and Obstetrics, University Hospital Center Zagreb. ThinPrep (Cytyc Corp., Boxborough, Massachusetts, USA) cervical cytology samples (N=120) were prepared according to the instruction detailed in the ThinPrep 2000 operator's manuals within 6 weeks of the specimen collection date. Cell sample obtained by Cervex-Brush (Rovers Medical Devices B.V., Oss, The Netherlands), from exocervix and endocervical canal was thoroughly washed in a vial containing PreservCyt solution. At the laboratory, the PreservCyt sample vial is placed into a ThinPrep 2000 Processor and a gentle dispersion step breaks up blood, mucus, non-diagnostic debris, and thoroughly mixes the cell sample. The cells are then collected on a ThinPrep Pap Test Filter specifically designed to collect diagnostic cells. The Processor constantly monitors the rate of flow through the filter during the collection process in order to prevent the cellular presentation from being too scant or too dense. A thin layer is then transferred to a glass slide in a 20 mm-diameter circle, and the slide is automatically deposited into a fixative solution. One slide was stained with Papanicolaou and analyzed using modified Bethesda cytologic classification named »Zagreb 2002«[29].

Immunocytochemistry

A second ThinPrep slide, prepared from the same sample, was fixed for at least 48h in 96% ethyl alcohol, followed by postfixation in 50% ethyl alcohol and acetone, and then immunostained for $p16^{INK4a}$ using CINtec $p16^{INK4a}$ Cytology Kit (DakoCytomation, Denmark). According to its protocol, immunocytochemistry was performed as follows: »cooking« in Epitope Retrieval Solution at 96 °C; cooling down in dark chamber; discarding of excessive Epitope Retrieval Solution; and rinsing in diluted Wash Buffer. Then the slides were placed in the DakoCytomation Autostainer for the following automated procedure: peroxidase blocking (peroxidase blocking reagent), addition of the mouse anti-human $p16^{INK4a}$ and visualization reagent, rinsing and immersion in the substrate-chromogen solution (DAB). Upon immunocytochemistry staining in the Autostainer, the samples were counterstained with hematoxylin, standard embedded, and analyzed by microscopy. Brown-colored cells (nu-

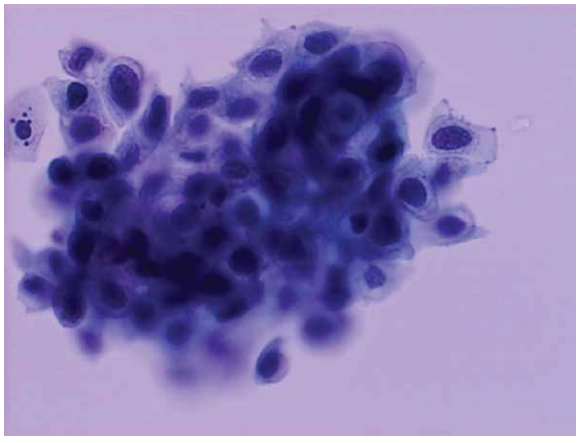

Fig. 1. High-grade squamous intraepithelial lesions (HSIL) in ThinPrep specimen (cervical intraepithelial neoplasia /CIN/ III on follow up histology), Papanicolaou, X200.

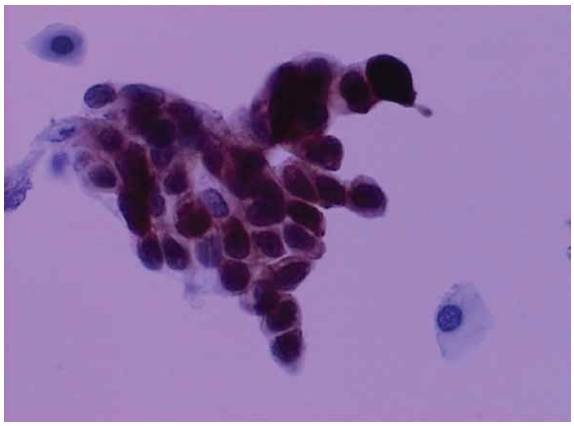

Fig. 2. p16^{INK4}a positive staining of high-grade squamous intraepithelial lesions (HSIL), X200.

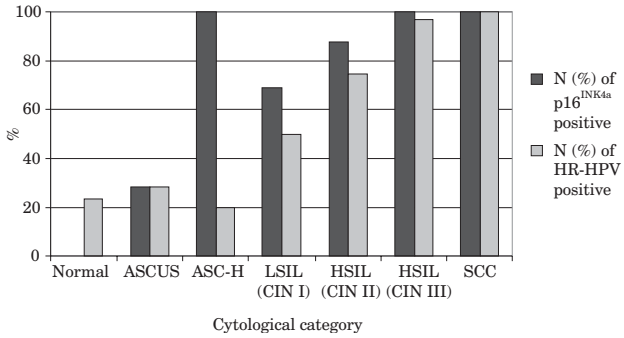

Fig. 3. p16^{INK4a} test and high-risk human papillomavirus (HR HPV) test according to cytologic diagnosis. ASCUS – atypical squamous cells of undetermined significance, ASC-H – atypical squamous cells – HSIL cannot be excluded, LSIL – low-grade squamous intraepithelial lesions, HSIL – high-grade squamous intraepithelial lesions, CIN – cervical intraepithelial neoplasia, SCC – squamous cell carcinoma.

clear and/or cytoplasmic staining) indicated p16^{INK4a} overexpression. Their number varied from case to case. Figure 1 provides representative images of high-grade SIL (HSIL) or CIN III on ThinPrep specimen and Figure 2 strong nuclear staining by the p16^{INK4a} immunocytochemistry assay.

HPV testing

Residual material was used for HR HPV-DNA detection by the PCR based AMPLICOR HPV test (Roche Molecular Systems). The test is designed to amplify HPV DNA from 13 high-risk genotypes (16,18,31,33,35,39, 45,51,52,56,58,59 and 68). The test amplifies 165 bp long nucleotide sequence within the polymorphic L1 region of the HPV genome with a master mix containing biotin labeled primers. An additional primer pair is added to allow for simultaneous amplification of the human ß-globin gene, which allows for assessment of cellular adequacy.

Results

A total of 120 ThinPrep Pap tests were included in this study representing the following cytologic diagnosis: 17 within normal limits, 17 atypical squamous cell (ASC) (7 ASC of undetermined significance /ASCUS/ and 10 ASC of HSIL cannot be excluded /ASC-H/), 26 low-grade SIL (LSIL) – CIN I, 57 HSIL – 24 CIN II and 33 CIN III and 3 squamous cell carcinoma (SCC). All CIN III (n=33) and SCC (n=3) specimens expressed p16^{INK4a} immunoreactivity, whereas the HR HPV test was positive in 97% (32/33) of CIN III and 100% (3/3) of SCC specimens. The p16^{INK4a} biomarker was positive in 87.5% (21/24) of CIN II and 69% (18/26) of CIN I, while HR HPV was positive in 75% (18/24) of CIN II and 50% (13/26) of CIN I. In ASCUS cytology, p16^{INK4a} and HR HPV showed an identical rate of positivity (28.5%; 2/7). Expression of p16^{INK4a} was detected in all cytologic (10/10) ASC-H lesions, in contrast to HR HPV detected in only 20% (2/10) of ASC-H cases (Table 1 and Figure 3). Table 2 summarizes the overall results for p16^{INK4a} and HR HPV status within the cytologic categories of ASCUS and LSIL. Positivity of both tests was found in 14.3% (1/7) of ASCUS and in 33.3% (8/24) of LSIL lesions, while both tests were negative in 57.1% (4/7) of ASCUS and 12.5% (3/24) of LSIL cases (Table 2).

Histopathologic verification (biopsy) performed in 35% (42/120) of abnormal cytologic findings confirmed HSIL (CIN II and CIN III) in 73.3% (22/30) of cases; CIN I was detected in one case; and negative biopsy finding was recorded in 7 cases. Out of 23% (6/26) of cytologic findings of CIN I, biopsy confirmed it in 3 cases; CIN II was verified by histopathology in one case; and the remaining two biopsy specimens were negative. Out of 4 cytologic findings of ASC-H (4/10) submitted to histopathology,

TABLE 1
p16^{INK4a} TEST AND HR-HPV TEST ACCORDING TO THE CYTOLOGICAL DIAGNOSIS

	Total N	p16^{INK4a} positive N (%)	HR-HPV positive N (%)
Normal	17	0 (0.0)	5 (23.5)
ASCUS	7	2 (28.5)	2 (28.5)
ASC-H	10	10 (100.0)	2 (20.0)
LSIL (CIN I)	26	18 (69.0)	13 (50.0)
HSIL (CIN II)	24	21 (87.5)	18 (75.0)
HSIL (CIN III)	33	33 (100.0)	32 (97.0)
SCC	2	2 (100.0)	2 (100.0)
Total	120	86 (71.6)	74 (61.6)

HR – high-risk, HPV – human papillomavirus, ASCUS – atypical squamous cells of undetermined significance, ASC-H – atypical squamous cells – HSIL cannot be excluded, LSIL – low-grade squamous intraepithelial lesions, HSIL – high-grade squamous intraepithelial lesions, CIN – cervical intraepithelial neoplasia, SCC – squamous cell carcinoma

TABLE 2
CORRELATION BETWEEN p16^{INK4a} TEST AND HR-HPV TEST

p16^{INK4a}	HR-HPV	ASCUS N (%)	LSIL N (%)
Negative	Negative	4 (57.1)	3 (11.5)
Positive	Negative	1 (14.3)	10 (38.5)
Negative	Positive	1 (14.3)	5 (19.2)
Positive	Positive	1 (14.3)	8 (30.8)
Total		7	26

HR – high -risk, HPV – human papillomavirus, ASCUS – atypical squamous cells of undetermined significance, LSIL – low--grade squamous intraepithelial lesions

CIN III and microinvasive SCC (MIC) were found in one case each, whereas the remaining two findings were negative.

Discussion

LBC has been compared with conventional cytology in many studies and several systematic reviews, and their conclusions have been disparate[5,6,12–15,30–32]. Studies comparing test positivity rates for low-grade cytologic abnormalities often yielded more favorable results for LBC[12,14], whereas in studies focusing on accuracy for biopsy-confirmed CIN, no significant differences were found between the conventional Pap and LBC[30,31]. In some countries, LBC has been officially adopted for cervical cancer screening because studies conducted in those countries showed LBC to be more often satisfactory than Pap smears. This is the case, for instance, in the NICE report from UK, where 7–10% of the conventional Pap smears were unsatisfactory[33]. In our country, Pap smears are taken by trained gynecologists and we have very few unsatisfactory smears (0.04% at our Department, 2005). Although there is no evidence that LBC is more accurate than conventional cytology, equivalent performance might be sufficient if LBC has other advantages, such as reducing the reading times, if it is more appropriate for automated screening devices and offers an opportunity for concurrent HPV DNA testing and immunocytochemistry. Morphological interpretation of ASCUS and LSIL shows significant inter-observer discrepancies, so additional methods could be really useful for triage of patients with these cytologic categories[34,35]. HPV testing is more sensitive, but less specific for detecting cervical neoplasia than conventional cytology[36,37]. HPV testing is less specific than cytology because many infections regress without developing high-grade lesions. Previous studies have reported that the introduction of the p16^{INK4a} biomarker together with HR HPV testing can increase specificity while maintaining sensitivity[7–9,26–28,38]. p16 overexpression has been shown to be associated with progression to CIN III or cancer[39,40].

A total of 120 ThinPrep specimens with another ThinPrep slides prepared from the same LBC samples immunocytochemically stained for p16^{INK4a} were evaluated at our Department for the first time in Croatia. Using this protocol, we obviated destaining and repeat immunocytochemical staining of the conventional Pap smear for p16^{INK4a}. Namely, ThinPrep specimens applied in a thin layer and with a lower content of blood, inflammatory cells and mucus are by far more suitable for analysis and reaction reading off[41]. The remaining LBC material left over after cytologic analysis was adequate for HR HPV PCR typing in all cases.

Our results pointed to good correspondence between positive p16^{INK4a} staining and grade of intraepithelial lesion (the rate of positivity increased from 69.0% in CIN I through 87.5% in CIN II to up to 100% in CIN III) and good correlation with HR HPV PCR typing (50% in CIN I, 75% in CIN II, and 97% in CIN III), which is consistent with literature reports[7–9,42], where the rate of p16^{INK4a} positivity ranged from 42% to 78% in LSIL and from 81% to 96% in HSIL. Unlike some of these reports[42], in our ThinPrep LSIL specimen total positive expression of p16^{INK4a} was higher than HR HPV typing (69% *vs*. 50%). In our three SCC cases, we found diffuse and highly positive reaction to p16^{INK4a} and presence of HR HPV[8,9,26–28,41]. Our cytologically negative ThinPrep specimens also showed negative reaction to p16^{INK4a}, in contrast to positive HR HPV typing in 23.5% of the same specimens. In comparison with other studies reporting on up to 10% of positive HR HPV findings in normal cytologic findings[43], the higher results obtained in the present study could be explained by the majority of our patients to have previously had one or more positive cytologic findings, i.e. these were not subjects recruited from primary screening. According to literature data, up to 8% of negative findings show positivity for p16^{INK4a}, which is attributed to the morphologically recognizable positively stained atrophic, metaplastic, endocervical columnar or endometrial cells[7–9,38,41], whereby this positivity is weak and recorded in a small number of cells in the group. The lack of correspondence between p16^{INK4a} expression and HR HPV status in the ASC-H lesions under study could be explained by difficulties in the interpretation of p16^{INK4a} stained atypical and immature metaplastic cells[42], although CIN III lesion was verified on biopsy in one case of positive p16^{INK4a} finding and negative HR HPV result.

Conclusion

Based on our initial results, on detecting HSIL lesions and carcinoma of uterine cervix, immunocytochemical expression of the p16^{INK4a} biomarker in ThinPrep cervical specimens correlates closely with the HR HPV typed by the PCR method in the same sample. However, the true value of adjunctive methods is their use in the triage of patients with low-grade lesions and reduction in the number of repeat cytology testing, unnecessary biopsies and respective costs. We can assume that the combination of these tests can identify two groups within low--grade lesions, i.e. one with low risk for the development

of premalignant cervical lesions, for which both of these tests are negative (57.1% ASCUS and 11.5% LSIL), and another group of women with both tests positive (14.3% ASCUS and 30.8% LSIL) and with the actual risk of squamous intraepithelial lesions. A prospective study with longer follow up may clarify their predictive values in the LSIL and ASC diagnosis, especially in the groups that have different test results of $p16^{INK4a}$ and HPV tests.

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TEKUĆA CITOLOGIJA – NOVE MOGUĆNOSTI U DIJAGNOSTICI LEZIJA VRATA MATERNICE

SAŽETAK

Metoda tekuće citologije (engl. »liquid-based cytology«, LBC) omogućuje primjenu dopunskih metoda u dijagnostici i prognozi lezija vrata maternice. Cilj rada bio je usporedba imunocitokemijske ekspresije biomarkera $p16^{INK4a}$ u LBC-ThinPrep cervikalnim uzorcima u odnosu na HPV tipizaciju metodom polimeraze lančane reakcije (engl. »polymerase chain reaction«, PCR). Ukupno 120 ThinPrep (Cytyc, USA) cervikalnih uzoraka obojenih metodom po Papanicolaouu primarno su se analizirali primjenom citološke klasifikacije »Zagreb 2002«, a na drugom ThinPrep preparatu istoga uzorka napravljena je procjena ekspresije biomarkera $p16^{INK4a}$ pomoću CINtec $p16^{INK4a}$ Cytology Kit (DakoCytomation,

Denmark). Povećana ekspresija onkogena E6 i E7 humanog papilomavirusa visokog rizika (engl. »high-risk human papillomavirus«, HR HPV) rezultira porastom ekspresije proteina $p16^{INK4a}$, čiji se pozitivitet očitava pojavom smeđih granula u jezgri i/ili citoplazmi abnormalnih stanica. Preostali LBC uzorak rabio se za HR HPV-DNA tipizaciju metodom PCR (AMPLICOR, Roche). Citološki je klasificirano: 17 negativnih nalaza, 17 atipičnih pločastih stanica (ASC) (7 ASC -neodređenog značenja /ASCUS/ i 10 ASC- ne može se isključiti skvamozna intraepitelna lezija visokog stupnja /ASC-H/), 26 skvamoznih intraepitelnih lezija niskog stupnja (LSIL) tj. cervikalnih intraepitelnih neoplazija (CIN) I, 57 SIL visokog stupnja (HSIL) koji uključuju 24 CIN II i 33 CIN III, te 3 karcinoma pločastih stanica. Imunocitokemijska reakcija na $p16^{INK4a}$ bila je pozitivna u svim CIN III lezijama (11/11) i karcinomima (3/3), dok je HR HPV tipiziran u 97% (32/33) CIN III i 100% (3/3) karcinoma. Citološki je identificiran $p16^{INK4a}$ u 87,5% (21/24) CIN II, te 69% (18/26) CIN I, dok je HR HPV bio pozitivan u 75% (18/24) CIN II i 50% (13/26) CIN I. ASCUS promjene imale su istu stopu pozitiviteta na $p16^{INK4a}$ i HR HPV (28,5%; 2/7), dok je u ASC-H promjenama $p16^{INK4a}$ bio pozitivan u svim slučajevima (100%, 10/10), a HR HPV je tipiziran u svega 20% (2/10) lezija. Na temelju naših prvih rezultata može se zaključiti da imunocitokemijska ekspresija biomarkera $p16^{INK4a}$ u ThinPrep cervikalnim uzorcima usko korelira s HR HPV tipiziranim metodom PCR iz istoga uzorka u otkrivanju HSIL lezija i karcinoma vrata maternice, dok su u procjeni LSIL i ASC lezija potrebna daljnja morfološko-molekularna praćenja.