Prognostic Value of KI6 Biomarker in Predict Short Time Prognosis of Low Grade Cervical Intraepithelial Neoplasia in HPV Negative and Positive Patients

Leila Mousavi Seresht[1], Noorieh Sharifi[2], Mona Najafi[3], Helena Azimi[1], Nooshin Babapour[1], Zohreh Yousefi[1]

[1] *Department of Obstetrics and Gynecology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*

[2] *Department of Pathology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*

[3] *Department of Socio-Medicine, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*

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Abstract- Screening of cervical cancer is the most common gynecologic cancer in developing countries. Despite being preventable, but we have still some problems with the screening of this cancer. Recently, many studies have been done on immunohistochemistry to improve screening of cervical intraepithelial neoplasia (CIN) as a precancerous lesion. But, the majority of the studies are based on cytological samples. The objective of this study was to analyze the correlation KI-67 biomarker and HPV infection in predict short time prognosis in CIN as an alternative or auxiliary method to the current screening method in a different geographic population. This descriptive cohort prospective study included 40 patients with a diagnosis of CIN based on cervical punch biopsy samples after colposcopy examination. They were referred to the department of gynecology and oncology of an academic hospital, Mashhad University of 2016 to 2017. All samples were investigated for HR- HPV DNA with Cobas test and immunostaining for KI-67 biomarker. Finally, after one year follows up, the prognosis for all patients was evaluated. Data were analyzed by SPSS software program version 23.0 and Mann-Whitney U test and Fisher's exact test. $P<0.05$ was considered significant. Significant difference was found between HR-HPV positive and negative tests in KI-67 expression ($P<0.001$), but no significant difference was observed in reactivity level ($P=0.5$), also no significant difference was found in KI-67 expression in the metaplastic and non-metaplastic epithelium ($P=0.88$). KI-67 biomarker is recommended as complementary screening tests not alternative for differentiating in high risks patients with CIN1. The patients with low KI-67 / HR-HPV positive test could be offered for a less aggressive follow-up protocol.

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Introduction

World Health Organization (WHO) in latest consensuses emphasis on study on cervical cancer outbreak and acknowledged the vacancy for a more effective method to predict CIN persistency or progression of disease (1,2). Current screening program (based on WHO and ASCCP (American Society of Colposcopy and Cervical Pathology) protocol) recommend periodic Pap smear (cytology) and high-risk HPV test (HR-HPV) (3,4). Each of them alone is associated with low sensitivity and high false negative rate that is dependent on the cytopathologist's experience (5). On the other hand, the majority of HR-HPV infection cases are highly transient, and regression potential, but a low percentage of them progress to cervical cancer (5). Prediction of which patients are really at risk of cervical cancer lead to high cost and patients' anxiety. With regard to the treatment of low-grade CIN (CIN1) cases, follow up all of the patients is recommended. Recently, several biomarkers have been introduced to design more effective follow-up- protocol with reduced costs and emotional stress. It has been made clear that after HR-HPV infected, the basal layer of epithelial cell integrated to host cell DNA and exacerbate oncogenic proliferation cycle which is the essential step in the transformation to high-grade lesion and cancer. It's important to find a reliable proliferation marker test which is capable to detect this phase of viruses persistent infection and can predict the real risk of pre-cancer to intensive disease and outcome

Corresponding Author: Z. Yousefi
Department of Obstetrics and Gynecology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
Tel: +98 915 1160750, Fax: +98 21 42910703, E-mail address: yousefiz@mums.ac.ir

of this patient (6,7). Nowadays, more studies have been conducted to predict active cellular phases with a lot of biomarkers including KI-67 biomarker. Biomarker KI-67 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 for follow up of patients especially in HR-HPV positive women (8, 9). Gustinucci *et al*. designed a population-based study to triage HR-HPV based cytology in combination with biomarkers KI-67 to increase screening sensitivity. They found combined strategies was with high sensitivity and allows longer intervals in HPV-positive, triage-negative women (10). The high sensitivity of combined strategies probably, triage-negative women. The high sensitivity of combined strategies probably allows longer intervals in HPV-positive, triage-negative women. Before that Solare and his coworker studied on a more simple triaging test for low-grade cytology (but normal histological biopsy) in predicting the risk of developing high-grade cervical lesions during one-year of follow-up; they also identified at higher risk of progression patient with higher biomarker such as KI-67 antigen (11). Unlike, at the same time, Rossi et al. showed the clinical performance of this immunostaining in cytological samples to assess their usefulness in predict risk of progression/regression, but the emphasise on the ineffectiveness of this method for the more personalized approach (7) .The objective of this study was to analyze the correlation KI-67 biomarker and HPV infection in predict short time prognosis in CIN1 histological sample as the gold standard of researches an alternative or auxiliary method to a current screening method for more appropriate individualize management in a different geographic population.

Materials and Methods

In this descriptive cohort prospective study, totally 40 formalin-fixed paraffin embedded cervical punch biopsy specimens of confirmed CIN1 were selected. The patients were 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 informed gyneco-oncologists, and all colposcopic finding (consist of satisfactory evaluation, abnormal vessel presence, and aceto white lesion) was documented. Also, two expert gyneco-pathologist assessed the samples. The inclusion criteria were: abnormal cytological results or HR-HPV positive test based on ASCCP guideline for colposcopy evaluation. The only positive genotype of 16 & 18 HR-HPV cases was selected, and patients were divided into two subgroups: group A) positive HR-HPV test , group B) abnormal. Pap smear and HR-HPV negative patients. The two groups were similar in terms of carcinogenic risk factor (including age, the start time of early intercourse, parity, oral contraceptive usage, and smoking). Excluding criteria were: multi-partner, immunocompromised, pregnant patients, and the cases with a history of any type of treatment for the cervical disease (conization, cryotherapy, Laser or hysterectomy, prior chemo/radiotherapy). Also, poor quality tissue blocks to process with the immunohistochemical study were rejected. In all cervical L.B of patients , in situ DNA hybridization HR- HPV DNA tests were performed by Cobas (Roche company) method . In cobas® HPV Test, at first barcode identification, homogenization and decapping of the PreservCyt vials using the p480 instrument were performed. The samples were transferred to the x480 system for DNA extraction and real time PCR setup using the sample preparation Kit (c4800 SMPL PREP) and Liquid Cytology Preparation Kit (c4800 LIQ CYT). Samples were homogenized again by automatic pipet prior to extraction. Processed sample vials were transferred back to the p480 system for recapping. Amplification, detection and HR-HPV typing (types 16, 18 and 12 "other" HR-HPV types) were done by real time PCR. All steps were performed according to the manufacturer's protocols (12, 13). Then for IHC study, all the prepared slides were incubated with 1:200 dilution of KI-67 antigen (Dako, Denmark) at room temperature for 2.5 hours based on manufacturer industry protocol and then staining with diaminobenzidine for 6 minutes (Envision kit, Novocastra, Newcastle, UK). Alongside with standard positive and negative controls, two blind pathologists interpreted them to increase the reliability of the study and reduce inter-observer variability. The KI-67 biomarker staining was categorized into 3 grades due to the proportion of cells with positive nuclear staining analysis. In grade I (low stained) less than 5% of epithelial cell nuclei was stained brown by this antigen, grade II (intermediate stained) consists of 5 up to 30% of cells staining, and grade III or high stained show staining in >30 % of cells. Immunoreactivity distribution and staining pattern between low to high layer texture in nonmetaplastic and metaplastic epithelium were evaluated to confirm the importance of other fixture of KI-67 antigen staining (in three levels of low, high and nonsignificant). Then, participants were followed up after one year by cytology and HPV test for evaluated the possibility of KI67 biomarker in prediction the risk of progression for CIN1 patient In short time follow up.

Ethics

The study was approved by the institutional Ethics Committee. All of the participants were verbally informed about the purpose of study when they were invited to follow up.

Statistical analysis

All statistical analysis was performed by SPSS software (version 16.0) (SPSS Inc., Chicago, IL, USA). Data normality was verified using the Kolmogorov-Smirnov test. Student t-test or Mann-Whitney U test was used to evaluate the significant difference. Fisher's exact test was used for qualitative variables. $P<0.05$ was considered significant.

Results

The study included 40 patients with confirmed diagnosis of CIN1 cervical tissues. The mean age of patients was 35.85±9.02 years (19-52y). Twenty patients (50%) was integrated into *group A*, and 20 (50%) in group B and KI-67 expression in three grading was analysis in all CIN1 histologic CIN1 samples. Demographic data for CIN 1 samples are shown in table 1 and figure 1.

Table 1. Demographic Result of CIN1 tissue samples

Feature	Status		Result	
Age*			35.85 +/- 9.02	
CIN1**	HR-HPV positive(*group A*)	20 (50)	<30 years	6
			>=30 years	14
	HR-HPV negative(*group B)*	20 (50)	<30 years	4
			>=30 years	16
KI67 expression **	Grade I		17 (42.5)	
	Grade II		8 (20)	
	Grade III		15 (37.5)	

*(mean +/- standard deviation)
**frequency (%)

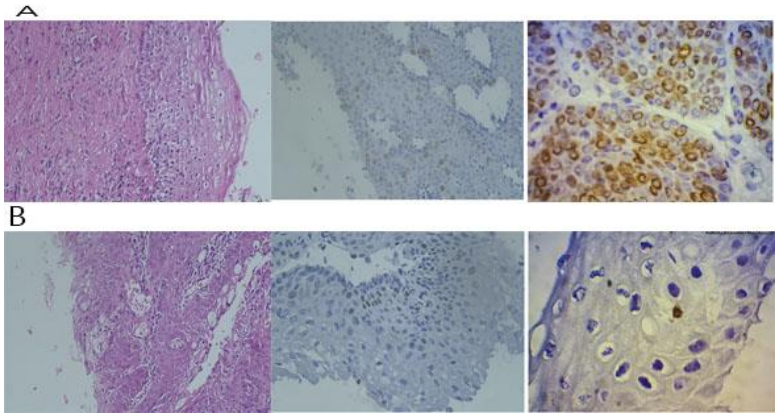

Figure 1. H AND E and IHC staining for KI67 expression in correlation with HR-HPV status

A) HR-HPV positive sample and high G staining (100 &400×)

B) HR-HPV negative sample and low G staining (100 &400×)

Frequency and immunoreactivity level of KI-67 expression in cervical epithelial nuclei had been compared between two groups. There was a significant difference between *group A and B* in KI-67 expression analysis ($P<0.001$) (Table 2, Figure 2).

Initial abnormal colposcopy was detected in 34 patients (85%). There was no statistical difference between KI-67 expression and worsen colposcopic findings by a gynecologist ($P=0.44$ ANOVA test).

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

Immunohistochemistry		HR-HPV+	HR-HPV-	*P*
KI67 expression*		38(2-55)	2(0-25)	<0/001
	high	2(10)	1(5)	-
Immunoreactivity level in Cervical epithelium**	**Low**	7(35)	18(90)	
	none	11(55)	1(5)	

*Median (min- max) with Mann Whitney u test.
**frequency (%)

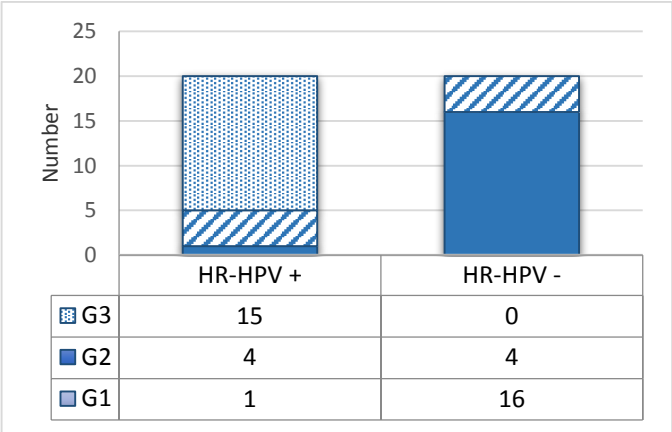

Figure 2. The relationship between KI-67 expression grading and HR-HPV status

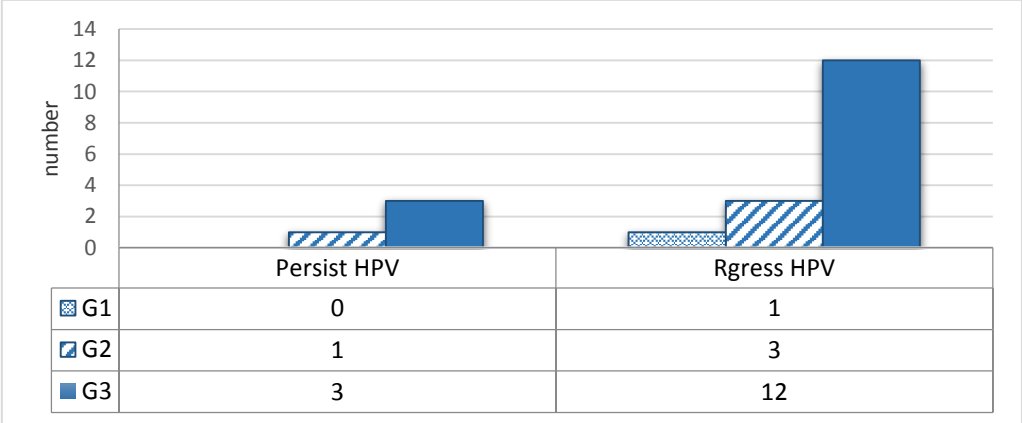

Figure 3. KI-67 expression grading in correlation with short time follow up persistence and regression

After one year follow-up of all patients; 4 patients of group A were "*persistent*" for HR-HPV test; none of the test persistent patients' were under age 30 years , there wasn't statistical difference between age and persistency due to limited persist patient upper 30 years old (4 of 30 cases >= 30y ,(13.3%)) (*P*=0.56, Fisher's exact test) . In *persistent group*, no one had GI of KI-67 staining, 3 cases had GIII and 1 had GII and); and in the other 16 regress HR-HPV test patient 12 cases (75%) had G III of KI-67 expression (*P*= 0.95,Mann Whitney test) (Figure 3) which could be interpreted that CIN1 samples with KI-67 staining fewer than 5 percent could be expected to regress during the time however high G KI-67 expression cases wouldn't necessarily persist or progress over the time. All group B members regressed on pap smear after one year except one who even had GI KI67 staining, and none of the patients in this group had high-grade KI-67 expression (*P* =0.62, Mann-Whitney test).

It is important to explain that cut point of 5, and 30 % for KI-67 staining was found based on 'under ROC curve area' with sensitivity and specificity of (95.5 & 85% vs. 70 and 100%) between two groups.

Although the cut point for KI-67 antigen for predicting the probability of HR-HPV presence in one person is 18.5

% with 90 %sensitivity and 95 % specificity.

Discussion

Recent studies demonstrated that the validity of KI-67 biomarker is a detector of pre-clinical cellular dysregulation in relation to HR-HPV statues in cases of CIN1. Actually, histological confirm CIN1 cases is likely to regress than persist or progress to high-grade lesion over time. The risk of progress CIN1 towards high-grade lesion is estimated at about 10% (14). The study of White in 2016 showed the persistency rate of 20% for a low-grade lesion (by cytology) in short time follow up, which was similar in the present study. On the other hand, in the white study as the present study had been recommended less intensive follow up for HR-HPV positive patients with a negative biomarker (15). It's important to find a reliable method which can predict the outcome of these patients. It is suggested that biomarker KI-67 as a proliferative biomarker can be used on the basis of proliferation activity in cervical dysplasia. Based on the literature review, the mean KI-67 expression in CIN1 lesion is reported 22-71% with different criteria for positive staining (1,6). For the first time, Dellas revealed KI-67 expression difference between HPV positive and negative patients that was correlated with our study (8). However, due to its capability, it can be used to follow up of patients especially in HR-HPV positive women (9,16,17). Rossi et al showed similar KI-67 expression in regress and persistent patients and no prediction possibility for this biomarker in two years' follow-up (7). Possati showed the highest sensitivity for this biomarker in proliferation at an older age (2). Šekoranja in 2017 reported similar results (18). In the current study, as there was no patient under 30 years who had persisted KI-67 expression; age couldn't be evaluated as a possibility to prognosis prediction. Supporting that proliferator's index could be an auxiliary method for HPV activity. Higher KI-67 index can predict the outcome of HR-HPV positive patients in short-term follow-up (one year in the current study), and showed no statistical difference between higher KI-67 index and probability of progression or even persistence risk of infection. In other studies, non-CIN 1 patients with HR-HPV positive with low-grade KI-67 staining were persisted after one year just as predicted (4,19). In addition, we demonstrated a correlation between colposcopic appearance and this biomarker and prognosis over time; no correlation was found between colposcopic findings and risk of persistence.

Limitation of this study was small number of cases, high number of inadequate histological samples for KI-67 analysis, and short-term follow up. We recommended more studies on the assessment of potential high-grade lesions and reducing side effects overtreatment.

In conclusion, KI-67 biomarker is recommended as complementary screening tests not alternative for differentiating in high risks patients with CIN1. The patients with low KI-67 / HR-HPV positive could be offered for a less aggressive follow-up protocol.

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