Original Article

Role of p16^{INK4A}/Ki-67 Dual Immunostaining on Cell Blocks in Detecting High-Grade Cervical Intraepithelial Lesions

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Abstract

Background: P16/Ki-67 dual immunostaining has been confirmed as a sensitive and specific test for human papillomavirus positive women. In the present study, we evaluated cell blocks (CBs) with p16^{INK4A}/Ki-67 biomarkers to detect high-grade cervical intraepithelial neoplasia (CIN). **Materials and Methods:** Samples for CB preparation were taken from females with abnormal Pap smears, who also underwent colposcopic guided biopsies, P16^{INK4A} and Ki-67 staining were performed on CBs and tissue biopsies, histopathology with p16^{INK4A} expression was considered the gold standard. Sixty-five specimens were included in the study. **Results:** The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy (AC) of CB + p16^{INK4A}/Ki-67 in detecting CIN2 when considering only cytology specimens with the low-grade squamous intraepithelial lesion (LSIL) were 86.67%, 100%, 66.67%, 89.66%, and 82.93%, respectively. The sensitivity, specificity, PPV, NPV, and AC of CB + p16^{INK4A}/Ki-67 in detecting CIN2 when considering only cytology specimens with atypical squamous cells of uncertain significance/LSIL were 75%, 85%, 60%, 91.89%, 82.69%, respectively. Rates of positive staining for p16 ^{INK4A}/ki-67 were enhanced according to increased pathologic grade and differed statistically between CIN1 and CIN2 as well as squamous cell carcinoma. **Conclusion:** CB preparation technique with p16^{INK4A} and Ki-67 immunostainings have improved the diagnostic AC of Pap smear in detecting high-grade CIN.

Keywords: *Cellblock, cervical intraepithelial neoplasia, Ki-67, p16 INK4A, Pap smear*

Introduction

Cervical cancer is the fourth most common cancer in women. In 2020, an estimated 604,000 women were diagnosed with cervical cancer worldwide and about 342,000 women died from the disease.[1] High-risk HPVs are important risk factors for human cervical cancer, approximately 96% of cervical cancers score positive for hrHPVs.[2,3] HrHPVs were present in 95% of invasive cervical cancers in Syrian women.[4] The main aim of cervical cancer screening is to detect and treat high-grade cervical intraepithelial neoplasia (CIN) to prevent its progression into invasive cancer, hence a screening test should have optimal sensitivity and specificity for detecting these lesions.[5] Recently, cell block (CB) preparation has been used as a diagnostic technique to complement liquid-based, monolayer cervicovaginal specimens.[6,7] Furthermore, many dysplasia-associated biomarkers have been identified and used to improve the diagnostic accuracy (AC) of neoplastic and preneoplastic lesions of the cervix in histology and cytology.[8,9] Ki-67 (MIB-1) a marker of cell proliferation, and P16^{INK4A} a surrogate marker of hrHPV infection, have shown promising results as reflected by the relatively high volume of literature.[10] In this study, we evaluated the role of P16^{INK4A} and Ki-67 dual immunostaining on CBs to detect high-grade CIN.

Materials and Methods

Study design

Approval for the study was obtained from the Ethics Committee of the Faculty of Medicine of the University of Aleppo. Our prospective study was carried out from January to July 2020. All pap smears received from the Department of Gynecology within this period were reviewed, and the abnormal ones were included in the study. After obtaining informed consent, new samples for CB preparation were taken from all females with abnormal Pap smears, who also underwent Colposcopy referral, and multiple punch biopsies were taken, P16^{INK4A} and

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Ki-67 staining were performed on CBs and Tissue biopsies which is considered the gold standard according to the recent WHO recommendations.[11] We were able to prepare CBs sections from (97) women. We could follow (70) cases clinically and (5) specimens were excluded because of low cellularity. In result, the total number of cases that were included in the study is (65). Both Pap smears and CBs were analyzed by two Cytopathologists according to the Bethesda 2014 system.[12]

Sample collection and cell block preparation

First, samples were taken with a sterile wooden spatula from the transformation zone of the cervix by 360-degree rotation around the cervix. Then spatula was placed into centrifuge tube containing normal saline solution and shaken. All tissue particles attached to the spatula were dislodged by toothless forceps into the normal saline solution which is then transformed into another small plastic centrifuge tube and centrifuged at 2000 PRM for 15 min. The supernatant fluid was poured off. 10% of neutral buffered formalin was added gently along the tube wall to the remaining sediment and allowed to fix for 24–28 h.

After fixation Eosin drop was added and the sediment was wrapped in filter paper and processed as a routine histopathology specimen. 4 μm sections were cut from the CBs and stained with Hematoxylin and Eosin for morphologic evaluation.

Immunohistochemical stain protocol

Immunohistochemical stains were done manually by an experienced technician using the manufacturer's standardized protocol (69 Santa Felicia Dr., Santa Barbara, CA 93117, USA). We used P16^{INK4A} (Bio SB, USA, Clone 16p04, JC2) and Ki-67 (Bio SB, USA, Clone Ep5) and Bio SB envision system as detection Kit. From each CB we took two sections, and we applied the formerly mentioned biomarkers to each one separately. For each batch we stained a positive and negative control, using cervical cancer as the positive control and the primary antibody as the negative one.

Biomarker's reporting

Both biomarkers (P16^{INK4A} and Ki-67) were assessed separately for:

The number of epithelial cells that were stained, which were counted as percentage to the total number of epithelial cells. For P16^{INK4A} at least 10 cells stained were considered positive to prevent nonspecific background staining, and only nuclear and nuclear with cytoplasmic immunostaining were considered positive. For Ki-67 nuclear immunostaining was considered positive.

Statically analysis

Demographic characteristics and variables of interest were summarized by using descriptive statistics: Mean (standard deviation) for continuous variables and frequency (proportion) for categorical variables. A $P < 0.05$ was used to detect the statistical significance. Chi-square and T independent tests were used as a test for statistical significance. Analysis was performed using IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY, USA: IBM Corp. We calculated the sensitivity and specificity of the detection of high-grade dysplasia: P16/Ki-67 dual stain. A true positive test result would identify patients with CIN grade 2 or worse CIN2+ on histology while a true negative result would identify patients with CIN grade 1 or normal histology. Sensitivity and specificity were calculated through MedCalc Statistical Software version 19.7.2 (MedCalc Software by, Ostend, Belgium; https://www.medcalc.org; 2020).

Results

Sixty-five women were included in this study which was conducted at the cytopathology department of Aleppo University Hospital. The ages of patients ranged between 20 and 63 years old, with mean age (41.29 ± 8.640) years old, and median, 43. There's no statistical significance between patients regarding age ($P = 0.081$), but we noticed that advanced ages correlated with the highest grades of malignancy on cytology.

To evaluate the AC of CB and Pap smear diagnosis, the 65 specimens were subjected to tissue follow-up, 48 (73.8%) were incisional Biopsies, 11 (16.9%) were cone biopsies, and 6 (9.2%) were from the hysterectomy. Histological study of the biopsies showed the dominance of CIN1 (49.2%) and the less frequent pattern was squamous cell carcinoma (SCC) (7.7%).

Pap smear specimens were diagnosed as (atypical squamous cells of uncertain significance [ASCUS]) were 11 (16.92%), 41 (63.08%) were (low-grade squamous intraepithelial lesion [LSIL]), 13 (20%) were high-grade squamous intraepithelial lesion (HSIL) two of them show features of invasion [Figure 1].

In this study, there were 10 cases of ASCUS with CIN1 or lesser degree of abnormality CIN1-on histology. For the 41 cases with LSIL on Pap smear, 30 were CIN1-on histology while 11 were CIN2+. For the 13 hIL specimens on Pap smear, 6 were CIN2 and 5 were SCC.

Correlation between Pap smear and the result of the biopsy was tabulated in [Table 1].

Positive cases for p16/Ki-67 dual immunostaining on CBs categorized according to the pap smear result are summarized in Table 2.

The sensitivity and specificity of CB+ P16, CB+ Ki-67, CB+ P16/Ki-67 in detecting CIN2 when considering only cytology specimens with LSIL are tabulated in Table 3. The sensitivity and specificity of CB+ P16, CB+ Ki-67, CB+ P16/Ki-67 when considering only cytology specimens with ASCUS/LSIL are tabulated in Table 4.

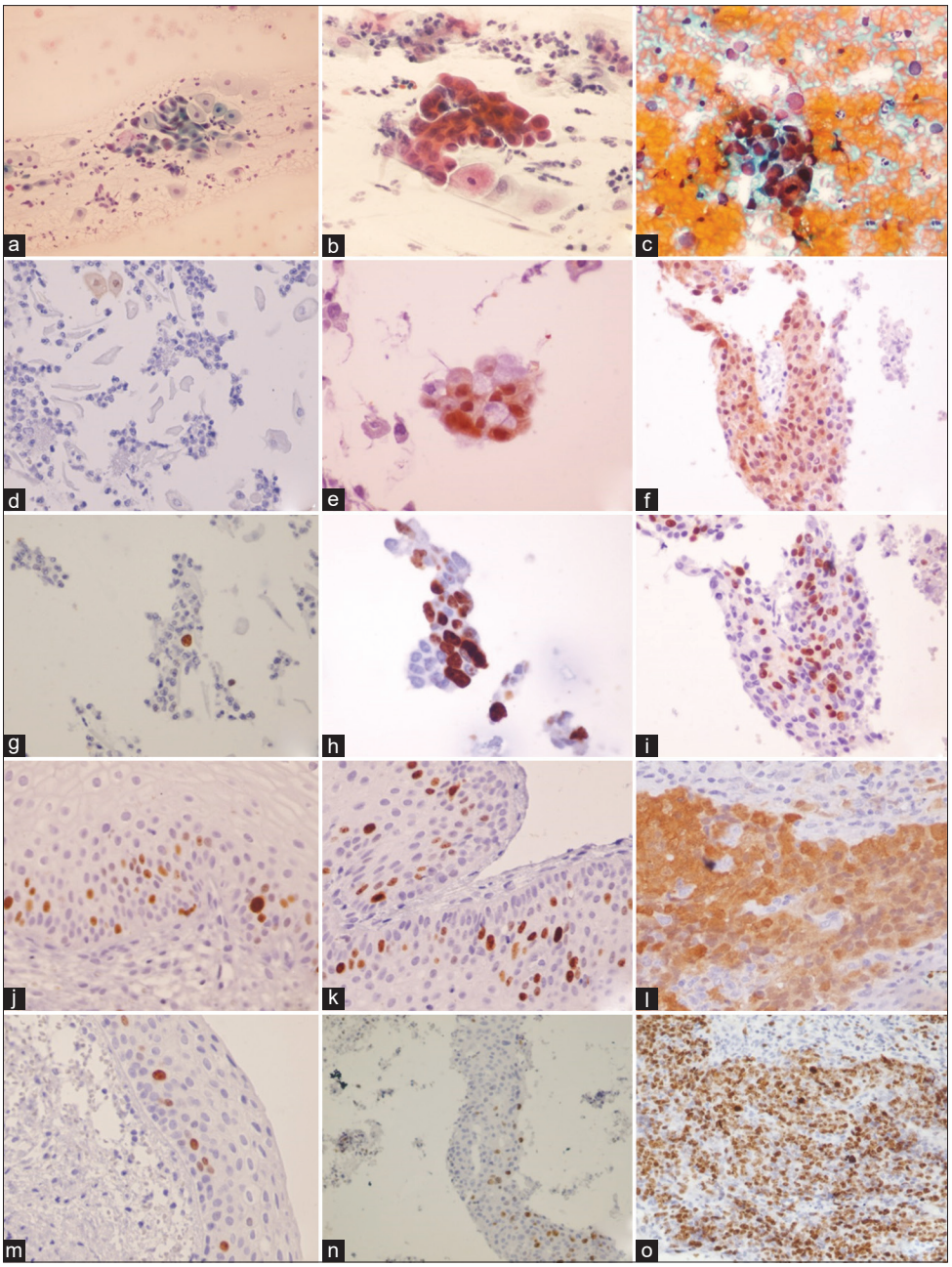

Figure 1: A group of photomicrographs (×40) showing different cervical lesions on Pap smears, and Expression of p16INK4A/Ki-67 on cell blocks and tissue sections. (a) (Low-grade squamous intraepithelial lesion) Pap smear; (b) (high-grade squamous intraepithelial lesion) Pap smear; and (c) (high-grade squamous intraepithelial lesion) with features of invasion Pap smear. (d) Cell block +P16 (low-grade squamous intraepithelial lesion); (e) cell block +P16 (high-grade squamous intraepithelial lesion); (f) cell block +P16 (squamous cell carcinoma); (g) cell block +Ki-67 (low-grade squamous intraepithelial lesion); (h) cell block +Ki-67 (high-grade squamous intraepithelial lesion); and (i) cell block +Ki-67 (squamous cell carcinoma). (j) Biopsy +P16 (cervical intraepithelial neoplasia 1); (k) Biopsy +P16 (cervical intraepithelial neoplasia 2); (l) Biopsy +P16 (squamous cell carcinoma). (m) Biopsy +Ki-67 (cervical intraepithelial neoplasia 1); (n) Biopsy +Ki-67 (cervical intraepithelial neoplasia 2); (o) Biopsy +Ki-67 (squamous cell carcinoma)

Table 1: Correlation between pap smear and the result of biopsy

Pap smear	Result of biopsy				Total
	CIN1, *n* (%)	CIN2, *n* (%)	SCC, *n* (%)	Negative, *n* (%)	
ASCUS	4 (12.5)	1 (5.0)	0	6 (75.0)	11 (16.9)
LSIL	28 (87.5)	11 (55.0)	0	2 (25.0)	41 (63.1)
HSIL	0	8 (40.0)	5 (100.0)	0	13 (20.0)
Total	32 (100.0)	20 (100.0)	5 (100.0)	8 (100.0)	65 (100.0)

ASCUS: Atypical squamous cells of uncertain significance, LSIL: Low-grade squamous intraepithelial lesion, HISL: High-grade squamous intraepithelial lesion, SCC: Squamous cell carcinoma, CIN: Cervical intraepithelial neoplasia

Table 2: Positive cases for p16/Ki-67 dual immunostaining on cell blocks categorized according to the pap smear result

Pap smear	Count (%)					
	Ki67		P16		Dual stain	
	Positive	Negative	Positive	Negative	Positive	Negative
ASCUS	4 (11.4)	7 (23.3)	3 (9.4)	8 (24.2)	3 (10.7)	8 (21.6)
LSIL	18 (51.4)	23 (76.7)	16 (50.0)	25 (75.8)	12 (42.9)	29 (78.4)
HSIL	13 (37.1)	0	13 (40.6)	0	13 (46.4)	0
Total	35 (100.0)	30 (100.0)	32 (100.0)	33 (100.0)	28 (100.0)	13 (100.0)

ASCUS: Atypical squamous cells of uncertain significance, LSIL: Low-grade squamous intraepithelial lesion, HISL: High-grade squamous intraepithelial lesion

Table 3: The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of cell blocks + P16, cell blocks + Ki67, cell blocks + P16Ki67 in detecting cervical intraepithelial neoplasia 2 when considering only cytology specimens with low-grade squamous intraepithelial lesion

Type of investigation	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AC (%)
CB + Ki67	81.82	70	50	91.3	73.17
CB + P16	72.73	73.33	50	88	73.17
CB + P16 + Ki67	86.67	100	66.67	89.66	82.93

PPV: Positive predictive value, NPV: Negative predictive value, AC: Accuracy, CB: Cell blocks

Table 4: The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of cell blocks + P16, cell blocks + Ki67, cell blocks + P16Ki67 in detecting cervical intraepithelial neoplasia 2 when considering only cytology specimens with atypical squamous cells of uncertain significance/low-grade squamous intraepithelial lesion

Type of investigation	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AC (%)
CB + Ki67	83	70	45.45	93.33	73.08
CB + P16	75	75	47.37	90.91	75
CB + P16 + Ki67	75	85	60	91.89	82.69

PPV: Positive predictive value, NPV: Negative predictive value, AC: Accuracy, CBs: Cell blocks

When detecting CIN2+ in LSIL cases (n = 41), the dual immunostaining showed high sensitivity and specificity with a left shift of the ROC curve [Figure 2]. This observation was supported by the area under the ROC curve.

In HSIL group, the sensitivity and specificity of CB + P16, CB+ Ki-67, CB+ P16/Ki-67 were 100%.

The rates of positive staining for p16 in CB preparation diagnosed as CIN1, CIN2, SCC were <24%, 24%, and 39%, respectively. The detecting rates for Ki-67 in CIN1, CIN2, SCC were <19%, 19%, and 32%, respectively. The differences in positive staining rates and staining intensity for p16 between CIN1 and CIN2 and for SCC were statistically significant P = 0.011. Furthermore, the positive rates for Ki-67 between CIN1 and CIN2 on CB preparations differed significantly P = 0.027. Obviously, the intensity of P16/Ki-67 staining and the number of positive cells was enhanced according to increased pathologic grade [Figure 1].

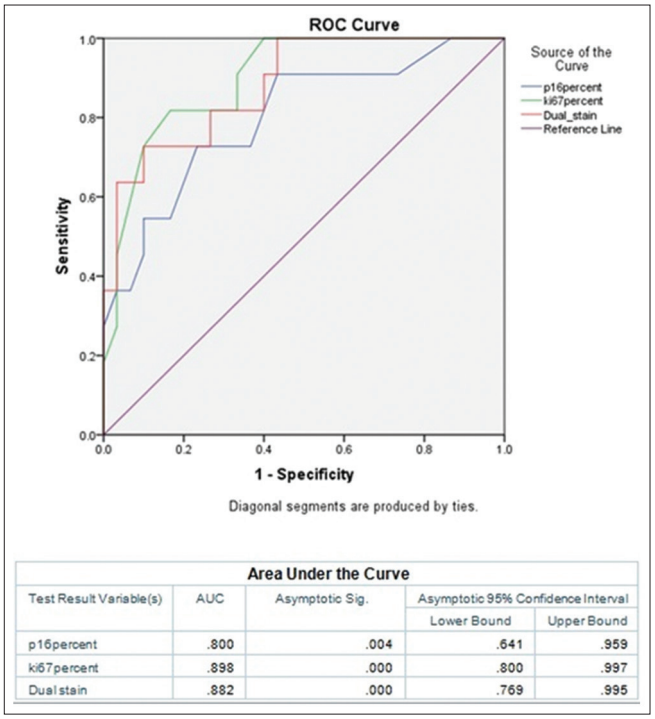

Figure 2: ROC curves in detecting CIN2+ in 41 LSIL cases. A left shift is seen in dual immunostaining

Discussion

The ability to predict the development of CIN is an important issue for cervical cancer prevention and

treatment.[13] Hence, increasing the AC of cytology diagnosis of cervical lesions, especially for HSIL and SCC, is pivotal. LSIL is the same as (CIN I) and represents a noncarcinogenic human papillomavirus (HPV) infection, which is generally resolved without treatment. HSIL (same as CIN 2 and 3) is a precancerous lesion and often requires surgical intervention to prevent further progression to SCC. HPV test is a sensitive way to detect CIN2+, but not specific because the test cannot differentiate between a transient infection, an early persistent infection that may develop to CIN2+, or prevalent CIN2+ disease.[14] P16/Ki-67 dual immunostaining has been confirmed as a sensitive and specific test for HPV-positive women.[15-17] The p16 protein can be used as an auxiliary complement for the screening of cases of LSIL, ASCUS, and with pap smear-negative results.[18] It has been suggested that the p16 protein may be useful to improve cytology and submit the colposcopy to a more detailed analysis. Furthermore, p16 staining can assist in the interpretation of results of Pap Smears or of histology in cases of atypical results.[19,20] The expression of Ki-67, differed between CIN1, CIN2, and CIN3, this study found that adding ki-67 to the CB raised the sensitivity in cases of LSIL.[21] In our study, in the ASCUS group: P16/Ki-67 dual staining on CB help in detecting CIN lesion and differentiate it from reparative atypia, especially in women who underwent cervical cauterization before the Pap Smear Test. In the LSIL group, our technique help picks out CIN2+ lesions, Consequently, selected candidates for additional examinations. In the HSIL group, our technique was similar to Pap smear in detecting high-grade lesions, but the rates of positive staining help in differentiating between CIN2/CIN3 and SCC. To our knowledge, the current study is one of the few that studies the value of p16^{INK4A}/Ki-67 dual immunostaining in cervical cancer screening. Until 2007, 61 studies have been published on p16 immuno-expression which included 27 studies on cytological specimens and 34 studies on cervical biopsies,[22] The analysis concluded that p16 immunostaining correlated with the severity of cytological/histological abnormalities. Recent studies focused in using p16^{INK4A}/Ki-67 on CBs to increase the diagnostic AC in detecting CIN lesions and as a complement Technique to the Traditional Screening Tests. Until 2021, many studies applied p16^{INK4A}/Ki-67 in cytology specimens either on CB or pap smear. Our sensitivity and specificity in detecting CIN2 in the LSIL group were similar to those reported by other authors.[23-26] In ASCUS/LSIL group our Technique was more specific but less sensitive in detecting CIN2 in comparison with Tay TK study.[27] Briefly, in our study, we compared the diagnoses in CB preparations with the diagnosis from Pap smear and tissue section; performed p16, and Ki-67 immunostains on CB and tissue sections; and analyzed the correlation of the expression of these biomarkers with the severity of cervical lesions.

Conclusion

Our data demonstrated That Pap smear test remains an essential screening method for detecting cervical lesions, and CB preparation technique with p16^{INK4A} and Ki-67 immunostainings have improved the diagnostic AC of Pap smear in detecting high-grade CIN.

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Conflicts of interest

There are no conflicts of interest.

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