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Original Research Article

A study on expression of p16INK4a by immunohistochemistry in cervical dysplasia and Invasive carcinoma of cervix

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ABSTRACT

Introduction: Cervical cancer is the most common cancer in developing country. Early diagnosis of intraepithelial cervical neoplasia and malignancy is important. Infection with Human Papilloma Virus (HPV) results in precancerous lesions and invasive cervical cancer. Interaction of HPV oncogenic proteins with cellular regulatory proteins leads to upregulation of p16INK4a, a cyclin dependent kinase inhibitor. Therefore, p16INK4a overexpression is a surrogate biomarker of HPV infection making it useful in evaluating HPV associated premalignant and malignant lesions of gynecological tract. In our study we have used p16INK4a IHC staining to find out its correlation with dysplasia grading and cervical carcinoma.
Materials and Methods: This retrospective descriptive case series for five years duration (from Jan 2014 to Dec2018) was conducted in the Department of Pathology, IGMC & RI, Puducherry, India. A total of 43 cervical biopsies and hysterectomies were collected out of which 17 cases were of carcinoma cervix and 26 cases were of cervical intraepithelial neoplasia (CIN). All biopsies were subjected to p16INK4a staining.
Results: The Immuno expression of p16INK4a of CIN I was 13.3%, CIN II was 20%, CIN III was 50% and Carcinoma cervix was 100%. In normal (non dysplasia) cervical epithelium it showed, 100% negative association.
Conclusion: IHC expression of p16INK4a in cervical biopsy is directly related to degree of histological dysplasia and malignancy, so it may be used as a adjunct with routine histopathological examination in identifying cervical dysplasia and also has prognostic value in the management of cervical lesions.

1. Introduction

In developing countries, cervical carcinoma is one of the common cancers. In women, it is the second most common cancer in India.[1] Researchers have identified that infection by high risk type Human Papilloma Virus namely type 16,18,31,33 lead to most of these cervical carcinomas over a period of time.[2] Oncogenic potential of HPV are related to the two viral proteins E6 and E7. These proteins interact with a variety of growth-regulating proteins encoded by proto-oncogenes and tumor suppressor genes. The E7 protein binds to the RB protein and displaces the E2F transcription factors that are normally sequestered by RB, promoting progression through the cell cycle. p16INK4a, is a cyclin dependent kinase inhibitor and is upregulated by interaction of certain HPV proteins and cellular regulatory proteins. The protein p16 is integral to Rb (retinoblastoma) mediated counters of the G1-S phase transition of the cell cycle by inactivating cyclin dependent kinases that phosphorylate Rb protein. Interference of the viral oncoproteins with cellular proteins involved in cell cycle regulation indicates overexpression of p16. Over expression of p16 is an indirect evidence of infection with high risk HPV and may be useful as a biomarker for lesions like cervical intra epithelialneoplasia and carcinoma of the cervix.[3] There are limited number of studies from India correlating the expression pattern of p16 with preneoplastic and neoplastic lesions of the cervix. If significant correlation is found, pathologist can use p16 as a additional evidence of presence of these preneoplastic

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lesions in doubtful cases. Therefore, the present study was conducted to assess the expression of p16INK4a in premalignant and malignant lesions of cervix.

2. Materials and Methods

Study settings and duration: This is a retrospective descriptive study conducted in the Department of Pathology, IGMC & RI, Puducherry, India.

2.1. Type of study

Retrospective descriptive study.

2.2. Inclusion criteria

All cases reported as cervical dysplasia or invasive carcinoma of cervix by histopathology in Department of Pathology during the period from January 2014 to December 2018.

2.3. Exclusion criteria

1. Cases with inadequate available tissue.
2. Cases with recurrence of neoplasm.
3. Cases that are already treated.

Ethical consideration & permission: This study was carried out after obtaining due permission from Institute Ethics Committee (IEC No.3/173/IEC/PP/2019). As it is a retrospective and lab data based in nature, no ethical issues involved.

2.4. Data collection procedure

Patient clinical data like age and history of recurrence was collected from requisition form. For this study, cases which are reported either as CIN or invasive carcinoma of cervix are included. Their H&E slides were retrieved from the archives of pathology department and reviewed. Paraffin blocks were obtained, followed by 3-4micrometer sections were cut and Immunohistochemistry (IHC) for p16 was done.

A total of 43 cases were included in the study fulfilling the inclusion criteria. Out of these cases, 17 cases were carcinoma cervix and 26 cases were CINs.

To know the expression of p 16 in non dysplastic epithelium, 20 randomly selected cases of normal (non dysplastic) cervical epithelium were studied (control group).

Cervical dysplasia was categorized using CIN (Cervical intraepithethelial neoplasia) classification. Cervical carcinoma was classified according to WHO histological classification of invasive carcinomas of uterine cervix.[4,5]

2.5. ImmunoHistoChemistry (IHC) Procedure

The paraffin tissue blocks were cut at 3-micron thickness. The sections were charged on Poly-L – lysine coated slides and incubated at 60–70C for 1 hour. Then the slides were deparaffinized in xylene; thereafter hydrated through descending grades of alcohol. Slides were washed in distilled water two changes, 2 minutes each. Antigen retrieval for 15 – 20 minutes in citrate buffer at pH 6 using pressure cooker. Washed in distilled water two changes two minutes each. Then washed in Tris Buffer Saline for 2 minutes. Endogenous peroxide was blocked by adding H2O2. After that, primary antibody was added and kept for 30 minutes which was followed by addition of Poly excel Target binder reagent for 12 minutes, followed by Polyexcel HRP for 12 minutes. Then DAB Chromogen was added and counterstained with Hematoxylin for 30 seconds blued. Then, the slides were serially dehydrated in alcohol, cleared in xylene, and thereafter mounted using DPX. After drying, slides were examined and P16INK4A status was recorded. All the slides were assessed by two trained pathologists and appropriate grading system was used.

2.6. Interpretation of P16INK4A Staining

P16INK4A Immunostaining was evaluated using a semi-quantitative IHC scoring system.[6] Points were given according to intensity of staining and proportion of cells stained.

Intensity of staining was categorized as no staining, weak staining, moderate staining and strong staining and given scores as 0, 1, 2 and 3 respectively.

According to proportion of cells stained in IHC scores were given as : 0 = no staining; 1 = $<$1%; 2 = 1-10%; 3 = 11-33%; 4 = 34-66%; 5 = $>$66% of cells. Total score was calculated by adding the points for intensity and points for proportion of cells stained with p16INK4a immunostain. By this scoring system, highest score was 8 and lowest score was zero. Cases with scores from zero to five were taken as low p16 expression while scores from 6-8 were taken as over expression.[6,7] Expression pattern (either low or over expression) in various histological types was studied.

Cervical dysplasia was categorized as CIN I, CIN II and CIN III, while cervical cancers were categorized into squamous cell carcinoma (SCC) and adenocarcinomas. In this study no other categories of carcinomas were seen. Expression pattern of P16INK4A in each category were graded according to the scoring system mentioned above.

Data analysis: Data analysis was done with the help of professional statistics package EPI Info 7.0 version. Categorical variables were expressed using frequency and percentages. Quantitative variables were expressed using mean. Data regarding age of the patient was expressed as mean $\pm$ SD and percentages.

3. Results

3.1. Demography

The age of the patients ranged from 3 2-74 years with mean age of 53.6 years. Four (9.3%) patients were aged between 30-40years, 15(34.88%) were between 41-50 years, 14(32.5%) were between 51-60 years and 10(23.2%) were between 61-70 years. Out of total 43 cervical specimens, 33 were cervical biopsies and 10 were hysterectomy specimens.

3.2. Histopathology Features

On H&E staining among the 43 biopsies, 15(34.88%) were reported as CIN I, 5 (11.6%) were reported as CIN II, 6(13.95%) were reported as CIN III and 17 (39.53%) were reported as Carcinoma cervix. Among the carcinoma cervix cases, 16 were squamous cell type histologically where as only one case was adenocarcinoma. Among squamous cell carcinomas,13 were well differentiated, one was moderately differentiated and 2 were poorly differentiated type.

3.3. Immunohistochemistry

Over expression of p16 was observed in 2/15(13.33%) cases of CIN I, 1/5(20%) cases of CIN II and 3/6(50%) cases of CIN III. Irrespective of the differentiation among the cervical carcinoma cases, there was a high immuno expression of p16INK4a in all 17/17(100%) cases Figures 1 and 2. Twenty randomly selected cases of hysterectomies done for leiomyoma were used to study the expression of p16INK4A in normal (non dysplastic) cervical epithelium and it showed 100% negative expression.

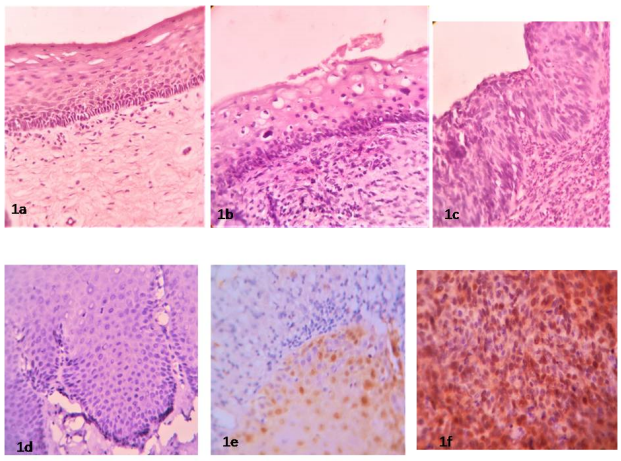

Fig. 1: 1a, 1b, 1c: Histological images of cervix showing CIN I, CIN II and CIN III changes respectively. (H&E, x40). Fig**1d**: control for p16 stain and fig **1e** and **1f** corresponds to low andoverexpression of p16 stain. (IHC, x40)

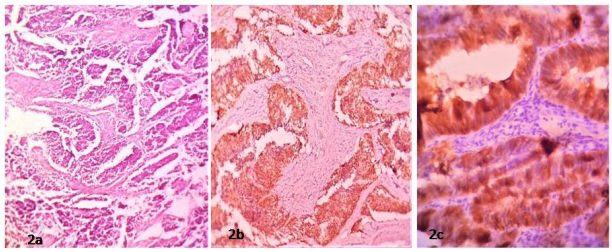

Fig. 2: Figure **2a** and **2b** : H&E picture showing Non-Keratinizing squamous cell carcinoma of cervix (2a-H&E,x10) and overexpression of p16INK4a stain(2b-IHC,x10). **2c**: Overexpression of p16INK4a stain in Adenocarcinoma of cervix (IHC,x40)

As shown in the Table 1, IHC stain over expression increases as the degree of the lesion advances. Non parametric Chi- Square test analysis showed that df was 6, X^2 value of 12.59 and p value 0.00015 which was stastically significant.

4. Discussion

In India, Pap smear is routinely done for cervical cancer screening. However, sensitivity and specificity of pap smear is sub optimal and false-positive and false-negative results are common. Colposcopic biopsy will be done in any suspicious-appearing lesions which subjects the patient to unnecessary surgical intervention. Availability of reliable IHC biomarker in pap smear could save the patient from surgical intervention and repeated testing.[8]

Histopathology is the current gold standard for diagnosis of cervical dysplasia and cervical cancer. However, it may have interobserver variation in making a quality judgement, which might be due to either 1) presence of mild nuclear atypia associated with underlying inflammation 2) reparative process is going on that may show nuclear hyperchromasia as well as cytoplasmic halos, 3) presence of binucleation or 4) post-menopausal squamous atypia. These can be overcome by doing immunohistochemistry.[9]

This study was conducted to understand the expression of p16INK 4A by IHC and to check whether it can be used as a diagnostic adjunct. Twenty cervical bits from hysterectomy specimens done for leiomyoma were used to study the expression of p16INK4A in normal (non dysplastic) cervical epithelium. None of these biopsies showed p16 expression ie 100% showed negative expression as observed in other studies Table 2.

The present study showed that the expression of P16INK4a in cervical biopsy is directly correlated with higher grade lesion, the result of which is comparable with data observed by Kanthiya et al.,2016.[10] Keating et al in their study reported that p16INK4a positivity is directly related to the presence of high-risk HPV types.[14]

Table 1: Pattern of p16 expression in different epithelial lesions

Content	No.of cases (n=43)	Overexpression of p16 (> 6) (%)	Low Experession of P16(<6) (%)	df	X^2	P
CIN I	15	2(13.33%)	13(86.67%)			
CIN II	5	1(20%)	4(80%)	6	12.59	0.00015
CIN III	6	3(50%)	3(50%)			
Carcinoma Cervix	17	17(100%)	0(0%)			

Table 2: Overall pattern of p16 expression in various studies

Study	Normal Epithelium	CIN I	CIN II	CIN III	Carcinoma Cervix
Kishore et al.,[2]	00	25%	50%	75%	100%
Lesnikova lana et al.,[6]	00	72.3%	91%	98.3%	98.5%
Srivastava et al.,[8]	00	80%	100%	100%	100%
Kanthiya et al.[10]	9.4%	10.4%	78.7%	78.7%	91.3%
Kory et al,[11]	00	0%	4.76%	50%	100%
Tsoumpou et al.,[12]	00	38%	68%	82%	100%
Gupta et al.,[13]	10%	50%	60%	70%	95%
Present study	00	13.33%	20%	50%	100%

Out of 17 cases of carcinoma cervix, 16 were squamous cell carcinoma and one was adenocarcinoma. All the cases showed high positive expression for p16INK4A which is similar to the studies by LesnikovaIana et al., (98.5%), Srivastava S (100%) and Kishore et al., (100%).[2,6,8]

A study done by Tsoumpou et al., (2009) reported that over expression of p16INK4a increased with the degree of cytological or histological abnormality, and showed immunoreactivity in 38% of CIN I, 68% of CIN II and 82% of CIN III cases, whereas the present study reported p16INK4a immunoreactivity in 13.3% of CIN I, 20% of CIN II, 50% positive stain in CIN III and (17/17)) 100% for invasive cervical cancer.[12]

Kory et al., studied 40 cases of cervical biopsy and found that p16 expression is directly related to higher grade lesions. The normal or non-neoplastic cervical tissue did not express p16 immunostain whose findings are similar to the present study.[11]

Keating et al observed positive scores for Ki-67, cyclin E, and p16 which were seen in 68.4%, 96.7%, and 100% of LSILs and 94.7%, 91.6%, and 100% of HSILs, respectively and concluded that cyclin E and p16 are complementary surrogate biomarkers. They are most sensitive for LSIL (Low Grade squamous Intraepithelial lesion) and HSIL (High Grade squamous Intraepithelial lesion).[14] In a study by Srivastava et al who correlated the grades of p16 and MIB 1 recommend that for LSIL, because the sensitivity of the p16 marker in their study is 80%, it must be evaluated together with MIB-1 or HPV test. For HSIL, the specificity and sensitivity of the p16 marker is 100% and hence it can be used as a stand-alone test.[8]

5. Conclusion

The result of the present study shows strong association of intraepithelial cervical neoplasia of different grades with staining pattern of p16INK4a. The staining intensity also increases with the increasing grades of the lesion which is strongly significant. Studying the expression of p16INK4a in biopsy would support the histopathological features of CIN in uncertain cases and may also predict the clinical behavior of the lesion and their possibility of progression to higher grade lesion.

Study contribution to existing knowledge: In our study no positive staining of p16 was observed in non-neoplastic cervical epithelium and also the immuno expression increases as the degree of dysplasia advances. Study findings suggest that p16 stain allow precise identification of even small CIN in biopsy sections and PAP smear and can also reduce interobserver variations.

5.1. Limitations

The limitation of the study is that only one biomarker is used. Combination of another markers like HPV and Ki67 would give a better understanding of proliferating potential of these lesions. The cases could not be followed up due to retrospective nature of the study.

6. Authors contributions

First author: Collected the data, Done HPE and IHC slide analysis, Data statistical analysis and article writing part.

Second author: Conception of study, getting necessary permissions, IHC slide analysis, Contributed to Data analysis, approved the submitted version and managing communications.

7. Source of funding

None

8. Conflicts of interest

None

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