Study of $p^{16ink4a}$ expression in cervical intraepithelial lesions and cervical carcinoma

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ABSTRACT

Background: Cervical cancer ranks as the second cause of female cancer in India. In India about 122,844 new cervical cancer cases are diagnosed annually. Many lines of evidence have demonstrated that HPV contributes to neoplastic progression through the action of two viral oncoproteins, namely E6 and E7, which interact with various cell cycle regulatory proteins. The $p^{16Ink4a}$ is a tumour suppressor protein and it's over expression highlights the possible potential of a marker for cervical intraepithelial lesions and cervical cancer.

Objectives: The present study was done to establish the ability of $P^{16INK4A}$ as a marker of dysplastic and neoplastic alteration in the cervical epithelium.

Materials & Methods: Surgical biopsy specimens of uterine cervix received at the Department of Pathology S.V. Medical College Tirupati from November 2014 to October 2016 were analyzed in a Prospective interventional study. 163 cases diagnosed as LSIL, HSIL, SCC, Adenocarcinoma on H&E stained sections were immunohistochemically stained for $p^{16Ink4a}$ using the BiogenexLifesystems Histology Kit. All sections that showed either strong nuclear and/or cytoplasmic staining were considered positive and graded qualitatively.

Also 20 random normal cervical biopsies were immunohistochemically stained for p16Ink4a and studied to confirm the negative association.

Results: Low Grade Squamous Intraepithelial (LSIL) is more associated with IHC grade 1, whereas High Grade Squamous Intraepithelial (HSIL) and Squamous cell carcinoma (SCC) were more associated with IHC grade 3.

Conclusion: The present study concludes that $p^{16Ink4a}$ is a specific marker for dysplastic and neoplastic alteration in the cervical epithelium and thereby significantly improves cervical precancer and cancer detection.

Keywords: Cervix Cancer, E7-Oncoprotein, HPV, IHC-$P^{16Ink4a}$.

INTRODUCTION

Cervical cancer ranks as the second cause of female cancer in India. In India about 122,844 new cervical cancer cases are diagnosed annually and 67477 deaths of this diagnosis were recorded according to 2014 statistics.[1]

Cytological screening for cervical cancer, using Pap smear testing is affected by a substantial rate of false negative test results.

As for cytology, histopathology is also affected by a substantial rate of inter-observer discrepancies and clearly decisive biomarkers would improve standardization and quality control of the histopathological diagnosis.[2]

Materials and Methods

Surgical cervical specimens, either hysterectomy or diagnostic biopsy of uterine cervix submitted for histopathological examination at the Department of Pathology, S.V. Medical College , Tirupati from November 2014 to October 2016 were analyzed in a prospective interventional study.

Then the representative tissue from hysterectomy specimens and entire tissue from cervical biopsy specimens were subjected to routine processing for paraffin embedding. 4-5 micron thick sections were cut and stained with hematoxylin and eosin (H&E) stain.

H&E sections were reviewed and those showing features of dysplasia and/or neoplasia were classified according to the criteria of the World Health Organization (WHO) into Low Grade Squamous Intraepithelial lesion (LSIL), High Grade Squamous Intraepithelial Lesion (HSIL), Invasive Squamous Cell Carcinoma (SCC) and Adenocarcinoma.

Collectively, 163 cases diagnosed as LSIL, HSIL, SCC, Adenocarcinoma were immunohistochemically stained for $P^{16Ink4a}$.

All sections that showed either strong nuclear and/or cytoplasmic staining were considered positive and graded

qualitatively according to the following arbitrary scale: 0 (no positive staining), 1 (<10% positive staining), 2 (>10% but <50% positive staining) and 3 (>50% positive staining).

The collected data was entered in excel sheets and analyzed using the following statistical methods:

1. Descriptive statistics

2. Chi-Square Test.

3. Cross tabs (contingency coefficient test)

The p value of < 0.05 was considered statistically significant.

All the statistical calculations were done using SPSS software for Windows version 16.0.

Only cases diagnosed as LSIL, HSIL or Invasive Carcinoma (SCC or Adenocarcinoma) of uterine cervix on histopathological examination were taken up for P16Ink4aimmunohistochemical study.

Sections that were

(a) Negative for LSIL, HSIL and Cervical cancer

(b) Showed extensive tissue necrosis and/or contained inadequate viable tissue on histopathological examination were excluded from the immunohistochemistry study.

RESULTS

The 163 specimens received comprised of 127 (77.9%) cervical biopsies and 36(22.1%) specimens.

163 cases on histopathological examination showed that 48(29.4%) cases were LSIL, 47(28%) cases were HSIL and 68(41.7%) were carcinoma of cervix. [Table1]

Table 1: Frequency of cervical biopsy and hysterectomy specimens in SIL and Carcinoma cervix cases

CATEGORY	BIOPSY	HYSTEREC-TOMY	Total
LSIL	25(15.3%)	23(14.1%)	48(29.4%)
HSIL	35(21.5%)	12(0.7%)	47(28.8%)
Carcinoma	67(41.1%)	1(0.006%)	68(41.7%)
Total	127(77.9%)	36(22%)	163(100%)

Out of 68 cases diagnosed as carcinoma cervix 64 were of squamous cell carcinoma, 3 were of adenocarcinoma and one case of adenosquamous carcinoma.

In the present study, a total of 64 cases of squamous cell carcinoma were evaluated, among which 49(76.5%) were of keratinizing type, 12(18.8%) were of non-keratinizing type and 3(4.7%) were of papillary type.

Out of 3 cases of adenocarcinoma studied in the present study one case was of usual type and the other two were one each of minimal deviation and clear cell type

In the present study comprising of 163 cases, all cases showed p16 immunopositivity except 9 cases of LSIL which showed negative staining for P16.

Among the 48 cases of LSIL, 23 (47.9%) cases showed grade 1 staining and 16(33.3%) cases showed grade 2 staining [Table2].

Table 2: Comparison of H&E with IHC grading

H&E			IHC GRADE				TOTAL
			0	1	2	3	
H&E	LSIL	NUMBER OF CASES	9	23	16	-	48
		% WITHIN H&E	19%	48%	33%	-	100%
	HSIL	NUMBER OF CASES	-	1	20	26	47
		% WITHIN H&E	-	2%	43%	55%	100%
	CARCINOMA	NUMBER OF CASES	-	-	-	68	68
		% WITHIN H&E	-	-	--	100%	100%
TOTAL		NUMBER OF CASES	9	24	36	94	163
		% WITHIN H&E	6%	15%	22%	58%	100%

47 cases of HSIL were studied in which 1(2.1%) case showed grade 1 staining, 20 (42.5%) cases showed grade 2 staining and 26 (55.3%) cases showed grade 3 staining. [Table 2].

68 cases of carcinoma cervix were studied and all 68(100%) cases showed grade 3 staining. [Table2].

Overall, grade 0 staining was observed in 9(5.5%) cases, grade 1 staining in 24(14.7%) cases, grade 2 staining in 36(22%) cases and grade 3 staining in 94(57.6%) cases.

Analysis of values in the table 2 showed that an association is observed between H&E and IHC grading where contingency co-efficient of .685 is found to be highly significant (p=. 000). This implies that LSIL is significantly associated with IHC grade 1, whereas HSIL and carcinoma cervix were significantly associated with IHC grade 3.

20 random normal cervical biopsy/ hysterectomy specimens were also studied for H&E and IHC to confirm the negative association established in previous studies that

P16Ink4a does not stain normal cervical epithelium. Similar statistical methods as employed for SIL and Carcinoma cervix cases were applied.

Among 20 random normal cervical biopsy/ hysterectomy specimens received, 3 (15%) were cervical biopsies and 17 (85%) were hysterectomy specimens.

DISCUSSION

The present study was undertaken to study the over expression of $P^{16Ink4a}$ in cervical intraepithelial neoplasia and cervical cancer. This was done to establish whether $p^{16Ink4a}$ over expression could be used as a marker of dysplastic/neoplastic alteration in the cervical epithelium. In the present study, CIN I was considered as LSIL. CIN II and CIN III combined were considered as HSIL. This is consistent with and in view of the latest revision of CIN I, II and III into LSIL and HSIL by WHO.

In the present study, all 20 (100%) cases of normal cervix showed $P^{16Ink4a}$

grade 0 immunostaining. Results of the present study are consistent with those of Klaes et al (2001) Murphy et al (2003), Redman et al (2008), Srivastava(2010) and Han et al (2011). All of these studies showed 0% $P^{16Ink4a}$ immunopositivity in normal cervix.

In the present study 39/48 (81.1%) LSIL cases were $P^{16Ink4a}$ immunopositive. These results are consistent with that of Mood et al (2012) whose study yielded 9/11(81.8%) rate of immunopositivity. Klaes et al (2001) observed 40/40 (100%) and study by Murphy et al (2003) yielded 38/38 (100%)rates of immunopositivity. On the contrary, study by Hu et al (2005) showed 40/45(88%), Zhang (2006) et al showed 81/157 (51%), Nam et al (2008) showed 2/12(16.6%), Ordi et al (2010) showed 56/86 (65.8%), Balan (2011) et al showed 81/157(51%), and Son et al (2013) showed 56/86(65.8%) rates of immunopositivity.[Table3]

Table 3: Comparative study of $P^{16Ink4a}$ Immunopositivity in LSIL

S. NO.	AUTHOR	$P^{16INK4A}$ IMMUNOPOSITIVITY LSIL CASES	
		P16 POSITIVE	PERCENT(+VE)
1.	Klaes (2001)	40/40	100
2.	Murphy (2003)	38/38	100
3.	Hu (2005)	40/45	88
4.	Zhang (2006)	51/157	32
5.	Nam (2008)	2/12	16.6
6.	Ordi (2010)	56/85	65.8
7.	Balan (2011)	20/32	62.5
8.	Mood (2012)	9/11	81.8
9.	Son (2012)	6/10	60
10	Tan (2010)	15/60	25
11	Swetha (2016)	1/1	100
12	Zouheir (2016)	11/22	50
13.	PRESENT STUDY(2016)	39/48	81.1

Study of Hu et al showed 40/45 (88%) rate of immunopositivity. He explained that HPV infection among $P^{16Ink4a}$ negative LSIL cases was transient and that viral DNA was not integrated into the host cells. Hence $P^{16Ink4a}$ immunopositivity was absent in these cases.[3]

In the study done by Nam et al, 2/12 (16.6%) cases were P^{16Ink4}aimmunopositive. He states that a possible reason for some significant lower rates of $P^{16Ink4a}$ immunopositivity in low-grade lesions may be because a certain percentage is thought to be caused by low-risk HPV types. Because the affinity of the E7 protein of low-risk HPV for Rb is much lower than that of high-risk HPV types, there would not be over expression of $P16^{16Ink4a}$.[4]

In the present study, 9 of the LSIL cases were $p^{16Ink4a}$ negative (18.75%). The negativity of $p^{16Ink4a}$ in CIN 1 may be due to latent or sub-clinical HPV infection with low viral load that may be insufficient for $P^{16\ INK4A}$ expression.

Ishikawa *et al*[5] found that over expression of $P^{16Ink4a}$ in CIN 1 was more common in cases with HPV 16 and HPV 52 infection. The other possible reason for lower expression of $p^{16Ink4a}$ in low grade lesions may be because a certain percentage is thought to be caused by low risk HPV types.

Present study showed 47/47 (100%) immunopositivity in HSIL cases. Results of the present study is consistent with that of Klaes et al (2001) who observed 92/92(100%) and Bolana et al (2003) whose study yielded 35/35 (100%) rates of immunopositivity. On the contrary, Murphy et al (2003) showed 78/79 (98.7%), Agoff et al (2003) showed 52/56 (92.8%), Guimaraes et al (2005) showed 13/18 (86.6%), Zhang et al (2006) showed 101/135 (74.8%), Cheah (2012) et al showed 24/27(88.9%), Mood et al (2012) showed 10/11 (90.9%) and Wei et al (2013) showed 26/36 (72.7%) rates of immunopositivity.[Table4]

Table 4: Comparative study of P16Ink4a Immunopositivity in HSIL

S. NO.	AUTHOR	$P^{16INK4A}$ IMMUNOPOSITIVITY HSIL CASES	
		P16 POSITIVE	PERCENT + VE
1.	Klaes (2001)	92/92	100
2.	Murphy (2003)	78/79	98.7
3.	Agoff (2003)	52/56	92.8
4.	Guimaraes (2005)	13/18	86.6
5.	Zhang (2006)	101/135	74.8
6.	Bolana (2007)	35/35	100
7.	Cheah et al (2012)	24/27	88.9
8.	Mood et al (2012)	10/11	90.9
9.	Wei et al (2013)	26/36	72.7
10.	Present study (2016)	47/47	100

In the study by Guimaraes et al 13/18 (86.6%) cases were $P^{16Ink4a}$ immunopositive. He explained that many studies have also reported a significant association between $P^{16Ink4a}$ overexpression and HSIL related to high-risk HPV types. It is possible that not all HPV types classified as high-risk possess the same potential for the cell cycle disruption or altered gene expression that leads to $P^{16Ink4a}$ up regulation. Thus, these results highlight the possible potential of $P^{16Ink4a}$ as a marker for type-specific HPV related HSIL and cervical cancer progression.[6,7,8,9]

In the present study 64/64 (100%) cases were $P^{16Ink4a}$ immunopositive.These results are consistent with that of Srivastava (2010) who observed15/15 (100%),Kumari et al (2013) whose study yielded 16/16 (100%) and Wei et al who showed 25/25 (100%) rates of immunopositivity. On the contrary, Klaes et al(2001) showed 52/53 (98.1%), Volgareva et al (2004)showed 20/21 (95.2%),Lesnikova et al (2009) showed 131/133 (98.4%), Tan et al (2010) showed 70/71 (98.5%), Cheah et al (2012)showed 46/53 (86.8%) and Mood et al (2012)showed 18/20 (90%) rates of immunopositivity. [Table5]

Table 5: Comparative study of $P^{16Ink4a}$ Immunopositivity in SCC

S. NO.	AUTHOR	$P^{16INK4A}$ IMMUNOPOSITIVITY LSIL CASES	
		P16 POSITIVE	PERCENT(+VE)
1.	Klaes (2001)	52/53	98.1
2.	Volgareva (2004)	20/21	95.2
3.	Lesnikova (2009)	131/133	98.4
4.	Srivatsava (2010)	15/15	100
5.	Tan et al (2010)	70/71	98.5
6	Gupta et al (2010)	19/20	95
7.	Cheah (2012)	46/53	86.8
8.	Mood (2012)	18/20	90
9.	Kumari (2013)	16/16	100
10.	Wei et al (2013)	25/25	100
11.	Present study (2016)	64/64	100

Volgareva et al stated that $p^{16Ink4a}$ negative squamous cell carcinomas had been detected. Substantial variability of the data may be due to small numbers of samples analyzed, to utilization of different types of monoclonal antibodies and to different criteria used by different research groups for the results interpretation.[7]

Tan et al observed that nearly all (98.6%) SCC cases showed $p^{16Ink4a}$ over expression. However, a few patients with cervical cancer had $p^{16Ink4a}$ negativity. Tan et al study shows that nearly all (98.6%) SCC lesions show $p^{16Ink4a}$ over expression, this further emphasizes the important causal relationship between HPV and cervical cancer. However, a few patients with cervical cancer had $P^{16Ink4a}$ negativity.[8]

Nieh *et al* showed that a proportion of their cervical cancer cases had neither HPV infection nor $P^{16Ink4a}$ expression. The possible explanation for the absence of $P^{16Ink4a}$ expression in these high grade lesions could be methylation of the $P^{16Ink4a}$ promoter resulting in silencing of the $P^{16Ink4a}$ gene.[9]

Gupta et al stated that the immunoreactivity for p16 in squamous cell carcinoma is unequivocally shown by all studies till date and has been confirmed by this study as well. However, they had only one case which was negative for p16 protein, which is in concert with a study by Volgareva et al. [30] who showed that $p^{16Ink4a}$ negative carcinomas did exist. The probable explanation could be p16 silencing through epigenetic mechanisms such as promoter methylation or through genetic mechanisms such as deletion or loss of heterozygosity.[10]

Tripathy et al (2003) from India, in a study on invasive cervical cancer, had shown p16 promoter hypermethylation and homozygous deletion in 6.5% and 8.7% samples respectively.[11]

The findings of the present study were similar to those of Branca *et al*[12] and Ozgul *et al*[13] which also found that $p^{16\ INK4A}$ expression was directly related to the increasing grade of CIN.

Present study showed 3/3(100%) immunopositivity in adenocarcinoma cases. The results of the present study are consistent with that of Swetha et al (2016) who observed 1/1 (100%) rate of immunopositivity. On the contrary, Klaes et al (2001) showed 6/7 (86%), Agoff et al (2003) showed 5/7 (71%), Houghton et al (2010) showed 54/63 (85.7%).

The present study has adapted the immunohisto chemical grading system which was used by Murphy et al. Results of $P^{16Ink4a}$ over expression in both studies were mostly similar.

In the study by Murphy et al, $P^{16Ink4a}$ over expression in all 21(100%) cases of normal cervical tissue showed grade 0 immunostaining. In 38 cases of LSIL, 3(7.8%) cases showed grade 1 staining, 11 (28.9%) cases showed grade 2 staining and 24 (63.1%) cases showed grade 3 staining. 79 cases of HSIL were studied in which 1(1.2%) case showed grade 0 staining, 12 (15.1%) cases showed grade 1 staining, 23(29.1%) showed grade 2 staining and 43 (54.4%) cases showed grade 3 staining. 10cases of SCC were studied in which 10 (100%) cases showed grade 3 staining.

The grades of $P^{16Ink4a}$ immunopositivity progressively increased from LSIL to HSIL to SCC in both studies. All SCC cases examined exhibited strong over expression of the $P^{16Ink4a}$ protein. Although a small number of LSIL cases showed exclusive nuclear staining, interestingly, the remaining LSIL, HSIL, and invasive cancer cases showed a combination of nuclear and cytoplasmic staining.

The presence of $P^{16Ink4a}$ in the cytoplasm may result from a type of post-transcriptional modification or, more simply, overproduction of the protein may force its transfer into the cytoplasm.

The present study establishes that $P^{16Ink4a}$ over-expression was restricted to LSIL, HSIL and SCC of cervix. No detectable $P^{16Ink4a}$ over expression was observed in normal cervical epithelium. The rates of immunopositivity increased from normal cervical epithelia to dysplasia of varying severity and to carcinoma. These findings clearly support previous studies in confirming that $P^{16Ink4a}$ is indeed overexpressed in dysplastic and neoplastic cells of the uterine cervix.

CONCLUSION

$P^{16Ink4a}$ is a widely available, robust, stable and strong predictive biomarker for evaluating and differentiating squamous intraepithelial lesions and cervical cancers. In the present study, $P^{16Ink4a}$ over-expression was seen in majority of the cases of LSIL, all cases of HSIL, SCC and adenocarcinoma of cervix and the proportion of immunohistochemical grading progressively increased in the same order.Significant association is observed between H&E and IHC grading. LSIL is more associated with IHC grade 1, whereas HSIL and SCC were more associated with IHC grade 3.Normal cervix showed IHC grade 0 staining in all cases. The present study concludes that $P^{16Ink4a}$ is a specific marker for dysplastic and neoplastic alteration in the cervical epithelium and thereby significantly improves cervical precancer and cancer detection.

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