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Original Research Article

Immunohistochemical expression of p16INK4a in premalignant lesions and malignant tumours of cervix

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ABSTRACT

Background: Cervical carcinoma is the third most common cancer in women worldwide and firth most common cause of cancer related deaths. Human Papilloma virus, the mostcommon cause of cervical cancer, causes functional inactivation of pRb, resulting in overexpression of p16INK4a. The overexpression of p16INK4a correlates well with the degree of cervical dysplasia and neoplasia. The present study is done to determine the IHC expression of p16INK4a so that it may be used as a biomarker for HPV and may play a role in the prevention, diagnosis and prognosis of cervical neoplasm.
Methods: The present study was conducted on 60 formalin-fixed, paraffin-embedded specimens of cervical neoplasms, which comprised 47 cases of squamous cell carcinoma followed by 8 cases of adenocarcinoma, 3 cases of HSIL and 2 cases of LSIL. These were then subjected to IHC by p16INK4a. Cytoplasmic and nuclear positivity for p16INK4a was noted.
Results: Overall 95% cases were positive for p16INK4a expression, 100% cases of adenocarcinoma, 98% cases of squamous cell carcinoma, 100% cases of HSIL and 0% cases of LSIL were positive for p16 expression.
Conclusions: The present study showed statistical correlation of immunoreactivity of p16INK4a with histological type was clinically significant (p value <0.0001). In our study, p16INK4a immunohistochemical expression increased with increasing degree of dysplasia and with presence of cervical carcinoma.

Keywords: p16INK4a, Cervical carcinoma, IHC, CIN, HSIL, LSIL

INTRODUCTION

Cervical cancer is the third most common cancer in women worldwide witha global prevalence of 11.7% and accounts as the fifth most common cause of cancer related deaths. Its annual estimated global incidence is 5,00,000 cases. With India accounting for approximately 1,00,000 cases.[1-3] Cervical cancer is the second most common cancer in underdeveloped countries.[1] The age range for cervical cancer is reported to be 27-80 years with mean age of 54.2 years; the maximum cases have been noted between 41-60 years of age.[1]

Almost all premalignant and malignant lesions (99%) are linked to infection with high-risk human papillomavirus (HPV), an extremely common virus transmitted through sexual contact.

Although most infections with HPV resolve spontaneously and cause no symptoms, persistent infection can cause cervical cancer in women.[4] Symptoms of cervical cancer include- Irregular spotting between periods in women of reproductive age, postmenopausal spotting, post coital bleeding,increased vaginal discharge, persistent back, leg or pelvic pain, weight loss, fatigue, loss of appetite, swelling of a leg or both lower extremities.[4]

Precursor lesions are classified as low grade squamous intraepithelial lesion (LSIL) and high grade squamous intraepithelial lesion (HSIL). LSIL does not progress directly to invasive carcinoma and in fact, most cases regress spontaneously; only a small percentage progress to HSIL. By contrast to LSIL, HSIL is considered to be at high risk for progression to carcinoma. In HSIL,there is progressive deregulation of the cell cycle by human papilloma virus (HPV), which results in increased cellular proliferation, decreased or arrested epithelial maturation, and lower rate of viral replication as compared to LSIL. Derangement of the cell cycle in HSIL may become irreversible and lead to a fully transformed malignant phenotype.[4]

Squamous cell carcinoma is the most common, present in approximately 80% of cases. The second most common tumor type is Adenocarcinoma, whichis present in about 15% of cervical cancer cases followed by Neuroendocrine carcinoma accounting for 5% of cases. All of the aforementioned tumor types are caused by high-risk HPVs.[4] Many lines of evidence have demonstrated a central role of specific types of HPVs in the pathogenesis of both dysplastic and neoplastic lesions of the cervix. High risk HPV types in particular HPV types 16 and 18 have been identified in more than 99% of cervical cancers.[5] Two viral oncogenes, E6 and E7 are consistently expressed in HPV associated cancer cells.[6-8]

The E6 oncoprotein, initiates premature degradation of the p53 tumour suppressor protein. Similarly, the viral E7 oncoprotein binds to the tumor suppressor protein pRb.[5] pRb is a tumor suppressor, which inhibits the progression of cells into S phase. pRb is regulated via phosphorylation by cyclinD1 complexed with cyclin dependent kinases for its optimal function.[9] When the HPV E7 protein specifically binds to and inactivates pRb, this mediates the release of E2F, a transcription factor to which pRb is bound. In turn, genes required for entry of cell into S phase of the cell cycle are activated.[5,9] The accumulation of E2F has also been found to be associated with an increase in INK4A gene transcription.[5] The INK4A gene product, the p16INK4a protein,is a tumor suppressor protein that inhibits cdk4 and cdk6 which phosphorylate the Rb protein. A reciprocal relation between p16 and pRb expression has been seen. p16 overexpression is demonstrated in cervical cancers as a result of functional inactivation of pRb by HPV E7 protein. This overexpression highlightsthe possible potential of p16 as a marker for cervical intraepithelial lesions and cervical cancer.[9]

p16INK4a (p16) is a 16kDa protein encoded by CDKN2A, within the INK4/ARF tumor suppressor locus on chromosome 9 (9p21.3). Its major function in cells isto inhibit cyclin-dependent kinases (CDK4 and CDK6) that are required tophosphorylate the retinoblastoma protein, pRb. This way it inhibits transversal of the G1/S checkpoint, resulting in blockade of the cell cycle. It is thereforemarker cellular senescence. p16 overexpression occurring in HPV-drivencancers is a result of its increased production due to transcriptional release from negative feedback control.[10] p16 is a good surrogate test for the presence ofa potentially transforming HPV infection in anogenital premalignant andcarcinoma.[11]

p16INK4a expression can be studied by immunohistochemistry which has both nuclear and cytoplasmic expression.[10]

Present study is an attempt to study the pattern of expression of p16INK4a in various premalignant and malignant cervical lesions.

METHODS

The present study was done over a period of one year from November 2021 to November 2022 on 60 cases of cervical neoplasms received in the department of pathology, Guru Gobind Singh Medical College, Faridkot.

The tissues were fixed and processed, and routine hematoxylin and eosin staining was done to know the histological type and grade of the tumor. This was followed by immunohistochemical staining with p16INK4a using a primary antibody. The methodology for p16INK4a IHC was as follows:

Sections of 3-5 µm were cut and mounted on poly-L-lysine coated slides. Slides were dried overnight at 37 degrees Celsius and dewaxed in xylene and hydrated.

Antigen retrieval was done by taking 1500 ml of citrate buffer solution, pH 6.0 was heated, till it boiled in a stainless-steel pressure cooker. The lid was covered but not locked. Slides were positioned into metal staining racks and lowered into the pressure cooker ensuring slides were 45 completely immersed in the unmasking solution. The lid was locked. When the pressure cooker reached operating temperature and pressure [after about 5 minutes] a timer was started for 1 minute. When the timer rang, the pressure cooker was removed from the heat source and run under cold water with the lid on. The lid was not removed until the indicators show that pressure had been released. Later lid was opened, slides were removed and placed immediately into a bath of tap water. Neutralization of endogenous peroxidase was done using peroxidase block for 5 minutes. Two washings in phosphate buffer saline/ tris buffer saline each for 5 minutes were given. Incubation with protein block for 5 minutes was done. Two washes in tris buffer for 5 minutes each were given. The primary antibody was put on the sections and sections were kept for 1 hour in the moist chamber. This was followed by 2 washes in tris buffer for 5 minutes each. The post-primary block was then applied for 30 minutes at room temperature. Again 2 washings of tris buffer were given for 5 minutes each. Incubation was done with polymer for 30 minutes. Again 2 washings were given with phosphate buffer for 5 minutes each with gentle rocking. Slides were then covered with DAB for 2-3 minutes. All the time slides

were kept in a moist chamber. Sections were washed in deionized water for 5 minutes. Haematoxylin counterstaining was done for 2-5 minutes and sections were washed under running tap water. Dehydration and clearing of the sections were done in propanol and xylene, respectively. Mounting was done by the mounting media DPX. Sections were viewed under the microscope.

Interpretation of p16 staining

All sections deemed adequate for evaluation were interpreted as per criteria defined by lower anogenital squamous terminology (LAST)- cases with diffuse, nuclear and/or cytoplasmic p16INK4a IHC staining were considered positive for p16INK4a IHC, whereas cases who showed either a focal p16INK4a staining or no staining were considered negative for p16INK4a IHC. [12]

Criteria for p16-staining pattern and interpretation and p16 IHC status was as shown in Table 1.

Table 1: Criteria for p16-staining pattern and interpretation and p16 IHC status.

P16 staining pattern	Description	p16 IHC status
Diffuse	Continuous staining of cells of the basal and parabasal cell layers of the cervical squamous epithelium, with or without staining of cells of intermediate, or intermediate and superficial layers	Positive
Focal	Focal staining pattern, defined as staining of isolated cells or shall clusters of cells	Negative
No staining	No immunoreactivity	Negative

Data analysis plan

The data related to clinical details was entered in the form of data matrix in Microsoft excel and analysis was done by using statistical package for the social sciences (SPSS) 23.0 ver. The descriptive statistics for categorical variables were represented in form of frequencies and percentages. The association between categorical variables was assessed by Pearson Chi-square test. A p value of <0.05 was considered as significant.

Ethical consideration

The study was carried out after seeking permission from institutional ethics committee of Guru Gobind Singh Medical College and Hospital, Faridkot. Written informed consents were taken from all the participating patients.

RESULTS

The present study was conducted on 60 histopathologically proven cases of premalignant lesions and malignant tumors of cervix in the department of pathology, Guru Gobind Singh Medical College, Faridkot.

The demographical variables, history of intercourse, contraceptives and histological types and differentiation were noted for all patients as described late (Table 2).

Table 2: Demographic observations with histological grade and types.

Variable	Frequencies (%)
Age (mean±SD in years) (years)	56.2±12.88
<40	20 (12/60)
41-60	38.3 (23/60)
>60	41.7 (25/60)
History of smoking	
Present	6.7 (04/60)
Absent	93.3 (56/60)
Presenting complaints	
Abnormal vaginal discharge	65 (39/60)
Abnormal per vaginal bleed	83.3 (50/60)
Post coital bleeding	10 (06/60)
Cervical growth	65 (39/60)
History of intercourse before 18 years	
Absent	78.3 (47/60)
Present	21.6 (13/60)
History of hormonal contraceptives	
Absent	85 (51/60)
Present	15 (9/60)
Histological types	
CIN I (LSIL)	3.3 (2/60)
CIN II (HSIL)	-
CIN III (HSIL)	5 (3/60)
Squamous cell carcinoma (SCC)	78.3 (47/60)
Adenocarcinoma	13.3 (8/60)
Tumor differentiation of SCC	
Well differentiated	6.4 (3/60)
Moderately differentiated	74.4 (35/60)
Poorly differentiated	19.2 (9/60)

p16INK4a staining was diffuse in 95% cases, focal staining in 1.7% cases and no staining in 3.3% cases as per LAST criteria. In this study, p16INK4a IHC was positive in 57 (95%) cases and negative in 3 (5%) of cases as per LAST criteria (Table 3).

Hence, our final observations were that both cases of LSIL (CIN I) were negative for p16INK4a expression. All the 3 cases (100%) of HSIL (CIN III) were positive for p16INK4a expression. The current study exhibited that 97.9% cases of squamous cell carcinoma were positive for

p16INK4a immunoreactivity. p16INK4a expression was positive in 100% cases of well differentiated squamous cell carcinoma, 97.1% cases of moderately differentiated squamous cell carcinoma and 100% cases of poorly differentiated squamous cell carcinoma. This study exhibited that p16INK4a immunohistochemistry was positive in 100% (8/8 cases) of adenocarcinoma (Table 4 and Figure 1).

Table 3: p16 staining patterns.

p16 staining pattern	Number of cases	Percentage
No staining	2	3.3
Focal staining	1	1.7
Diffuse staining	57	95

Table 4: Distribution of histological types and p16 expression.

Histological type	p16 negative		p16 positive		Total
	No. of cases	%	No. of cases	%	
CIN (premalignant lesions)					
CIN I (LSIL)	2	100	0	0	2
CIN II (HSIL)	0		0		0
CIN III (HSIL)	0	0	3	100	3
Carcinomas (malignant lesions)					
Squamous cell carcinoma	1	2.1	46	97.9	47
Adenocarcinoma	0	0	8	100	8
Total	3 (5)		57 (95)		60

P value <0.0001

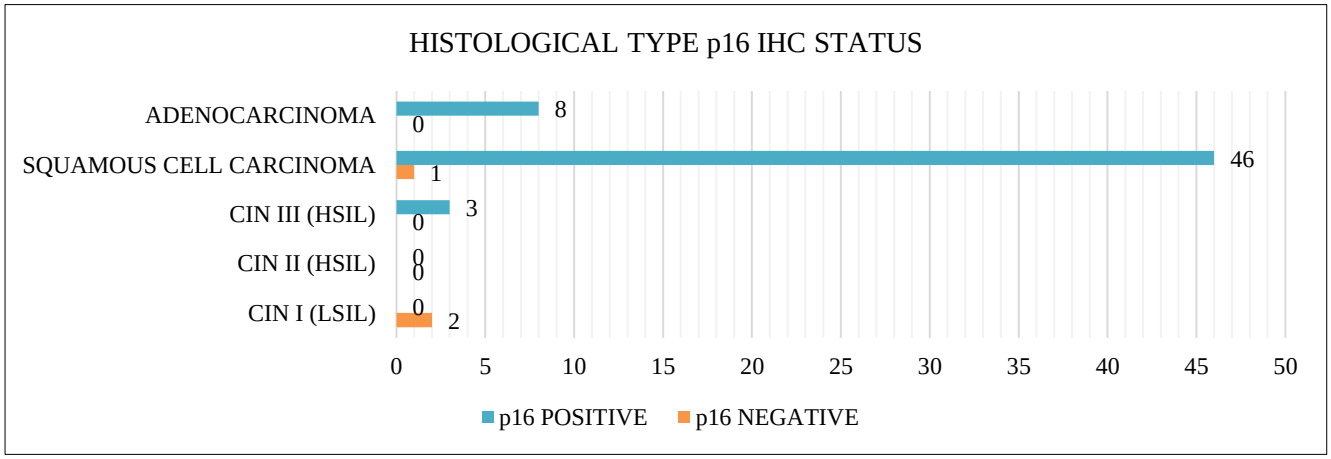

Figure 1: p16 expression in histological types.

The present study concluded that the statistical correlation of immunoreactivity of p16INK4a with histological type was clinically significant (p value <0.0001). In the current study, no statistically significant correlation was seen between p16INK4a expression and degree of tumor differentiation of squamous cell carcinoma (p value=0.868).

In the present study, 83.3% [10/12 cases] in age group of less than 40, exhibited block type positivity for p16INK4a while 95.6% [22/23 cases] in age group of 41-60 years were positive for p16INK4a and 100% [25/25 cases] were positive for p16INK4a in age group above 60 years. On correlation of age with status of p16INK4a expression, a statistically non-significant p value of 0.092 was observed.

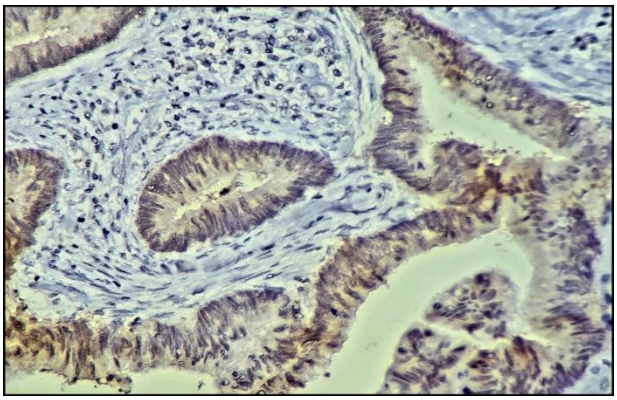

Figure 2: p16 in cervical adenocarcinoma.

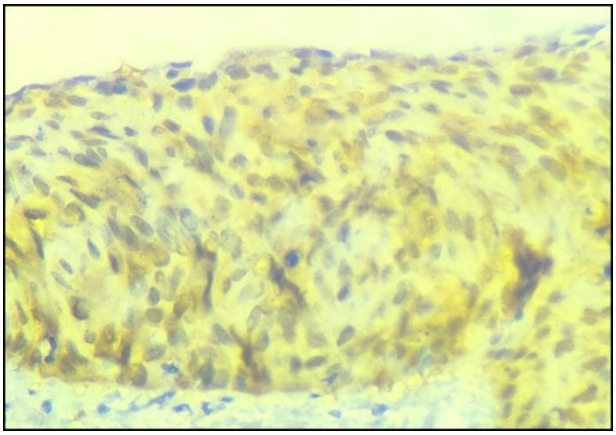

Figure 3: p16 in CIN III (HSIL).

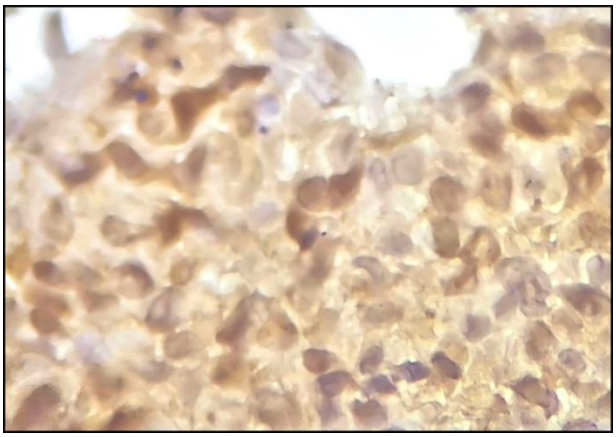

Figure 4: p16 in poorly differentiated squamous cell carcinoma cervix.

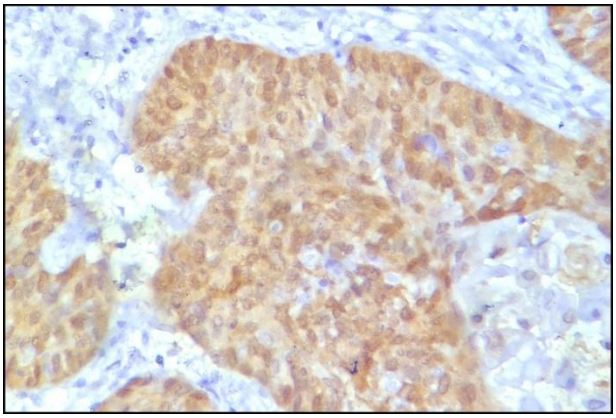

Figure 5: p16 in well differentiated squamous cell carcinoma cervix.

DISCUSSION

p16INK4a (p16) is a 16kDa protein encoded by CDKN2A, within the INK4/ARF tumor suppressor locus on Chromosome 9 (9p21.3). Its major function in cells is to inhibit cyclin-dependent kinases (CDK4 and CDK6) that are required to phosphorylate the retinoblastoma protein, pRb. This way it inhibits transversal of the G1/S checkpoint, resulting in blockade of the cell cycle. It is therefore a marker of cellular senescence. p16INK4a overexpression occurring in HPV-driven cancers is a result of its increased production due to transcriptional release from negative feedback control. p16INK4a expression can be studied by immunohistochemistry which has both nuclear and cytoplasmic expression.[12]

Correlation of p16 expression with histological types

Cervical intraepithelial neoplasia I [LSIL]

Current study reported only two cases of LSIL [CIN I] which were negative for p16INK4a expression. The findings of our results were concordant with studies conducted by Cheah et al in 2016 and Zabin et al in 2021.[13,14] Our observations differ from the results documented in the studies done by Izaadi-Mood et al in 2012, Kumari et al in 2013, Pandey et al in 2018 and Stoler et al in 2018.[12,15-17]

Cervical intraepithelial neoplasia II and III [HSIL]

The observations of present study reported that the all 100% [3/3cases] of HSIL [CIN III] were positive for p16INK4a immunohistochemistry. The results of our study were similar to studies done by Murphy et al in 2003, Lesnikova et al in 2009, Izaadi-Mood et al in 2012, Cheah et al in 2016, Pandey et al in 2018 and Stoler et al in 2018.[7,9,12,15,17] Our results differ from the findings documented in the studies done in 2010 by Gupta et al, Kumari et al in 2013 and Zabin et al. 2021.[16,18,19]

Squamous cell carcinoma

The current study recorded 97.9% [46/47 cases] of squamous cell carcinoma and all cases exhibited block type positivity for p16INK4a. Our study results were comparable to the studies conducted by Klaes et al in 2001, Murphy et al in 2013, Lesnikova et al in 2009, Shrivastava et al in 2010, Gupta et al in 2010, Kumari et al in 2013, Stoler et al in 2018, Kalyani et al in 2020 and Zabin et al in 2021.[5,7,9,12,14,16,18-20]

Adenocarcinoma

This study concluded that the p16INK4a expression was positive in 100% [8/8 cases] of adenocarcinoma. The findings were consistent with studies conducted by Murphy et al in 2013 and Pandey et al in 2018, who also reported p16INK4a expression in all the cases of adenocarcinoma.[7,17] Studies conducted in 2001 and 2012 by Klaes et al and Izaadi-Mood et al showed p16INK4a expression in 85% and 75% cases respectively, which was lower than our results.[5,15]

In our study, p16INK4a immunohistochemical expression increased with increasing degree of dysplasia and with presence of cervical carcinoma. However, no correlation was found between p16 expression and degree of

differentiation of squamous cell carcinoma. The cases of CIN I [LSIL] were negative which may due to infection by low-risk human papilloma virus. p16INK4a IHC can be used to identify the positive cells having the ability to recognize those lesions with an increased risk of progression to high grade lesions. Thus, proposing that p16INK4a is a promising biomarker for the diagnosis of cervical intraepithelial neoplasia and invasive cervical carcinoma. It can be used as a marker for progression of lesion from low grade to high grade i.e. CIN I to CIN III/carcinoma.

Limitations

As the sample size of our study was small, these observations cannot be attributed to the general population. No CIN2 case reported during the period of our study. Transformation of premalignant lesion to frank malignancy could not be commented upon due to short period of our study.

CONCLUSION

The present study was conducted on 60 histopathologically proven cases of premalignant lesions and malignant tumors of cervix. Majority of cases i.e. 95% were positive for immunohistochemical expression of p16INK4a. The present study showed statistical correlation of immunoreactivity of p16INK4a with histological type was clinically significant (p value <0.0001). In our study, p16INK4a immunohistochemical expression increased with increasing degree of dysplasia and with presence of cervical carcinoma. Concluding that p16INK4a is a surrogate marker of HPV E7 mediated pRb catabolism. This protein is not expressed in the normal cervical epithelium, but is overexpressed in dysplastic and malignant cervical epithelium. However, no correlation was found between expression of p16INK4a and degree of differentiation of squamous cell carcinoma. Also, its overexpression allows to specifically identify preneoplastic and neoplastic lesions and reduces interobserver disagreement of conventional histological tests. So, the immunohistochemical expression of p16INK4a should be incorporated into routine gynaecological pathology practice.

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Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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