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Original Research Article

Role of P16INk4a And Ki-67 Immunostaining as Specific Biomarker of Cervical Intraepithelial Lesions among Cervical Biopsy Samples: A Diagnostic Study

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Abstract:

Introduction: Cervical carcinoma, fourth most common cancer among women globally. The diagnosis on histopathology is considered as gold standard ,but pathologist faces diagnostic dilemmas not only because of overlap in morphological features among different grades of CIN but also due to intero bserver and intrao bserver variability especially during grading of CIN which affect the prognosis of the patient. For the prognostication and grading of tumours ,an application of IHC marker p16 and proliferative marker like Ki67 become imperative.

Aim: Role of p16ink4a and ki-67 immunostaining as specific biomarker of cervical intraepithelial lesions among cervical biopsy samples.

Method: Paraffin block of cases that fulfilled the inclusion criteria will be selected. Issued blocks will be cut serially at 3 to 5-micron thickness using rotatory microtome to prepare slides. Slides will be stained with routine hematoxylin and eosin stain and then mounted with DPX to review, after confirming and noting the diagnosis and microscopy details, sections will be taken for P16INK4a and Ki-67 staining.

Results: According to P16 IHC 72.86% of cases shows positive expression followed by 7.14% shows equivocal expression and remaining 20% cases shows negative expression. .According to Ki-67 IHC 78.57% cases shows positive expression and 21.43% cases shows negative expression . P16 IHC is 100% sensitive, 85.71% specific and 95.59% accurate in predicting positive results among cervical lesions. Ki-67 IHC is 96.49% sensitive, 92.31% specific and 95.71% accurate in predicting positive results among cervical lesions. P16 IHC and Ki-67 IHC shows almost perfect agreement in diagnosis malignant lesion among cervical biopsies with kappa value of 0.951.

Conclusion: Ki-67 and p16/INK4a can be used as complimentary tests for differentiating dysplastic and nondysplastic lesions. They also help in confirming the diagnosis in these cases as different lesions have specific treatment protocols based on the degree of dysplasia. The importance of p16/INK4a in cervix is that it is specific for HR-HPV associated dysplasia and is seen in high-grade lesions and few low-grade lesions with high tendency to progress to a higher grade.

Keywords- P16, Ki67, IHC, cervix, HR-HPV.

Introduction

Preinvasive disease typically lasts for a long time before an aggressive cervical cancer develops. The evolution from cellular atypia to different stages of dysplasia or cervical intraepithelial neoplasia (CIN) before advancement to cervical carcinoma is described microscopically as a spectrum of events.[1] The fourth most frequent malignancy in women worldwide is cervical cancer. The World Health Organisation forecasts 342 000 deaths and 604 000 new cases in 2020. In low- and middle-income nations, new cases and fatalities accounted for around 90% of global mortality in 2020.[2] Cervical cancer made up 18.3% (123,907) of new cases and 9.4% of all cancer cases in India in 2020.[3]

Most CIN cases don't require treatment since the immune system of the host either keeps them steady or eradicates them. A solid foundation for visual examination, colposcopic diagnosis, and comprehension of the therapeutic principles for these lesions is provided by having a thorough understanding of the aetiology, pathophysiology, and natural history of cervical intraepithelial lesions. Today, significant efforts are being done to find novel biomarkers with the goal of enhancing cervical cancer screening at the earliest stage feasible.

A categorization system based on IHC or biomarkers may increase the grading of CIN's accuracy and consistency, leading to the standardisation of diagnosis. The cyclin-dependent protein kinase inhibitor p16INK4a is a negative cell cycle regulator that often acts as a tumour suppressor or cell cycle brake.[4]

As a well-known indicator of cell proliferation, Ki-67 can be used to grade Cervical intraepithelial lesions (CIN)/SIL, in which the upper two-thirds of the epithelium are stained, as opposed to normal squamous epithelium, which often only exhibits modest staining in the parabasal cell layer. Immature squamous metaplasia, reactive/reparative processes, and atrophy are the other benign lesions that are most frequently confused for HSIL. These can be distinguished from HGSIL by using p16INK4a and Ki-67 immunostains, which are typically negative in them.[5]

Therefore, independent of the findings of the morphological examination, the simultaneous detection of tumour suppressor gene p16 overexpression and expression of the proliferation marker Ki-67 within the same cervical epithelial cell should indicate deregulation of the cell cycle and reveal real lesions. Previous research demonstrated that p16/Ki67 dual staining can successfully identify precancerous lesions and cervical carcinoma.[6]

Aim: to study p16ink4a and ki-67 immuno staining as specific biomarker for cervical intraepithelial lesions.

Methods: 50 cervical biopsies of patients for cervical lesions was examined in department of Pathology, Dr S.N medical college jodhpur, from January 2020 to January 2022. Staining was used was H&E stain and slides were studied in the presence of pathology. Block biopsy samples were included in the study, which were histologically identified by H&E as benign cervical lesions, LSIL, HSIL, squamous cell carcinoma of the cervix, and adenocarcinoma of the cervix. Inadequate material and poor fixation were excluded. Paraffin block of cases that fulfilled the inclusion criteria will be selected along with the permission to review the requisition form of these blocks biopsy specimen. Patient name, age, gender, registration number, path number, type of biopsy specimen and its gross feature will be noted.

Issued blocks will be cut serially at 3 to 5-micron thickness using rotatory microtome to prepare slides. Slides will be stained with routine hematoxylin and eosin stain and then mounted with DPX to review, after confirming and noting the diagnosis and microscopy details, sections will be taken for P16INK4a and Ki-67 staining. About 3–4 mm thick sections will be taken from formalin fixed paraffin embedded block from each case. P16INK4a immunohistochemical staining will be performed on 3-aminopropyltriethoxysilane-coated slides. Staining and evaluation using specific Rabbit monoclonal antibody to P16INK4a and Ki67 will be done. Chronic nonspecific cervicitis will serve as negative control and squamous cell carcinoma will serve as positive control. For negative control, primary antibody will be omitted while performing immune histochemical staining.

Measurement of Index

The results for p16/INK4a evaluated according the intensity and percentage of cells positivity. No staining (0,negative),1,2,3 (<1%,1-10%,11-33% ,weak and moderate) ,4 (34-66%,euivocal),5 (>66% ,strong) Ki-67 was scored according to the percentage of positive cells. score 0 (<10%),score 1 (10-30%),score 2 (30-50%) ,score 3 (>50%).

Results

The mean age of cases is 55.67 years with majority of cases are in age group ≥61 years (35.71%) followed by 28.57% in 51-60 years. In our study, 20% cases are benign and 80% are malignant in nature and out of total benign cases (14 cases) majority are in age group 41-50 years (35.71%). Similarly, out of total malignant cases (56 cases) majority are in age group ≥61 years (39.29%).

Table 1: Tumour types

Age Groups	Benign		Malignant	
	No of cases	%	No of cases	%
≤30 years	1	7.14	1	1.79
31-40 years	3	21.43	4	7.14
41-50 years	5	35.71	11	19.64
51-60 years	2	14.29	18	32.14
≥61 years	3	21.43	22	39.29

we found that most common type of diagnosis found in our study is squamous cell carcinoma (67.14%) followed by 15.71% Chronic non-specific cervicitis, 7.14% dysplasia, 4.29% cervical polyp and adenocarcinoma each and 1.43% adeno squamous cell carcinoma.

Table 2: Distribution of cases according to histopathological diagnosis

Histological Type	**No of cases**	**%**
Adeno squamous cell carcinoma	1	1.43
Cervical polyp	3	4.29
Dysplasia	5	7.14
Adenocarcinoma	3	4.29
Chronic non-specific cervicitis	11	15.71
SCC	47	67.14
Total	70	100.00

According to P16 IHC 72.86% of cases shows positive expression followed by 7.14% shows equivocal expression and remaining 20% cases shows negative expression. Here, according to P16 IHC, 45 cases of squamous cell carcinoma cases

followed by 3 cases of adenocarcinoma, 2 cases of dysplasia, and 1 case of adeno squamous cell carcinoma shows positive expression, 2 cases of squamous cell carcinoma and 1 case of dysplasia shows equivocal expression and 3 cases of cervical polyp, 2 cases of dysplasia, 9 cases of chronic non-specific cervicitis shows negative expression.

Table 3: Correlation between histo - pathological diagnosis and P-16 IHC diagnosis

Histological Type	P1-16 IHC											
	Grade 0		Grade 1		Grade 2		Grade 3		Grade 4		Grade 5	
	N	%	N	%	N	%	N	%	N	%	N	%
Adeno Squamous Cell Carcinoma	0	0	0	0	0	0	0	0	0	0	1	1.96
Cervical Polyp	0	0	1	100	2	28.57	0	0	0	0	0	0.00
Dysplasia	0	0	0	0	0	0	2	66.67	1	20	2	3.92
Adenocarcinoma	0	0	0	0	0	0	0	0	0	0	3	5.88
Chronic Non Specific Cervicitis	4	100	0	0	5	71.43	0	0	2	40	0	0
Suamous cell carcinoma	0	0	0	0	0	0	0	0	2	40	45	88.24

According to Ki-67 IHC 78.57% cases shows positive expression and 21.43% cases shows negative expression. Among different type of cervical lesion 47 cases of squamous cell carcinoma cases, 4 cases of dysplasia, 3 cases of adenocarcinoma, 2 cases of chronic non-specific cervicitis, and 1 case of adeno squamous cell carcinoma shows positive expression on Ki-67 IHC. And, 9 cases of chronic non-specific cervicitis, 3 cases of cervical polyp and 1 case of dysplasia shows negative expression on Ki-67 IHC.

Table 4: correlation between histopathological diagnosis and Ki-67 IHC diagnosis

Histological type	Ki-67 IHC							
	Grade 0		Grade 1		Grade 2		Grade 3	
	N	%	N	%	N	%	N	%
Adeno Squamous Cell Carcinoma	0	0	0	0	0	0	1	1.89
Cervical Polyp	1	10	2	40	0	0	0	0.00
Dysplasia	0	0	1	20	2	50	2	3.77
Adenocarcinoma	0	0	0	0	0	0	3	5.66
Chronic Non Specific Cervicitis	8	80	1	20	2	50	0	0.00
Suamous cell carcinoma	0	0	0	0	0	0	47	88.68

We found that P16 IHC is 100% sensitive, 85.71% specific and 96.23% accurate in predicting positive results among cervical lesions. The agreement of P-16 IHC and histopathological diagnosis is significant (P-value<0.00001).

We found that Ki-67 IHC is 96.49% sensitive, 92.31% specific and 95.71% accurate in predicting positive results among cervical lesions. The agreement of Ki-67 HIC and histopathological diagnosis is significant (P-value<0.00001)

Histopathological diagnosis and P16 IHC shows almost perfect agreement in diagnosis malignant lesion among cervical biopsies with kappa value of 0.904. The agreement of P-16 IHC and histopathological diagnosis is significant

(P-value<0.00001). Histopathological diagnosis and Ki-67 IHC shows almost perfect agreement in diagnosis malignant lesion among cervical biopsies with kappa value of 0.862. The agreement of Ki-67 HIC and histopathological diagnosis is significant (P-value<0.00001)

Table 5: correlation between benign and malignant lesion with P-16 IHC

	P16 IHC				
	Positive(>66)	**Equivocal (34-66)**	**Negative (up t0 33)**	**kappa**	**P-value**
Malignant	51	3	2	0.904	<0.00001
Benign	0	2	12		
malignant lesion with Ki-67 IHC					
	Positive		Negative		
Malignant	55		1	0.862	<0.00001
Benign	2		12		

P16 IHC and Ki-67 IHC shows almost perfect agreement in diagnosis malignant lesion among cervical biopsies with kappa value of 0.953. The agreement of Ki-67 HIC and P-16 IHC is significant (P-value<0.00001)

Table 6: Agreement of p-16 and Ki-67 IHC

		P16 IHC (%)		
		Positive (>66)	**Equivocal (34-66)**	**Negative (up to 33)**
Ki-67	Positive (grade-2,3)	51	5	1
	Negative (grade-0,1)	0	0	13
Kappa		0.953		
P-value		<0.0001		

1 case of adeno squamous cell carcinoma shows positive expression on P16-IHC, 3 cases cervical polyp shows negative expression on P16-IHC, 4 cases of dysplasia shows, negative (2case), equivocal (1 case) and positive (2 cases) expression, all adeno carcinoma and squamous cell carcinoma shows positive expression and chronic non-specific cervicitis shows negative (9 cases) and equivocal (2 cases) expression.

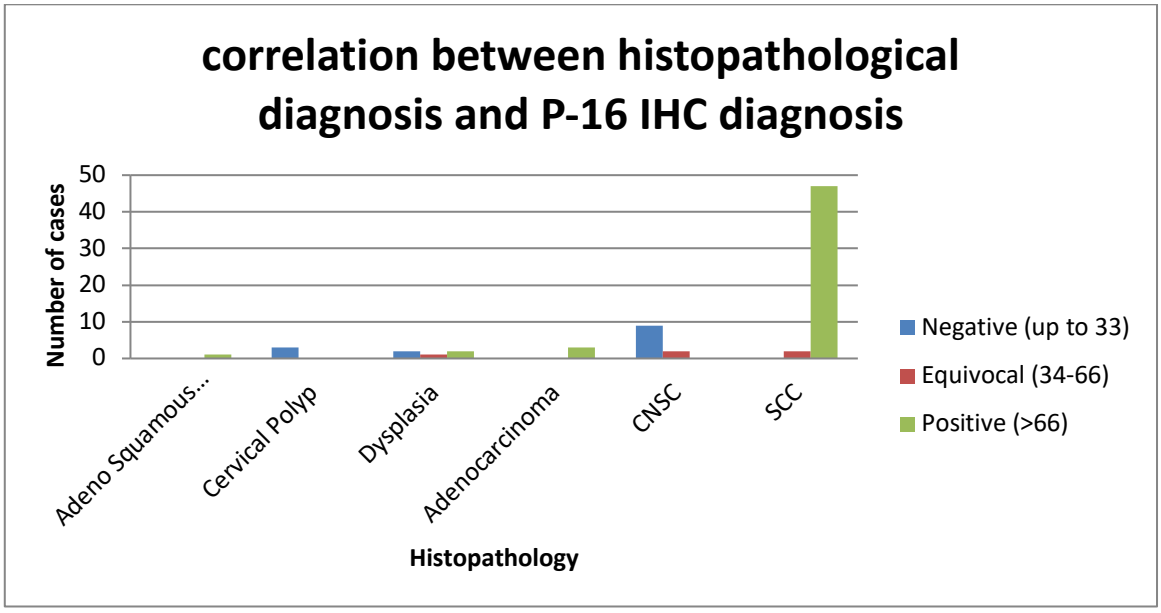

Figure: 1 correlation between histopathological diagnosis and P-16 IHC diagnosis

1 case of adeno squamous cell carcinoma shows positive expression on Ki-67 IHC, 3 cases cervical polyp shows negative expression, 4 cases of dysplasia shows positive and 1 case negative expression, all adeno carcinoma and squamous cell carcinoma shows positive expression and chronic non-specific cervicitis shows negative (9 cases) and positive (2 cases) expression.

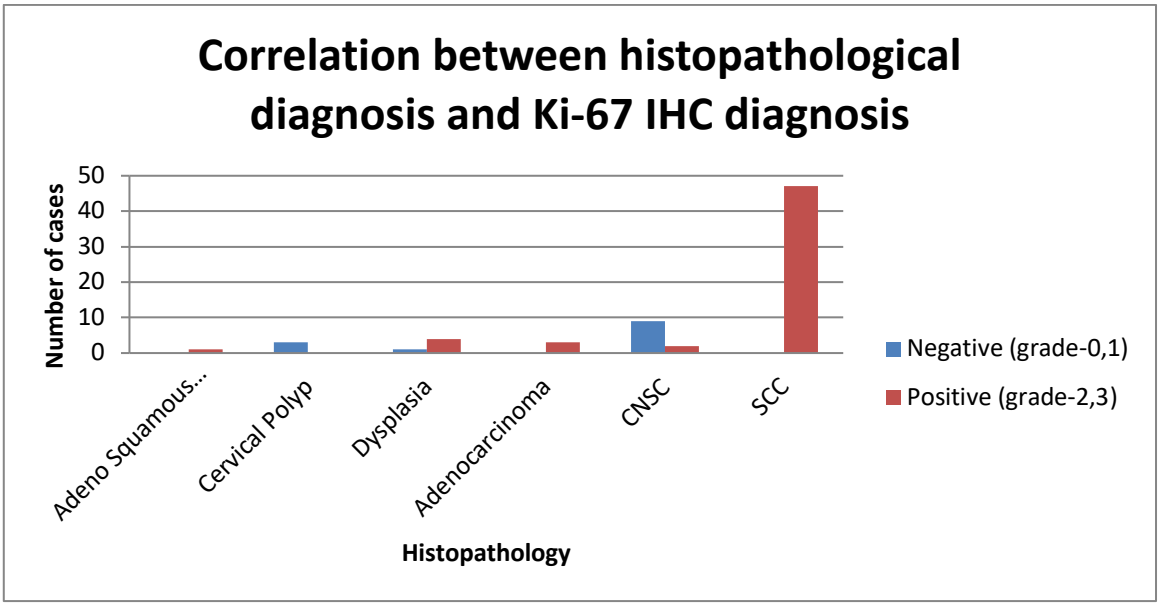

Figure 2: Correlation between histopathological diagnosis and Ki-67 IHC diagnosis

Discussion

In our study peak incidence was found after 4th decade of life with highest percent in age group ≥61 years (35.71%) followed by 28.57% in 51-60 years. The mean age of cases in our study was 55.67 years (27 - 95 years). In comparison to our results Sathiyamurthy et al[7] and Thirukumar and Ahilan[8] found similar age distribution.

In our study we found that out of total cases benign lesions were found in 20% cases and malignancy was found in 80%, similarly reported by Ezejiofor et al[9].

In our study we found that out of total benign cases (14 cases) majority are in age group 41-50 years (35.71%), similarly reported by Ezejiofor et al[9].

Here, we found that most common type of diagnosis found in our study is squamous cell carcinoma (67.14%) followed by 15.71% Chronic non-specific cervicitis, 7.14% dysplasia, 4.29% cervical polyp and adenocarcinoma each and 1.43% adeno squamous cell carcinoma, similarly reported by Juan et al[10].

In our study we found that Adeno Squamous Cell carcinoma and squamous cell carcinoma were having maximum incidence in age above 50 years, Cervical Polyp were more common in 3rd-4th decade, and chronic non-specific cervicitis are present in all age group with more common in 41-50 years, as also reported by Ezejiofor et al[9].

In our study, we found that according to P16 HIC 72.86% of cases shows positive expression followed by 7.14% shows equivocal expression and remaining 20% cases shows negative expression. And, out of them 45 cases of squamous cell carcinoma cases followed by 3 cases of adenocarcinoma, 2 cases of dysplasia, and 1 case of adeno squamous cell carcinoma shows positive expression, 2 cases of squamous cell carcinoma and 1 case of dysplasia shows equivocal expression and 3 cases of cervical polyp, 2 cases of dysplasia, 9 cases of chronic non-specific

cervicitis shows negative expression. Similarly, according to Ki-67 HIC 78.57% cases shows positive expression and 21.43% cases shows negative expression and, out of them 47 cases of squamous cell carcinoma cases, 4 cases of dysplasia, 3 cases of adenocarcinoma, 2 cases of chronic non-specific cervicitis, and 1 case of adeno squamous cell carcinoma shows positive expression on Ki-67 IHC. And, 9 cases of chronic non-specific cervicitis, 3 cases of cervical polyp and 1 case o dysplasia shows negative expression on Ki-67 IHC.

Here, we found that P16 IHC is 100% sensitive, 85.71% specific and 95.59% accurate and Ki-67 IHC is 96.49% sensitive, 92.31% specific and 95.71% accurate in predicting positive results among cervical lesions. The agreement of P-16 HIC, Ki-67 and histopathological diagnosis is significant (P-value<0.00001), similar results were seen by Das et al[11] Sripathi et al[12].

In our study we calculated interrater agreement using cohen's kappa value. We found that histopathological diagnosis shows almost perfect agreement with P16 IHC (Kappa-0.861) and with Ki-67 (Kappa-0.862). The agreement of P-16 HIC and Ki-67 with histopathological diagnosis is significant (P-value<0.00001) we also found that P16 IHC and Ki-67 IHC also shows almost perfect agreement in diagnosis malignant lesion among cervical biopsies with kappa value of 0.951, also reported by Waldstrøm et al[13].

Conclusion

For the purpose of differentiating dyplastic and nondysplastic lesions, Ki67 and P16INK4a may also be applied as complementary tests. In these cases, it helps to confirm the diagnosis because different lesions may have a particular therapy protocol depending on their degree of dysplasia. In cervix, it's important to note that p16INK4a is specific for HRHPV related dysplasia and has been seen in high grade lesions and a small number of low grade lesions which are highly predisposed to progressing to the higher grades.

There have been significant interobserver differences in histologic diagnosis of the biopsy specimens from Cervical Cancer. Therefore, there is a need for additional sensitive and specific biomarkers to improve cervical cancer screening which can improve standardization and quality control of histopathological diagnosis.

Ehical Approval

Yes Ehical approval is given by ehical committee of Dr S.N Medical College jodhpur,Rajasthan,india.

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