

# P16/Ki-67 Dual Staining Improve Detection of High-Grade Intraepithelial Lesions and Cervical Cancer.

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**Abstract--**Worldwide, cervical intraepithelial lesions are common in adult women , after the age of 30 years . The high grade lesions are precancerous and could transformed to in situ and invasive squamous cell carcinoma of uterian cervix .These transformation significantly can be reduced by screening program of the cervix ,namely : cytology, HR-HPV test , and colposcopy .

Women with cytology of utrian cervix that showed morphology of intraepithelial lesions ,especially high grade lesions , HR-HPV test should be done for these women . However , high prevalence of HPV infection can't predicate the risk of malignant transformation in these women , thus additional triage test , including biomarkers for transformed cells can be used to detect women need further evaluation , follow up and treatment .

In current study , p16/Ki-67 dual immunostaining , was used for cytology as biomarker for detection of cellular changes and can improve the diagnosis of precancerous lesions and cervical squamous cells carcinoma .

**Aim of the study:** To identify the role of p16/ki-67 dual immunostaining in predicting the presence of significant cervical lesions in women with mild cytological atypia .

**Patients , material and method:** A prospective study of 317 women , with average age 39 years , over a period of December 2017 to August 2019, in Bagdad city and Salahaden Government .Patients were referred from private gynecological clinics to private lab of Dr.Salim Rasheed Al-Obaidi in Baghgad city and Lab of Dr. Inas Abd Al Majed Rasheed in Salahaden Government. All cytological smears from these women that were showed cytological morphology of intraepithelial lesions , were tested for HR-HPV ( by PCR ) , and p16/ki-67 dual immunostaining of cytology followed by colposcopy-guided biopsy were done for 58 women .

**Results:** Positively staining cells for p16/Ki-67 dual staining showed dark red –red brown nuclei , with clear or brownish cytoplasm . Positive slides are defined by presences of one or more cervical epithelial cells that expressed simultaneously stained for both p16/Ki-67 markers.

It has been shown the sensitivity and specificity of immunostaining in diagnosis of CIN2/CIN3 for ASC-US and LSIL lesions were (100 and 73%), and (100-89.7%) respectively.p16/ki-67 immunostaining positivity also increased with cytological severity in corresponded to histological morphology.

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**Conclusion:** *In addition to cytological evaluation and HR-HPV testing in women with cervical intraepithelial lesions , p16/ki-67 dual immunostaining of useful value in triage of ASC-US and LSIL lesions , according to high sensitivity and specificity .*

**Key words--** *p16/ki-67 dual immunostaining, ASC-US , LSIL , LBC , colposcopy guided biopsy .*

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## I. INTRODUCTION

Cervical intraepithelial lesions are common all over the world , caused by infection with HPV , as this viral infection cause dysplastic or disorder maturation of the squamous epithelial lining of uterian cervix .The dysplastic changes of squamous epithelial lining can be divided into 3 grades : grade I(mild dysplastic changes, low grade ) , grade II and III , moderate and severe dysplasia ( high grade lesions). Severe dysplasia is considered carcinoma in situ of uterian cervix , and can be progress to invasive squamous cells carcinoma (1,2)

The cytological smears that revealed cellular dysplastic changes ,these women should tested for HR-HPV , (3,4) .

Regarding HPV genotypes , more than 100 different known genotypes had been found , and grouped as low risk (LR ) and high risk (HR) types. The most prevalent worldwide include HR-HPV genotype 16&18 , as they infect mucous membrane and cutaneous , causing wart lesions and dysplastic changes of the epithelial lining of uterian cervix (4,5).

Colposcopy is indicated for all women with abnormal cytological smears &/ or positive HR-HPV test (5,6) .To reduce over-referral colposcopy , p16/Ki-67 dual staining have been developed to stain dysplastic cells in cytology or histology of punch biopsy , to detect dysplastic changes and early diagnosis of cervical squamous cells carcinoma (7).

P16 is cell cycle regulatory protein that induce cell cycle arrest when DNA changes or damage have been taken place , to avoid abnormal cell proliferation (tumor suppressor gene), while Ki-67 is a cell proliferation marker .Normally , they don't co-expressed in the same cervical epithelial cell . The co-expression of both markers indicate a disordered cell cycle , mediated by HR-HPV infection .

The co-expression of p16/Ki-67 can be detected by , using antibodies against them .Immunostaining for p16 alone, resulting in brownish cytoplasm and nuclei , while red nuclei expressed by using ki-67 immunostaining alone .

Positive p16/ ki-67 dual staining , showed brown or clear cytoplasm with dark red-red brown nuclei in the same cells .Positive slides are defined by presences of one or more cervical epithelial cells that expressed simultaneously stained for both p16/Ki-67 markers .

## II. PATIENTS, MATERIAL AND METHOD

A prospective study of 317 women with average age of 39years old , was done in Baghdad city and Salahaden government in Iraq , over a period lasting form December 2016 to August 2019.

The included patients in the study , were referred from private gynecological clinics in Baghdad city and Salahaden government to private lab of Dr. Salim Rasheed Al-Obaidi, in Baghdad city and private lab of

Dr. Inas Abd Al Majed Rasheed in Samarra city-Salahaden government .All referred women were undergone cervical cytology ,HPV-test , and p16/ki-67 dual immunostaining .

Inclusion criteria include : patient over 30 years presented with postcoital bleeding , intermenstrual bleeding offensive vaginal discharge , pelvic pain , and dyspareunia .

Exclusion criteria for cytology include : pregnancy ,intrauterine device , hysterectomy , patients previously diagnosed as cervical neoplasia ,

By using liquid based cytology (LBC), which is a new technique for cervical cytology , in which cervical epithelial cells are collected by a small brush , in the same way as for a conventional smear test , but the sample is deposited into a vial of preservative liquid (SurePath®Preservative Solution, TriPath Imaging Inc., Burlington, NC27215, USA). The liquid is treated to remove mucous , blood and other debris , before a layer of cells is placed on a slides. Then , the slide is prepared and stained manually .LBC have an advantages over conventional cytology smear, include limitation or reduction of inadequate samples and increased the sensitivity of the cytological smears , and can be provide material for HPV test (7,9,10) .

The prepared slides then examined by cytopathologist and final diagnosis and report for each case was made according to Bethesda system of reporting cervical cytology (BTS).

Another set of cytological slides were prepared from harvested cells of cell mixture preserved in initial process of LBC technique for each patient with abnormal cytological smears .p16/ki-67 immunostaining of these slides was done , using CINtec®Plus Kit (Roche mtm laboratories Ag, Heidelberg , Germany ) according to the manufacture instructions. The primary antibody cocktail include :a mouse monoclonal antibody (clone E6H4) against p16 protein , and a rabbit monoclonal antibody (clone 274-11CA3) against ki-67 protein . Alcohol –free hematoxylin was used for counterstaining.

The slides were reviewed by two pathologist , positive slide was defined by presence of one or more cervical epithelial cells that expressed simultaneous brown-clear cytoplasm and dark red-brown nuclei , irrespective of cellular abnormalities .Slides without any cells expressed dual immunostaining were considered negative .

Patients with dysplastic cervical cytology and positive test for HR-HPV , were referred for colposcopy and biopsy at the gynecological clinic. Review of histopathological slides were done by two pathologist .

### Statistical analysis

Data analysis was done using Chi-square test for independence to evaluate the association of p16/ki-67 dual staining with cervical epithelial abnormality , with P value < 0.05 as statistical significance .

### III. RESULT

The study included 317 women of mean age of 39 years old , who were met inclusion criteria , 12 of these cases had unsatisfactory cytological smears , and were excluded from the current study .Of the remainder 305 cases , 247 cases were diagnosed as a nonspecific lesions (e.g cervical erosion , chronic –nonspecific cervicitis ) not need to follow up and monitoring , 16 (27.6%) had ASC-US , 14(24.1%) had LSIL , 19 (32.7%) had HSIL ,9 (%) had invasive squamous cell carcinoma .

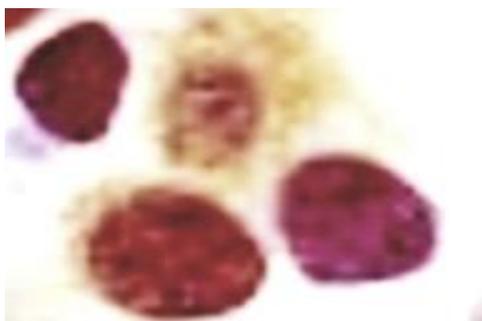
A total of 46 cases (79.3%) has positive p16/ki-67 dual immunostaining among 58 cases with cervical epithelial abnormalities .Those showed positive p16/ki-67 immunostaining(Fig-1) , were underwent biopsy later in the course of management .

Among the cases with ASC-US , 9 (56.3%) showed positive immunostaining , all were found to be CIN2/CIN3 in histology , 3(18.7%) cases showed negative immunostaining despite they having CIN2/CIN3 in histopathology .4 (25%) cases were found to be negative immunostaining and they had CIN 1 or no dysplastic changes in corresponding histology.

Regarding LSIL , 9(64.3%) cases had positive p16/ki-67 dual immunostaining with CIN2/CIN3 in histology , 2 (14.3%)of them were showed CIN2/CIN3 , and 1(7.14) had CIN1 on histology, with negative immunostaining .The rest 2(14.3%) patients had negative immunostaining , and were showed CIN1 and no dysplastic changes in corresponding histology .

For HSIL and squamous cell carcinoma , p16/ki-67 dual immunostaining were positive in 100% of the cases , corresponding with histology .

The current study had showed the sensitivity and specificity of p16/ki-67 dual immunostaining in detection of CIN2/CIN3 in women had ASC-US ,were 100 and 73 % respectively , and cases with LSIL were found to be 100 and 89.7% respectively .



**Fig 1:** Show positive p16/Ki-67 immunostaining.

#### **IV. DISCUSSION**

Cervical intraepithelial lesions are common in women above the age of 30 years .These lesions are categorized as low grade and high grade, according to dysplastic changes that taken place in cervical lining epithelial cells .The high grade can progress to carcinoma in situ and invasive squamous cell carcinoma .These lesions are mediated by HR-HPV infection that responsible to wart lesions and dysplasia of mucous membrane.

About more than 100 HPV genotype has been found , and further divided to low risk (LR ) and high risk (HR) HPV , the most prevalent subtypes include HR-HPV 16&18 .The infection transmitted through sexual contact . The integration of viral genome into host cells , resulting in abnormal regulation of cell cycle , and induce immune evasion of the infected host cells , which enable the virus to be undetectable for long period of time . The development of cervical carcinogenesis by HR-HPV infection is a multisteps process, ranging from persistent HR-HPV infection, different grades of intraepithelial lesions , and cervical cancer .

Thus , HR-HPV infection can produce heterogeneous molecular changes in the infected cells (3,6,7), and may difficult to be detected by cytological examination of the cervical epithelial cells , and additional triage test by biomarkers has been develop to increase the detection of dysplastic cellular changes .These biomarkers were included in current study , namely p16/ki-67 dual immunostaining of cervical epithelial cells.

The cervical lesions of ASC-US and LSIL remain difficult and of great challenge in clinical management .

In current study , the prevalence of CIN2+lesions in ASC-US women was 56.3%, which significantly higher than other studies , ranging from 5-22%,(3,15,16) ,These difference may be due to use of conventional cytological examination of the cervix as screening program and diagnostic tool in previous studies .Other studies have shown it to be around 53% ,(7).

Similarly , the prevalence of CIN2+ lesions in women with LSIL was found to be also higher than other studies and reached to 64.3%, while other studies were around 30-58.3%(3,17,18), but close to Diya Das et al study , which was 58.3%,(7).

## V. CONCLUSION

In current study , 79.3% of all cases with epithelial abnormalities , showed positive p16/ki-67 dual staining , which closely follow previously conducted studies(19,20,21) .It has been showed the dual staining of cervical epithelia have high sensitivity (100%) and specificity( 73%) in detecting CIN2+ lesions in ASC-US women , this follow previous study of Diya Das et al , which were (100 and 70%, respectively ) (7) ,but other conducted studies have shown wide range of sensitivity (64-98%)and specificity (43-81%) for dual immunostain in detection of high grade intraepithelial lesions in ASC-US populations (3,16,20,22).

Among women having LSIL in cytology , the sensitivity and specificity of p16/ki-67 dual staining to identify high grade intraepithelial lesions were 100 and 89.7%, respectively . Diya Das found the sensitivity 87.5% and specificity 100%(7), which close to current study .Consistent results have been achieved by other studies .(12,13,20,21,22,23)

The current study was limited by small population size , but the strengths lay in a well-defined population , with availability of histological diagnosis , and usage of same fresh cervical sample for both cytological diagnosis (LBC) and p16/ki-67 dual staining .

This p16/ki-67 dual immunostaining may potentially be a useful tool as a triage test for the ASC-US and LSIL group , as more studies have been shown a significant rate of immunostain positivity with high sensitivity and specificity for detection high grade intraepithelial lesions in these women .

In addition , over-referral colposcopy to women having abnormal cytological finding , can be reduced by use of p16/ki-67 dual immunostaining .

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Abbreviations:

ASC-US: Atypical squamous cell of undetermined significance.

BTS: Bethesda system

CIN: cervical intraepithelial neoplasia.

CIN1: cervical intraepithelial neoplasia, grade 1

CIN2: cervical intraepithelial neoplasia, grade 2

CIN3: cervical intraepithelial neoplasia, grade 3

CIN2+: cervical intraepithelial neoplasia, high grade (2&3)

HPV: human papillomavirus.

HR-HPV: high risk human papillomavirus.

HSIL: high-grade squamous intraepithelial lesion

LBC: liquid –based cytology.

LR-HPV: low risk human papilloma virus.

LSIL: low-grade squamous intraepithelial lesion.

Pap smear: Papinuclo stain smear.

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