

Investigation of the P16 and Ki67 Predictive Effect on the Progression of Cervical Intraepithelial Neoplasia Grade 1 in Shahid Motahari Hospital of Urmia, Iran

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ABSTRACT

Background & Objective: Cervical cancer is a common neoplasm in women, and the role of the HPV virus in the development of precancerous and cancerous cells has been established. There exist different strains of the HPV virus with varied functions. In the high-risk HPV strains, the p16 and ki67 proteins play a crucial role in regulating the cell cycle leading to cell proliferation and progression. P16 and ki67 proteins are positive in almost all lesions and indicate a high degree of malignancy. This study aims to investigate the predictive effect of p16 and ki67 on the progression of low-grade intraepithelial lesions to high-grade malignancy.

Materials & Methods: P16 and ki67 were measured on CIN1 lesions, and during the average two-year follow-up period, the outcome of positive cases was investigated. A total of 106 referred patients between the age of 15 to 75 years were examined from April 2015 to March 2019.

Results: Among the patients with progression of CIN1 to CIN2 and other severe lesions, p16 was positive in 14 cases (60.9%), and a significant difference between groups with positive and negative markers in the progression or regression of lesions was noticed. Ki67 frequently occurs in CIN2 and other severe lesions.

Conclusion: The use of p16 and ki67 as predictive markers is still under debate. In countries like the United States, these are not yet used separately for prediction but are being used in combination together. The authors of this study strongly recommend the conduction of further studies to assess the role of p16 in association with other markers and within a larger population so as to apply the functional role of p16 and ki67 in the clinical setting thereby effectively preventing it.

Keywords: CIN, HPV, Ki67, P16, Uterine cervical cancer



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Introduction

Cervical cancer is a prevalent issue among adult women. Many industrialized countries have achieved significant success in reducing invasive cervical cancer over the past six decades, with an annual prevalence of 4 per 100000 to 14 per 100000 cases. In developed countries, approximately 15000 new cases of cervical cancer and 5000 yearly deaths occur (1). Cervical cancer, a neoplasm caused by the HPV virus, originates from premalignant lesions inside the epithelium and is referred to as intra-epithelial lesions of the squamous cell or cervical intraepithelial neoplasm (CIN 1-LSIL) (CIN 2-3 -HSIL) that are the precursors of cervical cancer (2).

Nearly 80% of low-grade SIL lesions (LSIL-CIN1) regress spontaneously within one to two years. HSIL-CIN2-3 management generally involves biopsy and removal of the lesion and the transformed zone to prevent the lesion from progressing. While LSIL and CIN1 management is supportive and necessitates follow-ups, 10% to 15% of LSIL / CIN1 lesions progress to HSIL, CIN2-3 (3, 4). Unfortunately, the available clinical, cytological, and histological methods cannot determine the eventual transformation of the LSIL, CIN1 lesions to HSIL, CIN2-3. HPV has an etiological role in cervical cancer and is found in almost all premalignant and malignant lesions of cervical cancer (5).

Different types of HPV are broadly classified into two categories: high-risk and low-risk (6). High-risk HPV sequence accumulation in the cell genome plays an essential role in the progression of cervical neoplasia lesions with E2 gene disruption (7). It results in excessive expression of E6 and E7 oncogenes, which impairs P53 and RB suppressor activity (8) and eventually destroys cellular control. High-risk HPV in CIN1 lesions is important and common, but only 10% of CIN1 lesions progress to CIN2-3. P16, a tumor suppressor protein, inhibits CPK activation and regulates cell cycle and proliferation by PRB phosphorylation. Immunohistochemical expression of p16 may attenuate PRB activity in negative feedback.

The diffused and strong pattern of p16 expression is observed in CIN2-3 but cannot be seen in CIN1. P16 has varied expressions, and its overexpression leads to interference of viral oncoproteins in the cell cycle and ends up in cell cycle destruction. Tumor suppressor protein p16 demonstrates the substitute marker role in high-risk HPV lesions. P16 is valuable in the diagnostic process because it is distinguished nearly in all HSL and CIN2-3 lesions despite focal or negative staining in imitative reactions such as immature metaplasia or atrophy. So, P16 staining has a key role in the classification of progression of lesions to HSL, CIN2-3, or other severe lesions.

Studies carried out in recent decades concerning this issue suggest that LSIL, CIN1 lesions with high p16 expression progress to HSIL and CIN2-3; owing to the insufficiency of research articles pertaining to this issue, authentic validity, and value of p16 as a progressive marker in women remains unknown (9-11). On the other hand, ki67 usually is found in all active phases of the cell cycle but not in resting cells (12).

During HPV infection, the transition control between mitosis phase c1 and phase S1 is destroyed by the retinoblastoma protein; following this, retinoblastoma itself decomposes, resulting in excessive expression of HPV infection and, subsequently, over-expression of ki67 (13). Immunohistochemical analysis of ki67 in histological biopsy of CIN1-2 shows that it is an independent and strong predictor of CIN lesions (14).

Co-expression of p16 and ki67 in the cell occurs only under abnormal conditions (15). Since cervical cancer is one of the most important causes of death in women in developing countries, determining the progression of intraepithelial lesions in these patients is of primary concern. Therefore, the purpose of this study is to investigate the predictive effect of p16 and ki67 proteins in the progression of low-grade intraepithelial lesions of the cervix. The present study was conducted at Shahid Motahari Hospital, Urmia, Iran, from 2015 to 2019.

Materials and Methods

This descriptive cross-sectional study consisted of outpatients in the age bracket of 15 to 70 years, who were examined and biopsied for CIN1, and were referred to the gynecology oncology clinic of Shahid Motahari hospital from April 2015 to March 2018. A selection of 115 patients with CIN1 diagnosis was done, and their cervical biopsy samples were reviewed by a common pathologist. Out of these 115 patients, nine were excluded, among which six cases had distorted slides upon review, two due to hysterectomy with CIN1 pathology, one because of history of breast cancer, the other because of a history of the disease and the last due to pregnancy.

A total of 106 patients with a definitive diagnosis of CIN1 were selected, and p16 and ki67 dual-staining was done (DBS-USA). Cytological slides were examined by a common pathologist and reported according to the BETHESDA system. The results of the HPV high-risk test were confirmed from the patients' files, and the patients subsequently underwent histological examination as well as P16 and ki67 immunohistochemistry tests under the following steps: four-micron thickness tissue incisions were made and inserted into 1/50 diluted histogrip solution for one minute, kept at 60 to 80 C for one hour, and then paraffin-disinfected with xylol.

Then the alcohol dehydration was done from one to seventy percent. The ANTIGEN RETRIEVAL stage was performed by EDTA solution. The slides were placed in the EDTA buffer, heated to 90 C for two minutes, and finally rinsed with TBS (Tris Buffered Saline). In the chromogen complex stage, the antibody-antigen adherence and staining were performed. Diagnosis of CIN1 was made by staining with H&E. The identification criteria are based on the Female Reproductive System Tumor Pathology and Genetic classification criteria (WHO 2014). For each slide, a control slide with squamous cell cancer (SCC) detection and a negative control slide (normal cervical epithelial cells) were kept. According to the LAST project:

1. Positive finding: It is defined as nuclear or cytoplasmic staining, and in the case of positive p16, basal and parabasal cells are stained either with or without upper cell layers.
2. Negative finding: It is defined as resistance to staining or trace staining with a focal dye in a few cells.

In addition to the basal layer, the degree of positivity is divided into three groups: one to ten percent (+), ten to thirty percent (++), and more than thirty percent (+++).

Follow-up was done at an interval of at least 6 months and a maximum of 4 years after diagnosis. Patients underwent colposcopy, cervical biopsy, and hrHPV testing at each follow-up, with all slides being examined by the previous pathologist. Progression of lesions was defined as a histologic diagnosis of CIN2 or more severe lesions. Regression was defined as negative hrHPV testing and grade less than that of CIN1, and persistent was determined as CIN1.

Ethical Issues

The present research strictly observed the principles of the Helsinki Declaration of 1975, as revised in 1983. The study was approved by the Forensic Medicine Ethics Committee, Bushehr (Ir.umsu.rec.1398.313), and is extracted from Samira Jahangards' residency research thesis (#9648).

Statistical Analysis

After data collection, patients were divided into two groups based on the positive and negative p16/ki67 and age, following which comparisons were drawn in each

group concerning their frequency of outcomes. Descriptive statistical methods, including frequency-percentage and average \pm standard deviation, have been used for statistical analysis. Chi-Square test (and if necessary, Fisher's exact test) was employed to compare frequency. SPSS 20 (SPSS Inc., Chicago, Ill., USA) was also brought to use, and a statistical significance was observed when P -value <0.05 .

Results

In this descriptive-longitudinal research, 106 cervical biopsies in the ASCUS, LSIL, and HSIL triage were studied. The mean age of the patients was 37.40 ± 10.15 , with the age bracket being 23-68 years (Table 1). Frequency of markers in population was 33.9% (n=36) for p16, 66.03% (n=70) for ki67 and 28.3% (n=30) for co-expression of p16 and ki67. Accordingly, ki67 was observed to have more frequency than the other two groups (Figure 1).

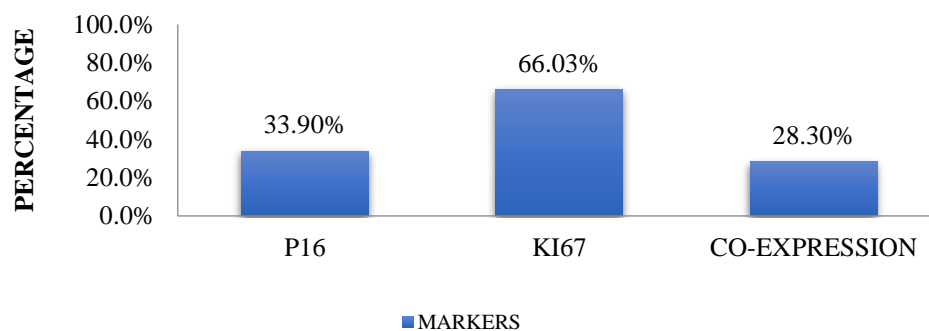


Figure 1. Frequency distribution of markers and co-expression of both markers

In terms of the frequency of progression of low-grade cervical lesions and based on the positive and negative p16 and ki67 in the two groups, progression or regression and persistency of the two groups in comparison to patients with negative p16 and ki67 in their biopsies, showed a significant difference and

transference to CINII and CINIII in patients in which both positive factors were significantly greater ($X^2=33.777$ $P=0.001$) (Table 1) and in those in which both negative factors were significantly greater ($X^2=22.277$ $P=0.001$) (Table 2).

Table 1. Frequency of low-grade progression cervical lesions based on the positive p16 and ki67 proteins

Variable	Both positive N (%)		X^2	P-value	
	Yes	No			
Patient biopsy status compared to previous biopsy	Normal	0 (0)	28 (36.8)	33.777	0.001
	CINII	14 (46.7)	9 (11.8)		
	CINIII	8 (26.7)	4 (5.3)		
	Fixed	8 (26.7)	35 (46.1)		

Table 2. Frequency of progression in low-grade cervical lesions based on the negative p16 and ki67 proteins

Variable	Both positive N (%)		X ²	P-value	
	Yes	No			
Patient biopsy status compared to previous biopsy	Normal	19 (57.6)	9 (12.3)	25.277	0.001
	CINII	3 (9.1)	20 (27.4)		
	CINIII	1 (3)	11 (15.1)		
	Fixed	10 (30.3)	33 (45.2)		

Table 3. Frequency of applied slides for pap smear in studied samples

Variable	Frequency	Percentage	
Applied slide for pap smear	ASCUS	47	44.3
	LSIL	52	49.1
	HSIL	7	6.6

Most of the applied slides for pap smear were LSIL (n=47), then ASCUS (n=52), and finally HSIL (n=7) (Table 3).

The highest frequency of negative P16 protein fell in normal and persistent groups – in these states p16 protein is less in number. Additionally, in 14 samples, p16 protein was observed in progression to CINII

(60.9%). In terms of the presence of this factor and progression or regression of disease, a significant difference among these groups was noticed (X²=27.585, P=0.001). The ki67 protein occurs more frequently in the CINII and CINIII groups, and a significant difference between these groups in terms of disease progression or regression has also been detected (X²=21.379, P=0.001) (Table 4).

Table 4. Frequency of progression of low-grade cervical lesions based on the positive or negative p16 and ki67 proteins

Variable	Biopsy of patients compared to previous biopsy N (%)				Statistics	
	Persistent	CINIII	CINII	Normal		
P16	Positive	14 (32.6)	8 (66.7)	14 (60.9)	0 (30)	X ² =27.585 P=0.001
	Negative	29 (67.4)	4 (33.3)	9 (39.1)	28 (100)	
Ki67	Positive	27 (62.8)	11 (91.7)	20 (87)	9 (32.1)	X ² =21.379 P=0.001
	Negative	16 (37.2)	1 (8.3)	3 (13)	19 (67.9)	

Discussion

Industrialized nations have significantly reduced invasive cervical cancer over the past six decades, with an annual prevalence of 4 per 100000 to 14 per 100000 cases. In developed countries, approximately 15,000 new cases of cervical cancer and 5000 annual deaths occur yearly (16). Cervical cancer is a neoplasm caused by the HPV virus that originates from premalignant lesions within the epithelium and is cervical cancer. Nearly eighty percent of low-grade SIL lesions (LSIL-CIN1) progress spontaneously within one to two years, so HSIL-CIN2-3 management generally involves biopsy and removal of the lesion and the transformed zone to prevent the lesion from progressing. LSIL and CIN1 management is supportive and consists of follow-ups, although 10% to 15% of LSIL / CIN1 lesions progress to HSIL, and CIN2-3.

In this descriptive-longitudinal study, 106 cervical biopsies with pap smear diagnoses of ASCUS, LSIL, and HSIL were examined. Colposcopy-guided cervical biopsies were taken and analyzed vis-a-vis the frequency of progression, regression, or state of persistence in a follow-up period of 6 months up to 4 years. The mean age of the study participants was 37.40 years, the lowest being 23 and the highest 68 years. In congruence with the results of our study, Xiaobo Zhang *et al.* confirmed a significant difference between patients positive for p16 and ki67 (mean age = 40.5 years) in terms of progression, regression and status of persistence, owing to the high progression of low-grade cervical lesions and the positive combination of p16 and ki67 proteins (which predict the progression of LSIL and CINI lesions), as compared to patients who were not p16 or ki67 positive.

A statistically significant difference was observed with regards to p16 and ki67 in all three groups. Also, in 24 cases that were p16 positive, the rate of progression was 14.56%, while in those with negative p16, it was 1.6%; a significant difference was also noticed with respect to the ki67 protein. The ki67 rate of progression in the group with less than 10% staining was 3.24 %.

According to Zhang *et al.*, ki67 can be considered a definitive predictor in the progression of CIN1 lesions (17). However, Mills *et al.*'s study has stated that P16 should be used selectively for problematic scenarios such as CIN2 cases wherein one struggles to differentiate CIN1, CIN2, and benign mimics of CIN3' (18). In a review study conducted by Saada Amed *et al.* in Nigeria, in 2017, the known risk factors for cervical cancer were as follows: persistent infection with high-risk HPVs, young age at the time of first sexual intercourse, multiple sexual partners, smoking, and weakened immune systems (especially in kidney transplant patients). They have further stated that the specificity and sensitivity of p16 to diagnose CIN2 and higher grade in the population is 90.09 %. Amed *et al.* concluded p16 and ki67 dual-staining was an effective screening method (1).

In the present study, progression to CIN 2 with positive p16 was seen in 14 samples (60.9%). A significant difference between the groups in the presence of p16 and disease progression or regression was also observed. Further, ki67 protein was found to be abundant in CINII and CINIII status. However, Pucchiurotti *et al.* have stated that P16 can be used as a biomarker for cases where ambiguity arises in the diagnosis, and a weak predictive effect for the progression of lesions exists (19).

Ammaro Filho *et al.* demonstrated that there is an excessive expression of p16 in uterine cervical cancer with a diffuse pattern; compared to the control group

CIN1 lesions with positive p16 swiftly change to CIN2 and other severe lesions (20). Razm Poosh *et al.* designed a study that matched IHC staining and DNA high-risk HPV with PCR and found that the high p16 appearance with the diffuse pattern was associated with a high-risk HPV suggesting that P16 cannot have high clinical value because P16 is a poor predictor for progression of CIN1 (21) which is contrary to the findings of the present study.

Conclusion and Recommendations

Ki67 protein was found in abundance in CINII and CINIII states. However, it cannot be used for prediction separately. The use of p16 and ki67 together as a predictor marker is still controversial, and in countries like the United States, it is not yet used for prediction separately. The authors of this study emphasize the conduction of further studies to assess the role of p16 in association with other markers and within a larger population to apply the functional role of p16 and ki67 in the clinical setting hence aiding its prediction.

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Conflict of Interest

The authors declared no conflict of interest.

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