Section: Pathology

Original Research Article

A STUDY ON IMMUNOHISTOCHEMICAL EVALUATION OF P16 REACTIVITY IN A SPECTRUM OF EQUIVOCAL AND NEOPLASTIC EPITHELIAL LESIONS OF UTERINE CERVIX

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Abstract

Carcinoma cervix ranks second among the cancers occurring in women of reproductive age group and is also the second leading cancer killer following carcinoma breast in India. Squamous Cell Carcinoma forms the bulk of cervical cancers, constituting 75-80% - according to WHO, is almost always preceded by CIN (Cervical Intraepithelial Neoplasia) lesions and is has a long latency period. Though histopathology is the gold standard in diagnosing of cervical lesions, interobserver variability is documented in few lesions. p16 is a tumour suppressor protein and it inhibits cyclin dependant kinase 4 & 6, which are regulatory proteins in cell cycle. p16 is a useful stand in/surrogate marker for infection with high-risk human papilloma virus (HR-HPV) mediated carcinogenesis in cervical epithelium. This study aims to evaluate the significance of P16INK4a biomarker using immunohistochemistry in cervical biopsies. This is a prospective study carried out in the Department of pathology in our institute. The study was carried out for a period of 24 months, i.e., from July 2022 to July 2024. The study was conducted on 50 patients who had undergone cervical biopsy. A total of 50 cases were included, out of which 22 cases were LSIL, 10 HSIL, 7 SCC, 4 Adenocarcinoma and 7 cases of equivocal lesions were studied. The p16 expression was seen to be increasing from low grade squamous intraepithelial lesion, high grade lesion, Squamous cell carcinoma and adenocarcinoma as 40.09%, 70%,85.71%, and 100% respectively. The p value for p16 IHC score was 0.01, which was statistically significant. This study concludes that p16 can be used as a diagnostic marker for epithelial dysplasia, which is especially useful in equivocal lesions. This marker aids in recognition of dysplasia caused by HR-HPV, which have higher tendency to progress to neoplasia.

INTRODUCTION

Carcinoma cervix ranks second among the cancers occurring in women of reproductive age group and also the second leading cancer killer following carcinoma breast in India.[1] Women of reproductive age group are preferentially affected with median age being 38 years. There is an estimated global incidence of 5,28,000 cervical cancers every year, among which India contributes 1,00,000 cases, i.e., one fifth of the world burden.[2] About 74,000 patients die from this preventable disease in India every year.[3] Virtually all cases of cervical cancer are caused by Human Papilloma Virus.[4,5]

It is a fact that Squamous Cell Carcinoma forms the bulk of cervical cancers, constituting 75-80% - according to WHO,[6] is almost always preceded by CIN (Cervical Intraepithelial Neoplasia) lesions and has a long latency period. The availability of screening procedures, makes it possible to diagnose cervical cancer in its pre-neoplastic stages. This has significantly reduced the burden of cervical carcinoma in population having increased risk for the same. Therefore, cervical cancer has become a

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preventable cause of cancer morbidity and mortality. There are 3 intervention strategies available in cervical cancer screening which includes colposcopy, cervical cytology and cervical biopsy.[7] Colposcopy has a high sensitivity but low specificity.[8] Thus, there is a problem of over diagnosing, resulting in false positive results. To increase the diagnostic accuracy of the screening procedures, the need for the use of biomarkers has arisen.

Though histopathology is the gold standard in diagnosing of cervical lesions, interobserver variability is documented in differentiating reactive atypia from LSIL and to distinguish equivocal lesions like immature squamous metaplasia, inflammatory atypia or atrophic squamous epithelium from high grade squamous intraepithelial lesion, warranting the need for biomarkers.

p16 is a tumour suppressor protein and it inhibits cyclin dependant kinase 4 & 6, which are regulatory proteins in cell cycle. p16 is a useful stand in/surrogate marker for infection with high-risk human papilloma virus (HR-HPV) – mediated carcinogenesis in cervical epithelium.[6,7]

This study aims to evaluate the significance of P16INK4a biomarker using immunohistochemistry in cervical biopsies. The primary focus is on improving the accuracy of cervical cancer screening and diagnosis. We seek to assess their diagnostic precision, ability to stratify patient risks, and potential advantages over traditional screening methods. Additionally, the study aims to understand the practicality of incorporating these biomarkers into routine diagnostic protocols, considering cost-effectiveness and patient outcomes. By addressing these aspects, the research aims to contribute valuable insights that may influence clinical practices and guidelines in the realm of cervical cancer diagnostics.

MATERIALS AND METHODS

This is a prospective study carried out in the Department of pathology in our institute. The study was carried out for a period of 24 months, i.e., from July 2022 to July 2024. The study was conducted on 50 patients who have undergone cervical biopsy. Patients of age 20-70 years were included in the study. The samples from patients of less than 20 years age, females vaccinated for HPV, autolysed samples, inadequate tissue biopsies and biopsies from pregnant females were excluded from this study.

All the patients fulfilling selection criteria were explained about the details of the disease process, options of treatment, ultimate outcome, possible effects, complications and chances of recurrence in this procedure and a written informed consent was obtained before enrolment. They were informed of their right to withdraw from the study at any stage.

A detailed clinical history and physical examination was carried out on patients followed by a thorough review of their hospital records. All the patients meeting inclusion criteria were included in the study. Cervical biopsy specimens were received in the Department of Pathology in 10% formalin. In every case the standard protocol for surgical grossing of resection specimens was followed. Specimen was kept for fixation for 24 hours. After a detailed specimen description, multiple sections were taken as per the standard protocol. After conventional processing, the specimen was embedded in paraffin wax; Sections of 4 to 5 µm thickness were cut using Leica RM 2125 and stained using haematoxylin and eosin for histopathological study. In addition, 4 µm sections were cut from paraffin blocks of tissue and taken on 2 poly L lysine coated glass slides for further immunohistochemical analysis to detect p16 expression. The H and E stained slides were studied and reported as per the standard reporting protocol. The immune-stained slides were examined for p16 expression. These values were recorded and noted down in the master charts. All the data was documented and analyzed by subjecting to statistical analysis.

Immunohistochemical Evaluation

The cells showing brown colored staining with p16 antibody in the nucleus, cytoplasm, or both will be considered positive staining. p16 immunohistochemistry will be scored using the standard scoring criteria which includes percentage of positive cells, Intensity of reaction and cellular reaction pattern. This scoring system states as follows. The cases showing no reaction were considered negative.

Features		**Score**
Percentage of positive cells	< 5	0
	5-49	1
	50-80	2
	>80	3
Intensity of reaction	No reaction	0
	Weak	1
	Variable	2
	Strong	3
Cellular reaction pattern	No reaction	0
	Focal	1
	Diffuse	2
Total score: 0-3= negative, 4-8= positive		

Statistical Analysis

The collected data was entered into Microsoft Excel Worksheet-2010 and data was taken into IBM SPSS Statistic for windows, version 24 (IBM Corp., Armonk, N.Y., USA) software for calculation of frequency, percentage, mean, standard deviation and probability value. P value less than 0.05 was considered statistically significant.

RESULTS

The present prospective study was conducted on 50 subjects who underwent cervical biopsy, to study

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the correlation of histopathological lesions with p16 immunohistochemistry.

Majority of the subjects belonged to the age group of 51-60 years (23/50; 46 %) with a mean age of subjects was 51.45 ± 4.87 years. Majority of the cases belonged to lower middle class (13/50; 26%) and lower class (11/50; 22%) as per modified Kuppuswamy classification.

Majority of the patients presented with Abnormal bleeding (46%) followed by, Discharge per vagina (28%), Bleed on touch (20%), postmenopausal bleeding (6%).

Most of the cervical biopsies were low grade squamous intraepithelial lesions (22/50; 44%) followed by high grade lesion (10/50; 20%), and carcinoma (11/50: 22%) including Squamous invasive carcinoma (7/50; 14%) and Adenocarcinoma (4/50; 8%). The study included 7 (14%) cases of equivocal lesions that had interobserver variability in diagnosis of these lesions. Among them, 2 were histopathologically diagnosed as atrophic squamous epithelium, 3 reactive atypia and 2 immature squamous metaplasia and p16 immunohistochemistry was performed.

The p16 immunohistochemistry was done and was scored as per the percentage of positively stained cells, from score 0 (<5% cells), score 1 (5-49% cells) and score 2 (50-80% cells). Highest percentage (>80% staining) of positive cells was seen from low grade squamous intraepithelial lesion to high grade lesion to Squamous cell carcinoma and adenocarcinoma (4.54%, 70%, 85.71 and 75%, respectively). Among the equivocal lesions one immature squamous metaplasia showed score 2 percentage of positive cells all the others had negative expression. [Table 2]

The p16 immunostaining was scored weak, moderate or strong based on the staining intensity and majority of the high-grade intraepithelial lesion, Squamous cell carcinoma and adenocarcinoma showed strong intensity of staining (60%, 71.42%, 75%, respectively). [Table 2]

Majority of high-grade squamous intraepithelial lesion, Squamous cell carcinoma and adenocarcinoma (70%, 85.71%, and 75%, respectively) showed diffuse pattern of positivity. [Table 2]

p16 IHC score was obtained by adding the scores of percentages of positive cells, staining intensity, and pattern of reaction and was interpreted as positive and negative p16 using a score of 3 as cut-off.

The p16 expression was seen to be increasing from low grade squamous intraepithelial lesion, high grade lesion, Squamous cell carcinoma and adenocarcinoma as 70%,85.71%, and 75% respectively [Table 2]. The p value for p16 IHC score was 0.01, which was statistically significant.

Table 1: showing Distribution of Histopathological categorisation of cervical lesions.

Squamous lesions	Number of cases n (%)
LSIL	22 (44)
HSIL	10(20)
SCC	7 (14)
Adenocarcinoma	4 (8)
Equivocal lesions	7 (14)
Total	50 (100)

Table 2: showing immunohistochemical features of p16 immunostaining

Feature (Score)	LSIL (22) (n) (%)	HSIL (10) (n) (%)	SCC (7) (n) (%)	Adenocarcinoma (4) (n) (%)	Equivocal lesions (7) (n) (%)	P value
Percentage of positive cells						
<5% (0)	12(54.54)	1 (10)	1 (14.28)	0(0)	6 (85.71)	
5-49 % (1)	6 (27.27)	2 (20)	0 (0)	0(0)	0 (0)	<0.001
50-80% (2)	3(13.64)	0 (0)	0 (0)	1 (25)	1 (14.28)	
>80% (3)	1 (4.54)	7 (70)	6 (85.71)	3 (75)	0 (0)	
Intensity of reaction						
No reaction (0)	11(50)	1 (10)	1 (14.28)	0 (0)	-	
Weak (1)	5 (22.72)	2 (20)		0 (0)	1 (14.28)	0.039
Variable (2)	4 (18.18)	1 (10)	1 (14.28)	1 (25)	-	
Strong (3)	2 (09.09)	6 (60)	5 (71.42)	3 (75)	-	
Cellular reaction pattern						
No reaction (0)	13(59.09)	2 (20)	0	0 (0)	-	
Focal (1)	3 (13.63)	1 (10)	1 (14.28)	1 (25)	-	0.06
Diffuse (2)	6 (27.27)	7 (70)	6 (85.71)	3 (75)	1 (14.28)	
p16 IHC score						
0-3 (Negative p16)	13(59.09)	3 (30)	1 (14.28)	-	6 (85.71)	
4-9 (positive p16)	9 (40.09)	7 (70)	6 (85.71)	4 (100)	1 (14.28)	0.01

Table 3: Comparison of p16 expression in LSIL with other studies

Previous studies	P16 immunoreactivity in LSIL cases		
	P16 positive cases	Total cases	Percentage
Nam et al,[23]	2	12	16.6
Zhang et al,[24]	51	157	32

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Balan et al,[25]	20	32	62.5
Son et al,[26]	6	10	60
Present study	9	22	40.09

Table 4: Comparison of p16 expression in HSIL with other studies

Previous studies	P16 immunoreactivity in HSIL cases		
	P16 positive cases	Total cases	Percentage
Wei et al,[27]	26	36	72.7
Zhang et al,[24]	26	36	72.7
Guimaraes et al,[28]	13	18	86.6
Present study	7	10	70

Table 5: Comparison of p16 expression in SCC with other studies

Previous studies	P16 immunoreactivity in SCC cases		
	P16 positive cases	Total cases	Percentage
Tan et al,[29]	70	71	98.5
Lesnikova et al,[30]	131	133	98.4
Present study	6	7	85.71

DISCUSSION

The screening of women by Pap smear has led to a remarkable decline in the mortality from cervical cancer; however, secondary to subjective criteria, interpretation of Pap smears is subject to marked inter- and intra-observer variability as well as having a relatively low sensitivity for cervical neoplasia on a single sample (as low as 66% sensitivity for biopsy-proven high-grade squamous intraepithelial lesions [HSIL]).[9,10] Recently, histology, which is thought of as the gold standard for the diagnosis of cervical neoplasia, has also been found to suffer from marked intra- and interobserver variability, and testing for high-risk human papillomavirus (HPV) by Hybrid Capture 2, which has been shown to be very sensitive in the detection of cervical neoplasia and useful in the triaging of ASCUS smears, has a low specificity for cervical neoplasia.[9,11] Thus, new biomarkers that are more sensitive and specific in the detection of cervical neoplasia and more reproducible than cervical cytology are needed.

Human papillomaviruses (HPV) are known to be a major causative agent in cervical neoplasia and invasive cervical carcinoma. Many different HPV types associated with cervical neoplasia have been discovered, and they have been subdivided into high- and low-risk categories based on their association with invasive cervical carcinoma.[12] This association is based, in part, on the relative affinity that the HPV-type specific oncoproteins E6 and E7 bind to cellular regulatory proteins, specifically, the p53 tumor suppressor protein and the retinoblastoma protein (Rb).[13] Inactivation of these factors, either by degradation (p53) or functional inactivation (Rb), leads to disruption of the cell cycle and increased proliferation, thought to ultimately give rise to carcinoma.

p16INK4a is a cyclin-dependent kinase inhibitor that regulates the activity of cyclin-dependent kinases 4 and 6 and is often inactivated in many cancers by genetic deletion or hypermethylation.[14] In non-HPV–associated tumors, this inactivation leads to increased cyclin-dependent kinase activity and inactivation of Rb. However, in HPV-associated tumors, inactivation of Rb by E7 leads to markedly increased levels of p16INK4a. Recent studies have documented overexpression of p16INK4a not only in cervical intraepithelial neoplasia (CIN) but in cervical cancer as well.[14-18]

The comparison of our study results with previous studies was done. [Table 3-5].

In the present study one immature squamous metaplasia showed p16 positivity. Keating and colleagues found that p16 positivity in lesions with squamous metaplastic atypia correlated significantly with presence of high-risk HPV type.[31]

CONCLUSION

The present study concludes that p16INK4a can be used as ancilliary tests in differentiating dysplastic and nondysplastic lesions and help in confirming the histomorphological diagnosis. This marker aids in recognition of dysplasia caused by HR-HPV, which have higher tendency to progress to neoplasia. This study concludes that p16 can be used as a diagnostic marker for epithelial dysplasia, which is especially useful in equivocal lesions. The potential of p16INK4a as a screening test warrants investigation. However, further research is advocated before the widespread use of this markers for screening of dysplasia.

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