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ORIGINAL ARTICLE

Evaluation Of P16 INK4a Status As A Biomarker For Uterine Cervical Neoplasms

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ABSTRACT

Uterine cervical cancer is the most common cancer cause of death in the developing countries. Human papilloma virus (HPV) infection is the most important risk factor for uterine cervical neoplasms. HPV oncogene expression can be detected by cellular protein p16 (INK4a) using immunostaining and it may assist in improving the reproducibility of diagnoses in cervical dysplastic and reactive lesions. This study aims to observe the frequency and distribution of p16INK4a protein expression and relating it to different histological grades of uterine cervical neoplasms. Of the 608 cases of uterine cervical neoplasms diagnosed during the study period, 60 cases were selected randomly for p16INK4a expression by immuno his to chemistry. It was observed that CIN1 constituted77% of the total intraepithelial neoplasms and squamous cell carcinoma is the most frequent type of malignancy accounting for 96% invasive tumors. P16ink4a immunoreactivity was positive in 100% of all cervical neoplasms included in the study ($p<0.0001$) and its intensity of staining, distribution increased with increasing grades of cervical neoplasms. This pattern of over expression demonstrates the potential use of p16 as a diagnostic marker for cervical squamous and glandular neoplastic lesions.

KEY WORDS : Uterine cervical neoplasms, Human papilloma virus and p16.

Introduction

Uterine cervical cancer is the second most common cancer among women across the globe and is the most frequent cancer among females of reproductive age group in India [1].

Cervical cancer preventive measures, early diagnosis and remedial therapies influences on mortality rates. Cervical cancer screening programmes with Pap smear, colposcopic biopsy has significantly reduced the incidence and mortality rate of cervical cancer. However, these tests have limitations due to subjective test criteria. This interobserver variability in diagnosis highlights the need to identify biomarkers for dysplastic epithelial cells in primary screening and diagnosis [2-3]. A potential biomarker should distinguish between cervical intraepithelial neoplasms (CIN) and other non-neoplastic cervical lesions.

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Human papilloma virus (HPV) being a prime cause for cervical carcinogenesis, its detection can be done using p16INK4a, a surrogate marker of HPV by immunohistochemical staining method. p16 is expressed only in dysplastic cervical epithelial cells, never in normal epithelium and is associated with high-risk HPV[2,4]. Recently, College of American pathologists included p16 immunohistochemistry in their revised nomenclature for lower genital tract lesions [4]. In order to evaluate the usefulness of potential biomarker in the diagnosis of cervical neoplasms, we have used p16 INK4a as a diagnostic marker to show the varied intensity and expression in increasing grades of uterine cervical neoplasms.

Materials and Methods

In this prospective study, uterine cervical neoplasms diagnosed from January 2013 to December 2013 in the Department of Pathology, Thanjavur Medical College Hospital, Thanjavur were included. Females above the age of 20 years were subjected to screening by visual inspection method (VIA/VILI) and punch biopsy was taken from abnormal areas visualized in colposcopy.

During this study period a total of 3198 cervical biopsies were received, among which 608 cases diagnosed as uterine cervical neoplasms were included in the study. All the specimens obtained were fixed in 10% buffered formalin and were submitted in Toto for routine tissue processing. Paraffin embedded tissues were sectioned and stained with Haematoxylin and eosin.

Immunohistochemistry

For Immunohistochemistry, paraffin embedded tissue sections were floated on chromalum coated slides. The antigen retrieval was done by heat using Tris buffer. The sections were stained using a standard peroxidase - antiperoxidase technique. The slides were incubated with primary monoclonal antibody - p16 (a mouse monoclonal anti-p16 antibody, Fremont, CA, Biogenex, USA) for one and half hour. The chromogen used was DiaminoBenzidine (DAB), after incubation with secondary polymer antibody for 30 minutes, counterstaining was done with haematoxylin.

Evaluation Of Immunohistochemical Marker-p16 INK4a

The immunostaining was considered positive when the nucleus and/or cytoplasm took chest nut brown colour in atleast 1% of tumor cells. Various studies have used different methods for scoring p16 immunostaining, but in this study the following four parameters were considered to increase the specificity of the results.

1. Percentage of proportion of positive tumour cells[5-9] were graded as 0, 1+, 2+, 3+ when 0%, 1-5%, 5-25%, >25% of tumour cell shows positivity respectively.
2. Intensity of staining [4-5,7,10] was graded between 0-3. (negative - 0, weak –1+, moderate – 2+ and Strong – 3+
3. Cellular reaction patterns[5,7-8,10] showing only cytoplasmic positivity, Nucleocytoplasmic positivity and nuclear positivity.
4. Patterns of p16INK4a staining expression within epithelium[5,8,11]as diffuse full thickness, Diffuse basal and Patchy.

Statistical Methods

Descriptive statistics were derived using mean and percentages. Fisher exact test and Chi square test were used to assess the association between their trends in their subgroups. Value

< 0.05 was considered as significant p value. All statistical analysis was done using SPSS version -18 (Statistical Package for Social Sciences) Inc., Chicago, USA.

Results

Uterine cervical neoplasms constituted 19% (608 cases) of the total cervix biopsies received during the study period. Uterine cervical carcinoma accounted for 10.38% (332 cases) and cervical intraepithelial neoplasia for 8.6% (276 cases). The tumors were typed according to the WHO classification system. Cervical intraepithelial neoplasia (CIN) were graded as CIN 1,2 and 3.

all tables

Immunohistochemical Analysis

p16 INK4a immunostaining was done in randomly selected 69 cases(Table.No.1).

Based on the parameters mentioned above, 69 cases were studied and the results of p16 immunoexpression are shown in the following Table no.[1,2] and in Fig.No. 1 a-h & 2.

From the above results it is evident that p16 immunostaining positivity found to be nuclear and/or cytoplasmic; chronic non-specific cervicitis cases was predominantly negative for p16 (7/9) immunostaining, where as all cases of intraepithelial and invasive neoplasms were positive for p16, with a progressive increase in staining intensity and proportion of positive cells for p16 expression with increasing grades of CIN and also from CIN to carcinoma. P16 was found to be positive in cervical glandular intraepithelial neoplasia (CGIN) and in adenocarcinoma variants.

p16 scoring was calculated as a product of % of positive tumor cells and staining intensity. The values obtained were 1,2,3,4,6 and 9. Cut off value of 4 was taken. 100% of chronic non specific cervicitis and70% of CIN1 had a p16 score <4; where as 70% of CIN2,70% of CIN 3 cases and all carcinoma cases had a p16 score >4.

Statistical Analysis

p Value was calculated to find the significant correlation of p16 over expression within the sub groups of cervical lesions by statistical analysis. Test used were Fisher exact test and Extended Mantel-Haenszel chi square test for linear Trend pValue 0.0001.

From the tabulated data, p value was calculated. The value obtained was 0.0001. Since the p value is smaller (<0.05), it is evident that rejecting the null hypothesis can be possible. This infers that p16INK4a expression can be directly correlated with the increasing grades of cervical intraepithelial neoplasia and carcinoma.

Discussion

In this study we report the strong association between p16 INK 4a expression and cervical neoplasia. Immunohistological expression of p16 was seen only in dysplastic / neoplastic cells and was never observed in normal epithelium. Thus p16 expression appears to be a specific and sensitive biomarker for cervical neoplasia as reported in many studies[1,12]

p16 ink 4a (inhibitor of kinase 4) is a tumor suppressor protein and inhibits CDK4 &6. In the hypophosphorylated form, retinoblastoma protein binds to transcription factors responsible for cell cycle progression. p16 inhibits CDK and prevents phosphorylation of Rb protein keeping it in active form. In HPV infection, viral gene E7 binds to Rb protein and causes functional inactivation resulting in accumulation of p16 protein because, normally, Rb protein inhibits the transcription of p16[2-3].

The following Table No.3 compares the present study with the recent studies done on p16 expression.

All precancerous lesions and carcinomas have shown positivity for p16 expression in

our study similar to the reports of Supriya et al[2] and Murphy et al[13]. As p16 positivity directly correlates with the HPV induced morphological changes and its expression is expected to be negative in normal epithelium and in inflammatory lesions. But, in our study we found weak p16 positivity in 2 /9 cases of non specific cervicitis. Similarly, Kumari et al[5], Klaes et al[6] and Gupta et al[7] have also reported p16 positivity in inflammatory lesions. Such cases were reviewed and classified into CIN 1. It infers the difficulty in distinguishing reactive atypia from CIN with usual haematoxylin and eosin stained slides. Hence, p16 immunostaining can serve as diagnostic tool in sorting out neoplastic lesions without subjective variation.

Murphy et al.[13] found p16 expression to be negative in all normal cervical tissue and 100% positivity in all cervical carcinomas. He reported nuclear staining of p16 in some CIN1cases. We encountered a case of Squamous cell carcinoma exhibiting nuclear staining. Murphy et al. found precancerous lesions showing predominantly cytoplasmic staining and all cancerous lesions, both squamous cell carcinoma and adenocarcinoma exhibiting strong nuclear and cytoplasmic staining for p16. p16 is basically a nuclear protein and hence immunohistochemical expression should show nuclear staining. However, in dysplasia both nuclear and cytoplasmic staining is observed possibly because of post-transcriptional modification or over production of p16 protein forcing its transfer into cytoplasm [2].

Supriya et al, reported that all normal cervical epithelium, metaplastic, reactive and inflammatory conditions were negative for p16 immunostaining, whereas all precancerous and cancerous lesions showed 100 % positivity. The present study showed results similar to Murphy et al and Supriya et al. [2].

We encountered a case of cervical glandular intraepithelial neoplasia(CGIN) which had strong p16 expression similar to the reports of Murphy et al and Klaes etal. p16 helps in distinction of carcinoma in situ from benign mimics like tubo-endometrial metaplasia, endometriosis and microglandular hyperplasia as it is positive only in dysplastic cells [8]. Gupta etal [7] reported a progressive increase in expression of p16 immunostaining from CIN to carcinoma with strong nuclear or Nucleo cytoplasmic positivity as noted in our study. The staining pattern within the epithelium in CIN cases correlated well with the reports of Kumari et al. Observed statistical analysis in this study showed p value <0.05 which was considered very significant and signifies p16 is ideal marker for cervical dysplasia.

Conclusion

This study highlights the increasing expression of p16INK4a in higher grades of CIN and cervical carcinoma concurrent with many studies. This finding emphasizes the role played by this marker in early carcinogenesis and progressive accumulation of nuclear protein as the tumour progresses. The association of this marker seen in high grade CIN and cervical carcinoma suggests their association with infection by high risk HPV types. The present study signifies the usefulness of an ideal immunomarker p16 INK4a as a diagnostic tool and emphasizes the importance of incorporating the HPV cotesting with p16 INK4a in the primary screening programme which will have a beneficiary effect on both the patient and the health care system.

Table. No. 1 : Status of p16 expression in cervical neoplasms.

Category	No. of Cases (n)	Proportion of positive cells: n (%)				Intensity of staining: n (%)			
		0% Grade0	1–5% Grade1	5–25% Grade2	>25% Grade 3	0 negative	1+ weak	2+ moderate	3+ strong
CNSC	9	7(78%)	1(11%)	1(11%)	0	7(78%)	2(22%)	0	0
CIN1	10	0	6(60%)	4(40%)	0	0	4(40%)	5(50%)	1(10%)
CIN2	10	0	2(20%)	8(80%)	0	0	3(30%)	4(40%)	3(30%)
CIN3	10	0	1(10%)	5(50%)	4(40%)	0	2(20%)	3(30%)	5(50%)
CGIN	1	--	--	--	1(100%)	--	--	--	1 (100%)
SCC	22	0	0	1(4%)	21(96%)	0	0	6(27%)	16 (73%)
AC	6	0	0	0	6(100%)	0	0	0	6 (100%)
ASC	1	--	--	--	1(100%)	--	--	--	1 (100%)

CNSC-chronic non specific cervicitis; CIN-cervical intraepithelial neoplasia; CGIN-cervical glandular intraepithelial neoplasia; SCC- squamous cell carcinoma, AC- adenocarcinoma, ASC- adenosquamous carcinoma.

Table. N. 2: P16 Staining Pattern In Different Groups

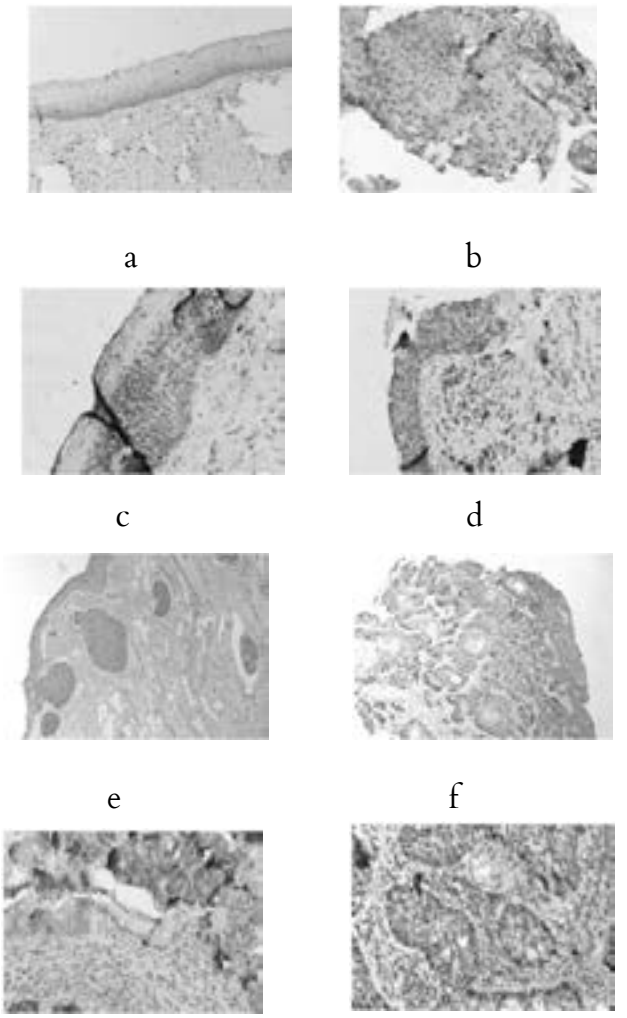
TYPES	CNSC	CIN				CA		
		CIN 1	CIN 2	CIN 3	CGIN	SCC	AC	ASC
Negative -Ve)	7	-	-	-	-	-	-	-
Positive (+Ve)	2	10	10	10	1	22	6	1
Cyto Plasmic Positivity	1	3	1	2	-	4	1	-
Nucleo:cyto Plasmic Positivity	1	7	9	8	1	17	5	1
NUCLEAR POSITIVITY	-	-	-	-	-	1	-	-

Table No. 3: Studies Published On P16ink4a Expression

Studies (year)	Normal %(n)	CIN1 %(n)	CIN2 %(n)	CIN3 %(n)	CGIN %(n)	SCC %(n)	AC %(n)
Klaes at al [6] (2001)	27 (13 /48)	85 (40 /47)	100 (32 /32)	100 (60 /60)	ND	98 (52 /53)	85 (6/7)
Murphy et a[13] (2003)	0 (0 /21)	100 (38 /38)	100 (33 /33)	98 (45 /46)	100 (5/5)	100 (8/8)	100 (2/2)
Gupta et a[7] (2009)	10 (2 /20)	50 (10 /20)	60 (12 /20)	70 (14 /20)	ND	95 (19 /20)	ND
Lesniko et al[4] (2009)	ND	72 (180 /249)	91 (212 /233)	98 (178 /181)	ND	98.5 (131 /133)	ND
Supriya et al[2] (2010)	0 (0 /15)	100 (15 /15)	100 (15 /15)	100 (3/3)	ND	100 (15 /15)	ND

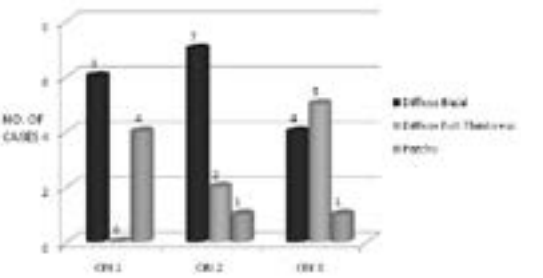
Kumari et al[5] (2012)	25 (4 /16)	63 (10 /16)	75 (12 /16)	81 (13 /16)	ND	100 (16 /16)	ND
Present study	22 (2 /9)	100 (10 /10)	100 (10 /10)	100 (10 /10)	100 (1/1)	100 (23 /23)	100 (6/6)

n- Number of cases; ND – not done



p16 immunohistochemical expression in cervix biopsies: Figure.No. 1: a- CIN 1 exhibiting diffuse basal positivity, score 1+(40X);b-CIN 1 focal weak patchy positivity-score 1+, (100X); c-CIN 2 diffuse basal positivity in lower 2/3 of epithelium, score 2+, (100X); d-CIN3 showing strong nucleo-cytoplasmic positivity in full thickness of the epithelium, score 3+, (100X); e-microinvasive carcinoma with strong positivity, (40X); f-keratinising SCC strong positivity (40X); g -CGIN- strong positivity in dysplastic glands(100X); h-strong positivity in endocervical adenocarcinoma(100X).

Fig.No.2: Pattern Of P16 Expression In Different Grades Of CIN



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