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Prognostic Value of KI6 Biomarker to Predict Short Term Prognosis of Low Grade Cervical Intraepithelial Neoplasia in Human Papilloma Virus Negative and Positive Patients

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Abstract

Background: Cervical cancer is the most common gynecologic cancer in developing countries. Although this malignancy is preventable, problems exist with screening this cancer. Numerous studies have researched immunohistochemistry methods, such as the KI-67 biomarker as a proliferation marker, to improve screening for cervical intraepithelial neoplasia as the precancerous phase of cervical cancer. These studies mostly screened cytological samples. In the current study, we sought to analyze the correlation between the KI-67 proliferative biomarker and HPV infection in order to predict short-time prognosis in cervical intraepithelial neoplasia as an alternative or ancillary method to current screening methods. Our assessment was based on histologic samples from a different geographic population.

Methods: This descriptive cohort prospective study included 40 patients diagnosed with low grade cervical intraepithelial neoplasia based on cervical punch biopsy samples after colposcopy examination. We enrolled patients who referred to the Department of Gynecology-Oncology of an academic hospital of Mashhad University of Medical Sciences from 2016 to 2017. All low grade cervical intraepithelial neoplasia samples were investigated for HR-HPV DNA with the Cobas test and immunostaining for the KI-67 biomarker. After a one-year follow-up, we evaluated the prognosis for all patients based on liquid based cytology and HR-HPV test. Data were analyzed by SPSS version 23.0 and the Mann-Whitney U and Fisher's exact tests. A P-value < 0.05 was considered significant.

Results: We observed a significant difference between HR-HPV positive and negative tests in KI-67 expression (P<0.001), but there were no significant differences in reactivity level of cervical epithelium (P=0.5) and in KI-67 expressions in metaplastic and non-metaplastic epithelium (P=0.88). After one year, most low grade cervical intraepithelial neoplasia cases in group A that had a low staining KI-67 biomarker had evidence of regression. On the contrary, all cases with high grade KI-67 expression didn't persist or progressed necessarily.

Conclusion: The KI-67 biomarker is recommended as a complementary screening test, but not an alternative for triage of high-risk patients with low grade cervical intraepithelial neoplasia. Patients with low grade cervical intraepithelial neoplasia/HR-HPV positive cervical samples and low staining KI-67 antigen could be offered a less aggressive follow-up protocol.

Keywords: KI-67 biomarker, HPV infection prognosis, Cervical intraepithelial neoplasia, Immunohistochemistry

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Introduction

The latest consensus of the World Health Organization (WHO) has emphasized the study of cervical cancer and acknowledged the lack of a more effective method to predict cervical intraepithelial neoplasia (CIN) persistency or disease progression.[1,2] The current screening program based on the WHO and American Society of Colposcopy and Cervical Pathology (ASCCP) protocol recommend a periodic pap smear (cytology) and high-risk HPV test (HR-HPV).[3,4] Each of these tests, individually, are associated with low sensitivity and a high false negative rate that is dependent on the cytopathologist's experience.[5] The majority of HR-HPV infections are highly transient and have regression potential; however, a low percentage of them could progress to cervical cancer.[5] Prediction of which patients are at risk for cervical cancer leads to high cost and patient anxiety. Follow-up is the recommended option for low grade CIN (CIN 1) cases. Recently, several biomarkers have been introduced with the intent to design a more effective follow-up protocol that has reduced costs and decreased emotional stress. After an HR-HPV infection, the basal layer of epithelial cells integrates to the host cell's DNA and exacerbates an oncogenic proliferation cycle, which is the essential step in transformation to a high grade lesion and cancer. It is important to find a reliable proliferation marker test that has the capability to detect this phase of the virus' persistent infection in order to predict the actual risk of transformation from pre-cancer status to active (invasive) disease and patient outcome.[6,7] Numerous studies have been used a number of biomarkers to predict active cellular phases, including KI-67. The KI-67 antigen, as a proliferative biomarker, can be used on the basis of proliferation activity in cervical dysplasia. However, due to its capability, it can be used in follow-up and particularly for HR-HPV positive women.[8,9] Gustinucci et al. have designed a population-based study to triage HR-HPV positive women according to cytology in combination with KI-67 biomarkers to increase screening sensitivity. They found that the combined strategies had high sensitivity and allowed longer follow-up intervals in HR-HPV positive, triage-negative women.[10] Previous to their study, Solares et al. reported a simpler triaging test for low grade cytology samples that had normal histological biopsies. They sought to predict the risk of developing high-grade cervical lesions during one-year of follow-up. These researchers identified patients at higher risk for progression with higher biomarker such as the KI-67 antigen.[11] At the same time, Rossi et al. studied the clinical performance of immunostaining cytological samples to assess their ability to predict the risk for progression and regression; they concluded the ineffectiveness of this method is due to inter-observer discordance.[7]

The objective of this study was to analyze the role of the KI-67 biomarker and its correlation with HPV infection in order to predict the short term prognosis of CIN 1 histological samples, as the gold standard of research, in a different geographic population. The result of such study, could be used as an alternative or ancillary method to current screening methods and lead to more appropriate individualized management.

Materials and Methods

In this descriptive cohort prospective study, we selected a total of 40 formalin-fixed paraffin embedded cervical punch biopsy specimens with confirmed CIN1. The samples were obtained from patients who referred to the Department of Gynecology and Oncology of an academic hospital, Mashhad University of Medical Sciences from 2016 to 2017. Colposcopy was performed by two expert gynecologic oncologists. All colposcopic findings that consisted of satisfactory evaluation, presence of abnormal vessels, and acetowhite lesions were documented. Two expert gynecologic pathologists assessed all samples. The inclusion criteria, based on American Society for Colposcopy and Cervical Pathology (ASCCP) guidelines for colposcopy evaluation, consisted of an abnormal cytological results based on liquid cytology or HR-HPV positive test by the Cobas system (only 16 and 18 genotypes). We divided

the patients into either group A (positive HR-HPV test results) or B (abnormal Pap smear and HR-HPV negative results). The two groups were similar in terms of carcinogenic risk factors of age, onset of early intercourse, parity, oral contraceptive usage, and smoking. Exclusion criteria consisted of multiple partners, immunocompromised, pregnancy, and history of any type of treatment for cervical disease (conisation, cryotherapy, laser, or hysterectomy, or prior chemo/radiotherapy), as confounding factors. In addition, we rejected any poor quality tissue blocks for immunohistochemical processing. We used an in situ DNA hybridization HR-HPV DNA test by Cobas® HPV test (Roche Molecular Diagnostics) to assess the cervical biopsies. After barcode identification, we used the p 480 instrument for homogenization and de-capping of the Preserve Cyt® vials. The samples were transferred to the x 480 system for DNA extraction and real-time PCR using the c4800 SMPL PREP Kit and a liquid cytology preparation kit (c4800 LIQ CYT). Samples were homogenized again with an automatic pipette prior to extraction. Processed sample vials were transferred back to the p 480 system for recapping. Amplification, detection, and HR-HPV typing (types 16, 18, and 12 additional HR-HPV types) were performed by real-time PCR. All steps of the

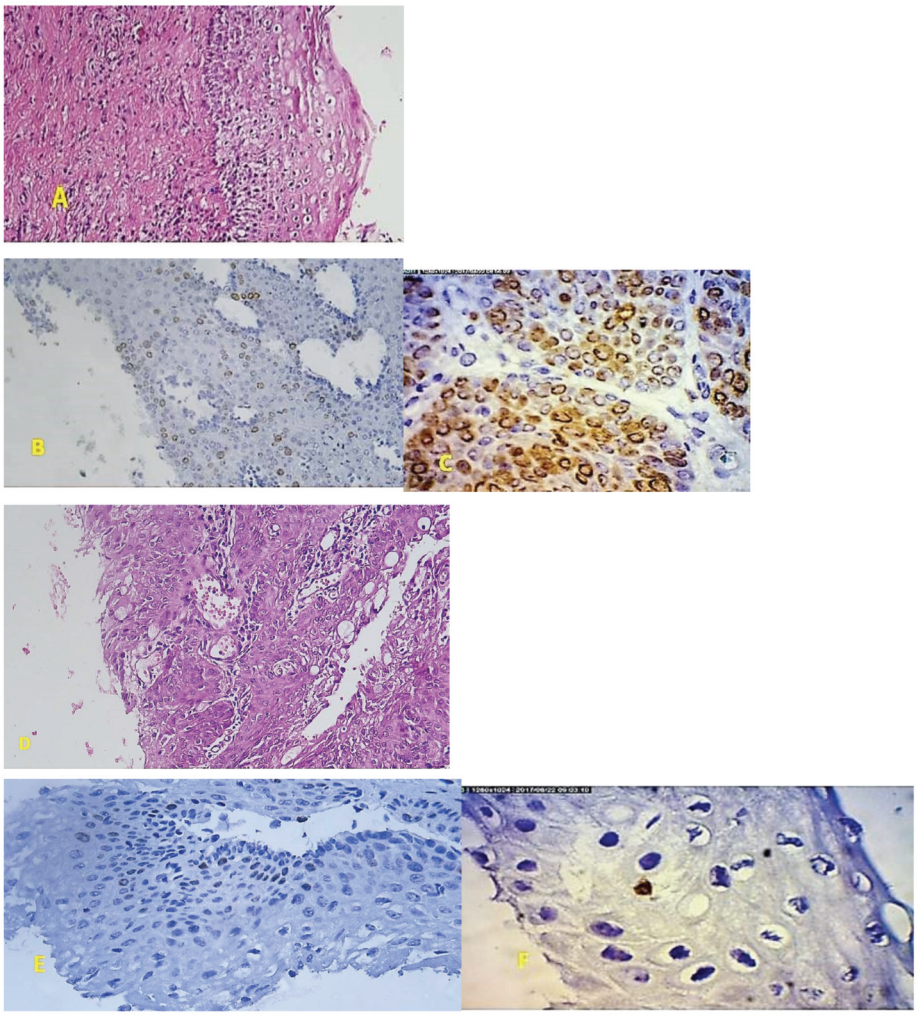

Figure 1. H & E and immunohistochemistry (IHC) staining for KI-67 expression in correlation with high-risk HPV (HR-HPV) status. A) H & E staining of HR-HPV positive sample (100×). B) IHC staining of HPV positive sample; High grade expression (100×). C) IHC staining of HPV positive sample; High grade expression (400×). D) H & E staining of HR-HPV negative sample (100×). E) IHC staining of HPV negative sample; Low grade expression (100×). F) IHC staining of HPV negative sample; Low grade expression (400×).

Table 1. Demographics of the CIN 1 tissue samples.

Features	Status	Results	
Age (years)*		35.85 ± 9.02	
CIN 1**	HR-HPV positive (group A patients)	20 (50)	6 "<30 years"
			14 "≥30 years"
	HR-HPV negative (group B patients)	20 (50)	4 "<30 years"
			16 "≥30 years"
KI-67 expression **	Grade I	17 (42.5)	
	Grade II	8 (20)	
	Grade III	15 (37.5)	

* Mean ± standard deviation; ** Frequency (%), "the frequency based on age"; HR-HPV ; High risk HPV.

IHC study were performed according to the manufacturers' protocols.[12,13] The prepared slides were incubated with a 1:200 dilution of KI-67 antigen (Dako, Denmark) at room temperature for 2.5 h according to the manufacturer's protocol followed by staining with diaminobenzidine for 6 min (Envision kit, Novocastra, Newcastle, UK) for IHC analysis. In addition to the standard positive and negative controls, two pathologists who were unaware of the study objectives interpreted the results for increased reliability and reduce inter-observer variability. The KI-67 biomarker staining was categorized into 3 grades according to the proportion of cells nuclei with positive staining (brown color). Grade I (low) had less than 5% of epithelial cell nuclei that expressed KI-67 antigen; grade II (intermediate) 5%-30% of cells stained brown; and grade III (high) had staining in >30% of cells.

Participants were followed after one year by liquid based cytology and HPV analyses to evaluate the potential for the KI-67 biomarker to predict the risk of progression in a short follow-up period for CIN 1 patients.

Ethics

The Mashhad University of Medical Sciences Ethics Committee (IR.MUMS.fm.REC.1396.630) approved this study. All participants were verbally informed about the purpose of study when were re-invited for follow-up.

Statistical analysis

All statistical analysis was performed by SPSS software (version 16.0, SPSS Inc., Chicago, IL, USA). Data normality was verified by the Kolmogorov-Smirnov test. We used either the

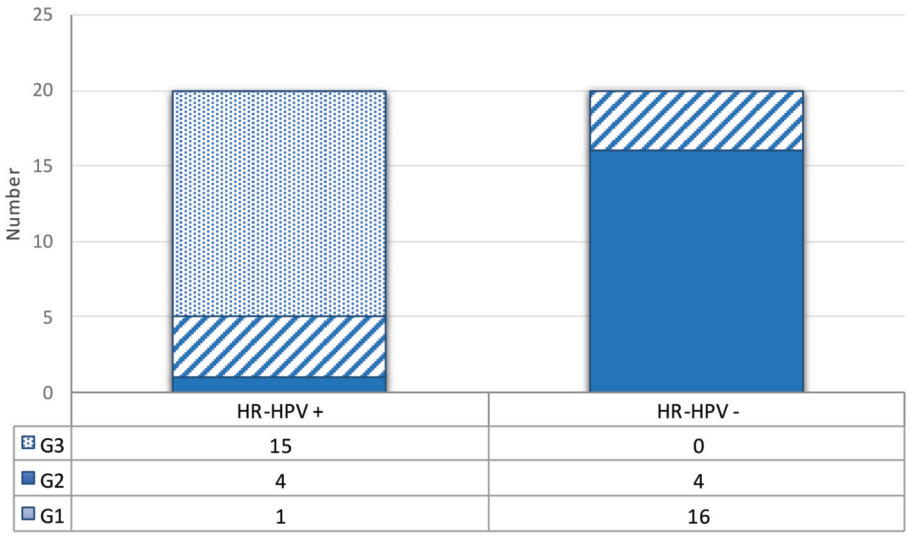

Figure 2. Relationship between the grades of KI-67 expression and high-risk HPV (HR-HPV) status. G: Grade.

Table 2. Frequency of KI-67 expression in cervical epithelium and level of immunoreactivity in relation with HR-HPV status.

Immunohistochemistry (IHC)		HR-HPV positive	HR-HPV negative	*P*-value
KI-67 expression*		38 (2-55)	2 (0-25)	<0.001
Immunoreactivity level in cervical epithelium**	High	2 (10)	1 (5)	-
	Low	7 (35)	18 (90)	
	None	11 (55)	1 (5)	

*: Median based on Mann-Whitney U test (min-max); HR-HPV: High-risk HPV; **: Frequency (%)

student's t-test or Mann-Whitney U test to evaluate significant differences. Fisher's exact test was used for qualitative variables. A *P*-value <0.05 was considered significant.

Results

The study included 40 patients with confirmed diagnosis of CIN in cervical tissues. The mean age of patients was 35.85 ± 9.02 years (19-52 years). There were 20 (50%) patients in group A and 20 (50%) patients in group B. We assessed KI-67 expressions according to 3 grades for all histologic CIN 1 samples. Table 1 and figure 1 lists the patients' demographic data for the CIN 1 samples.

We compared the frequency and immunoreactivity level of KI-67 expression in the cervical epithelial nuclei between both groups. There was significant difference between the groups in KI-67 expression (*P*<0.001; Table 2, Figure 2).

There were 34 (85%) patients who had initial abnormal colposcopy findings. There was no statistical difference between KI-67 expression and more abnormal colposcopic findings as assessed by an oncologist (*P*=0.4, ANOVA).

After the one-year follow up of all patients, we noted that 4 patients from group A were persistent for the HR-HPV test. None of these patients were under the age of 30 years. There was no statistical difference between age and persistence due to the limited number [4 out of 30 (13.3%); *P*=0.56, Fisher's exact test] and age (≥30 years) of these patients. There were no patients with grade I KI-67 expression in the persistent group; however, there was one case of grade II and 3 cases of grade III. In the other 16 cases that had regression according to the HR-HPV test, 12 (75%) had grade III KI-67 expression (*P*=0.95, Mann-Whitney U test) as seen in figure 3. It could be stated that CIN 1 samples with grade I (<5%) KI-67 expression could be expected to regress during this time; however, patients with high grade KI-67 expression would not necessarily persist or progress over time. All group B patients had regression according to Pap smear results

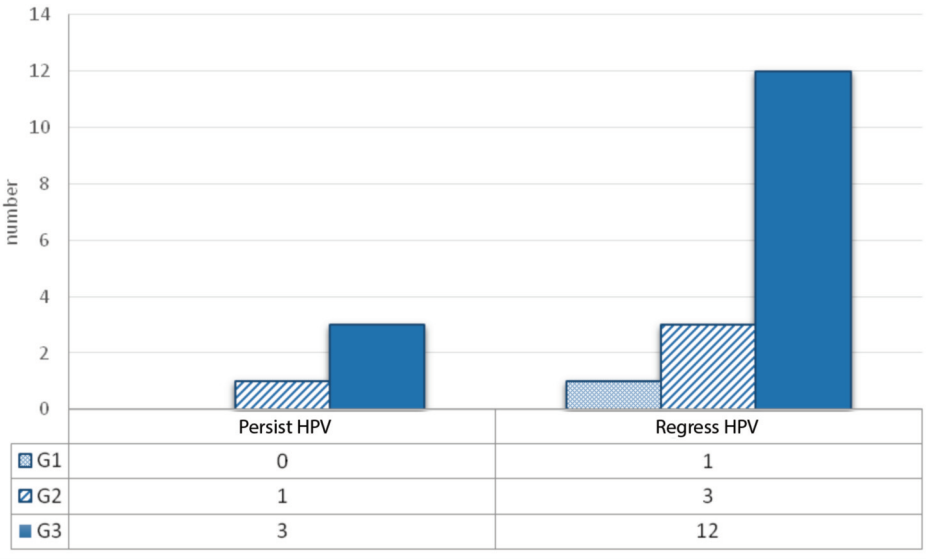

Figure 3. Grades of KI-67 expression correlated with short time follow-up. Persist: Persistence; Regress: Regression; G: Grade.

after one year, with the exception of one case who had grade I KI-67 expression. None of the group B patients had high grade KI-67 expression (P=0.62, Mann-Whitney U test).

For the KI-67 immunostaining expression cut-off determination in present study, the sensitivity and specificity of 5% KI-67 expression in comparison with 30% under the ROC, was 95.5% and 85% for the cut-off of 5% versus 70% and 100% for the cut-off 30% in regards.

The cut-off for the KI-67 antigen to predict the probability of HR-HPV in one case was 18.5% with a 90% sensitivity and 95% specificity

Discussion

Recent studies demonstrated the validity of the KI-67 biomarker as a detector of pre-clinical cellular dysregulation in relation to HR-HPV infection status in patients with CIN 1 lesion. Nowadays, we know that, the CIN 1 lesion would regress mostly instead of progress or even persist over the time. The risk of progression of CIN 1 to a high grade lesion is estimated to be approximately 10%.[14] White et al. have reported a persistency rate of 20% for low grade lesions by cytology in a short-term follow-up, which was similar to the present study results. They have recommended a less frequent follow-up for HR-HPV positive patients with negative biomarkers.[15] It is important to find a reliable method that can predict the outcome of these patients. The KI-67 biomarker, as a proliferative biomarker, has been proposed on the basis of proliferation activity in cervical dysplasia. Based on literature review, the mean KI-67 expression in CIN 1 lesions was reported to be 22%-71% with different criteria for positive staining.[1,6] For the first time, Dellas et al. reported the difference in KI-67 expression between HPV positive and negative patients, which was confirmed by the current study.[8] However, due to KI67 antigen capacity it could be used to follow patients, particularly HR-HPV positive women.[9,16,17] Rossi et al. observed similar KI-67 expressions in regression and persistent patients, with no prediction capacity for this biomarker during two years of follow-up.[7] Possati reported the highest sensitivity for the KI-67 biomarker in proliferation in older patients.[2] Šekoranja, in 2017, reported similar results.[18] In the current study, there was no patient under 30 years of age who had persistent KI-67 expression, so the role of age in prognosis prediction remain in doubt. The study result determined the benefits of this proliferator's index in prediction the outcome of HR-HPV positive patients in short-term follow-up, one year. There was no statistical difference between a higher KI-67 index and probability of progression or persistence risk of infection. In other studies, patients that were not CIN 1, but HR-HPV positive with low grade KI-67 staining were persistent after one year, as predicted.[4,19] We demonstrated a correlation between colposcopic appearance, KI-67, and prognosis over time. There was no correlation observed between colposcopic findings and risk of persistence.

The limitation of this study was the small numbers of cases, the high numbers of inadequate histological samples for KI-67 analysis, and short-term follow-up. Additional studies should be conducted to assess the potential high-grade lesions in order to reduce the side effects of the treatment.

Conclusion

We recommend the KI-67 biomarker as a complementary screening test, but not an alternative for triaging in high-risk patients with CIN 1. Patients with CIN 1/HR-HPV positive cervical samples with low expression of KI-67 antigen could be offered a less aggressive follow-up protocol.

Conflict of Interest

None declared.

References

1. Kanthiya K, Khunnarong J, Tangjitgamol S, Puripat N, Tanvanich S. Expression of the p16 and KI-67 in cervical squamous intraepithelial lesions and cancer. *Asian Pac J Cancer Prev.* 2016;17(7):3201-6.
2. Possati-Resende JC, Fregnani JH, Kerr LM, Mauad EC, Longatto-Filho A, Scapulatempo-Neto C. The accuracy

of p16/KI-67 and HPV test in the detection of CIN2/3 in women diagnosed with ASC-US or LSIL. *PLoS One.* 2015;10(7):e0134445. doi: 10.1371/ journal.pone. 0134445.
3. Alshenawy HA. Evaluation of p16, human papillomavirus capsid protein L1 and KI-67 in cervical intraepithelial lesions: Potential utility in diagnosis and prognosis. *Pathol Res Pract*. 2014;210(12):916-21. doi: 10.1016/j.prp.2014.07.007.
4. Korolczuk A, Orzel M, Wozniak S, Smolen A, Caban K. P16/ Ki67 dual immunostaining in conventional cytology in women with positive papanicolau test. *J Cytol Histol.* 2015; 6(5):1-5. doi:10.4172/2157-7099.1000358.
5. Luttmer R, Dijkstra MG, Snijders PJ, Berkhof J, van Kemenade FJ, Rozendaal L, et al. p16/KI-67 dual-stained cytology for detecting cervical (pre) cancer in a HPV-positive gynecologic outpatient population. *Mod Pathol*. 2016;29(8):870-8. doi: 10.1038/ modpathol.2016.80.
6. Wentzensen N, Schwartz L, Zuna RE, Smith K, Mathews C, Gold MA, et al. Performance of p16/KI-67 immunostaining to detect cervical cancer precursors in a colposcopy referral population. *Clin Cancer Res.* 2012;18(15):4154-62. doi: 10.1158/1078-0432.CCR-12-0270.
7. Rossi P, Borghi L, Ferro R, Mencarelli R. A population of 1136 HPV DNA-HR positive women: expression of p16 (INK4a)/KI-67 dual-stain cytology and cytological diagnosis. Histological correlations and cytological follow-up. *Pathologica.* 2015;107(3-4):185-91.
8. Dellas A, Schultheiss E, Almendral AC, Torhorst J, Gudat F. Assessment of EGFR and TGF−alpha expression in relationship to HPV status and KI−67 distribution in cervical intraepithelial neoplasms. *Int J Cancer.* 1996;69(3):165-9.
9. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin*. 2011;61(2):69-90. doi: 10.3322/caac.20107.
10. Gustinucci D, Giorgi Rossi P, Cesarini E, Broccolini M, Bulletti S, Carlani A, et al. Use of cytology, E6/E7 mRNA, and p16INK4a-Ki-67 to define the management of human papillomavirus (HPV)-positive women in cervical cancer screening. *Am J Clin Pathol.* 2016;145(1):35-45. doi: 10.1093/ajcp/aqv019.
11. Solares C, Velasco J, Alvarez-Ruiz E, Gonzalez-Fernandez L, Encinas AI, Astudillo A, et al. Expression of p16/KI-67 in ASC-US/LSIL or normal cytology with presence of oncogenic HPV DNA. *Anticancer Res.* 2015;35(11):6291-5.
12. Heideman DA, Hesselink AT, Berkhof J, van Kemenade F, Melchers WJ, Daalmeijer NF, et al. Clinical validation of the cobas 4800 HPV test for cervical screening purposes. *J Clin Microbiol.* 2011;49(11):3983-5. doi: 10.1128/JCM.05552-11.
13. Lindemann ML, Dominguez MJ, de Antonio JC, Sandri MT, Tricca A, Sideri M, et al.. Analytical comparison of the cobas HPV Test with Hybrid Capture 2 for the detection of high-risk HPV genotypes. *J Mol Diagn*. 2012;14(1):65-70. doi: 10.1016/j.jmoldx. 2011.09.005.
14. Lim S, Lee MJ, Cho I, Hong R, Lim SC. Efficacy of p16 and KI-67 immunostaining in the detection of squamous intraepithelial lesions in a high-risk HPV group. *Oncol Lett.* 2016;11(2):1447-52.
15. White C, Bakhiet S, Bates M, Keegan H, Pilkington L, Ruttle C, et al. Triage of LSIL/ASC−US with p16/Ki−67 dual staining and human papillomavirus testing: a 2−year prospective study. *Cytopathology.* 2016;27(4):269-76. doi: 10.1111/cyt.12317.
16. Ancuţa E, Ancuţa C, Cozma LG, Iordache C, Anghelache-Lupaşcu I, Anton E, et al. Tumor biomarkers in cervical cancer: focus on KI-67 proliferation factor and E-cadherin expression. *Rom J Morphol Embryol.* 2009;50(3):413-8.
17. Scholzen T, Gerdes J. The Ki−67 protein: from the known and the unknown. *J Cell Physiol.* 2000;182(3):311-22.
18. Šekoranja D, Fokter AR. Triaging atypical squamous cells-cannot exclude high-grade squamous intraepithelial lesion with p16/KI-67 dual stain. *J Low Genit Tract Dis*. 2017;21(2):108-111. doi: 10.1097/ LGT.0000000000000297.
19. Schmidt D, Bergeron C, Denton KJ, Ridder R; European CINtec Cytology Study Group. p16/ki-67 dual-stain cytology in the triage of ASCUS and LSIL papanicolaou cytology: results from the European equivocal or mildly abnormal Papanicolaou cytology study. *Cancer Cytopathol.* 2011;119(3):158-66. doi: 10.1002/cncy.20140.