

ROLE OF P16 INK4A EXPRESSION IN LIQUID BASED CERVICAL ASPIRATE SAMPLES AS AN EFFECTIVE SCREENING TOOL IN BIDI ROLLING WOMEN FOR DETECTION OF EARLY CERVICAL DYSPLASIA

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ABSTRACT

Background: Cervical cancer is a leading cause of mortality in India. HPV infection is one of the major risk factors for developing carcinoma cervix. Also, there is an increased incidence of cervical cancer among rural women with genotoxic exposures. All HPV infected dysplastic cells express viral oncoprotein E7 which inactivate the Retinoblastoma (Rb) gene leading to overexpression of p16INK4a. This study evaluates p16INK4a as a surrogate marker for high-risk HPV in a high-risk occupational group. **Materials and Methods:** A prospective study of 60 bidi-rolling women (aged 25–70) was conducted. Conventional Pap smears were obtained and cell blocks were made cervical fornical aspirates using Liquid-Based preservative fluid vial and tissues were analysed. IHC for p16INK4a was performed on cell block sections. **Results:** Dysplasia was detected in 13% of cases by Pap smear. p16INK4a positivity was observed in 12% of the population. When comparing the pap smear cytology with p16 expression, among 48 cases with no epithelial abnormality in pap smear 3 showed p16 positivity (SM+IS-2 cases and NILM-IS -1 case) and 4 dysplasia cases in pap smear (LSIL-2 and HSIL-2) also showed positivity in p16 expression. There was a significant association between pap smear and p16 expression. Significant associations were found between advanced age, high parity (>3), and longer exposure to bidi rolling with increased incidence of dysplasia. **Conclusion:** p16INK4a expression significantly increases in cervical dysplasia, serving as an effective screening tool to reduce interobserver variation and identify high-risk cases in resource-limited settings.

INTRODUCTION

Carcinoma of the uterine cervix is the second most common malignancy among women worldwide. It accounts for approximately 5,30,000(8.8%) new cases, per year and 2,75,000 deaths per year.^[1] India accounts for 15.2 per cent of cervical cancer deaths in the world. The incidence rate is higher among poor socio-economic women and in rural areas, this makes cervical cancer a major public health problem.^[2] Cervical cancer is preceded by squamous intraepithelial lesions which can be detected by effective screening programmes.^[3] Cervical cancer if detected at the earliest stage, the 5-year survival rate is around 92%.^[4]

HPV infection is one of the major risk factors for developing carcinoma cervix.^[5] The vast majority of HPV infection will regress spontaneously, only 10 to 20 % of HPV infected cases will progress to invasive carcinoma in 10 to 15 years.^[6] Therefore, Detection of cervical premalignant lesions is a crucial component to reduce the associated morbidity and mortality.

Pap smear is an important screening test initially done by Dr. George N. Papanicolaou in 1928,^[7] which is easy to perform, cost effective.

All HPV infected cells will express viral oncoproteins E6 and E7 which causes erratic cell division that leads to enhanced expression and cellular accumulation of p16INK4a in the dysplastic

epithelial cells, which can be detected by immunohistochemistry.^[8] P16 overexpression is thus a surrogate marker for high-risk HPV infection.

In high-risk occupational groups like bidi-rolling women, chronic exposure to tobacco dust, which contains potent genotoxins can exacerbate DNA damage and increase the risk of cervical dysplasia. While the Pap smear is a traditional, cost-effective screening tool, it is subject to interobserver variation. This study explores the use of p16INK4a, a cyclin-dependent kinase inhibitor that becomes overexpressed in response to HPV-mediated E7 oncoprotein activity, as a more objective diagnostic adjunct.^[9,10]

MATERIALS AND METHODS

After obtaining Institutional ethical committee clearance the study was conducted at mukoodal village of Tirunelveli district and Department of Pathology, Tirunelveli Medical College, Tirunelveli for a period of 2 years from 2019 to 2021. Line listing of ever married female bidi rolling workers of mukoodal village of Tirunelveli district in the age group of 25 to 70 years was made and 60 cases who are willing for the study were included and consent was obtained. The data including case name, age, age at marriage, the obstetric history, sexual history, abnormal vaginal discharge, any chronic drug intake, and other clinical data were documented.

Exclusion criteria: Pregnant women, Women with Active vaginal bleeding, Post hysterectomy patients, known case and treated cases of carcinoma cervix are excluded from the study.

Conventional pap smear and cervical aspirate collection:

The procedure was well explained to the participants. The willing patients were brought to nearby Mukoodal primary Health centre. Patient was placed in lithotomy position. Speculum examination of cervix was done. Conventional Pap smear was collected using Ayres spatula. Ayres spatula was inserted into cervix and rotated for 360 degrees. Then the sample was smeared onto a dirt free, clean glass slide and fixed immediately in 95 % ethyl alcohol for 30 minutes. After fixation for 30 minutes to 1 hour, smears were stained with PAP stain. Also, a sample for cell block was collected using EZI PREP A Liquid Pap test vial, which contains 14ml of preservative solution (PreserveCyt- methanol based) with a cervix cyto broom that can collect the cells from both ectocervix and endocervical region. The cyto broom was inserted into the endocervical canal and using pencil pressure its rotated in clockwise direction for 5 times to obtain optimal amount of material. Then, the brush was rinsed in the Ezi prep LBC preservative fluid vial immediately by pushing the broom into the bottom for 10 times, and then swirling around vigorously to further release the collected material into the preservative fluid. The vials are properly sealed and labelled.

Cell block preparation & processing

1. The contents of the vial were transferred to test tubes and spun in centrifuge at a rate of 3500 rpm for 10 minutes.
2. Cell pellet was formed at the bottom of the vial.
3. The supernatant is discarded.
4. 5 drops of bovine thrombin were added and mixed properly.
5. The contents were allowed to stand undisturbed for 30 mins by placing vertically in test tube rack for cell button formation
6. The cell button is wrapped in filter paper and placed into cassette and fixed in 10% neutral buffered formalin for 3-4 hours.
7. After adequate fixation the cell button is processed in automatic tissue processor through different stages: dehydration, clearing, impregnation, embedding.
8. After tissue processing the two thin ribbon sections of size 2-3 microns are cut using semi-automated microtome.
9. One section will be stained with routine Hemotoxylin and eosin, the other subjected to IHC technique for p16INK4a by DAKO envision method based on the guidelines with a positive control of squamous cell carcinoma cervix

Interpretation

Conventional pap smears were analysed and reported based on 2014 BETHESDA SYSTEM OF REPORTING CERVICAL CYTOLOGY and compared with H & E-stained cell block sections.

The p16INK4a expression is analysed based on:

Negative: When no cells stained or only Weak Cytoplasmic staining

Positive: Both cytoplasm and nuclear staining present.

The percentage of positive cells were assessed semi quantitatively:

Grade 1: positive cells >0 – 5%, Grade 2: -positive cells >5 – 25%, Grade 3: -positive cells >25%

The intensity of staining: Graded subjectively into three categories:

Weak staining +, Moderate ++, Strong +++

Statistical analysis:

Descriptive statistical analysis was done using the statistical package for social sciences (SPSS) for windows software (trial version 20; SPSS Inc, Chicago). Bar charts and pie charts were used for visual representation of data. To find association between two categorical variables chi square test was used. Level of significance was set at 0.05.

RESULTS

This study included 60 females who are bidi rollers at mukoodal village. Among these 32% belonged to 20-40 years, 55% belonged to 41-60 years and 13% are >60 years. Majority of our participants belonged to Upper lower socio-economic category (63%), followed by lower category (32%) only 5% belonged

to lower middle category. Around 70% of them are Multiparous women, and 25% have >3 parity and only 5% are Nulliparous. 63% of study population were of reproductive age group and 37% were postmenopausal. And 12 % of women took oral contraceptive pill. Majority of participants had complaints of white discharge (57%), 20% had lower abdominal pain, 8% had abnormal uterine bleeding and 15% were asymptomatic.

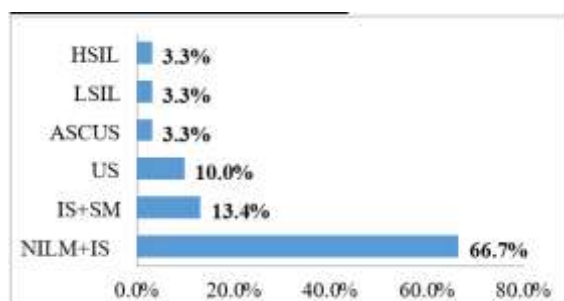


Figure 1: Pap smear report analysis

Figure 1 shows Pap smear report analysis. Majority of patients (66.7%) presented with NILM+IS in pap smear, followed by IS+SM (13.4%) had, US- (10%) had, ASCUS – (3.3%), LSIL (3.3%) and HSIL (3.3%).

Abbreviations: NILM- Negative for intraepithelial lesion or Malignancy, IS- Inflammatory smear, SM- squamous metaplasia, US- unsatisfactory smear, ASCUS- Atypical squamous cells of undetermined significance, LSIL- Low grade squamous intraepithelial lesion, HSIL - High grade squamous intraepithelial lesion.

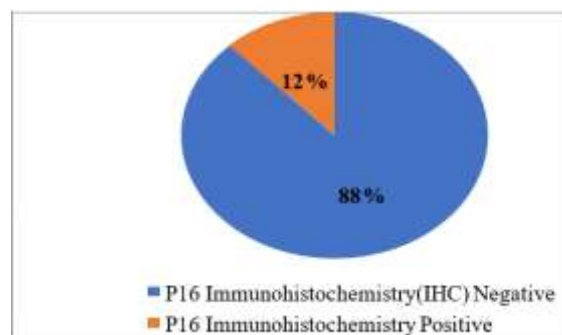


Figure 2: p16 IHC report analysis

Figure 2 shows p16 IHC report analysis: Negative p16 Immunohistochemical expression was observed in 88% of cases, while 12% demonstrated p16 positivity.

Table 1: P16 IHC Staining intensity & grading among study population

Feature	Category	Number	percentage
Intensity of P16 staining	Weak	1	14%
	Moderate	3	43%
	Strong	3	43%
Grading	Grade 1	0	
	Grade 2	4	57%
	Grade 3	3	43%

Table 1 shows P16 IHC Staining intensity & grading among study population:

Among P16 positive cases, 43% had strong intensity, 43% demonstrated moderate staining intensity, and only 14% had weak intensity. Furthermore 57% were grade 2 and 43% were grade 3.

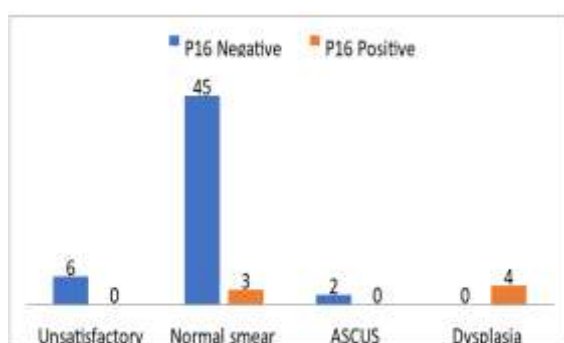


Figure 3: Distribution and association of P16 IHC and Pap smear results

Figure 3 shows Distribution and association of P16 IHC and Pap smear results: P16 expression was positive in 12% of the study population. All six cases with unsatisfactory Pap smear cytology were negative for p16 expression. Among the 48 cases with normal cytology (no intraepithelial lesion or malignancy), three demonstrated p16 positivity (squamous metaplasia, n = 2; NILM with immunostaining, n = 1). Additionally, all four cases with cytological dysplasia (LSIL, n = 2; HSIL, n = 2) showed p16 positivity. Statistical analysis demonstrated a significant association between Pap smear cytological findings and p16 expression.

Table 2: Distribution and association of demographic factors with Pap smear

Demographic factors	Pap Smear		Total	Chi square value	P value
	ASCUS/ Dysplasia	Normal/US			
Age group					

20-40 years	0 (0%)	19 (100%)	19 (100%)	4.276	0.118
41-60 years	4 (12%)	29 (88%)	33 (100%)		
>60 years	2 (25%)	6 (75%)	8 (100%)		
Exposure to Bidi occupation					
0-5 years	1 (4.2%)	23 (96%)	24 (100%)	1.512	0.387
>5 years	5 (14%)	31 (86%)	36 (100%)		
Total	6 (10%)	54 (90%)	60 (100%)		

Table 2 shows Distribution and association of demographic factors with Pap smear: Greater age group had more dysplasia in pap smear however this difference was not statistically significant with p value=0.118 which is >0.05. Though more years of exposure to bidi occupation had higher dysplasia this difference was not statistically significant with P=0.387 which is >0.05.

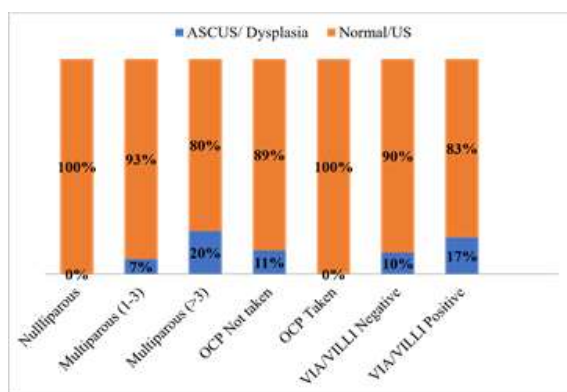


Figure 4: Distribution of study variables with Pap smear

Figure 4 shows Distribution of study variables with Pap smear

All nulliparous women (100%) had normal Pap smear cytology. Among multiparous women with 1–3 pregnancies, 7% showed cytological dysplasia and 93% had normal cytology. In the grand multiparous group (>3 pregnancies), 20% demonstrated dysplasia while 80% had normal findings.

Although higher parity correlated with an increased rate of dysplasia, this association was not statistically significant p value=0.304 which is >0.05.

Among non-OCP users 11% had dysplasia and 89% had normal pap smear whereas 100% of OCP users demonstrated normal cytology. Non-users displayed a slightly higher rate of dysplasia; however, the difference was not statistically significant p value=0.348 which is >0.05.

Cytological dysplasia was present in 10% of VIA/VILI-negative cases (90% normal) compared to 17% in the VIA/VILI-positive group (83% normal). While dysplasia was more frequent in VIA/VILI-positive women, this difference did not reach statistical significance p value=0.547 which is >0.05.

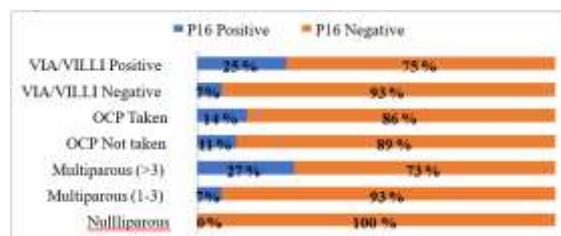
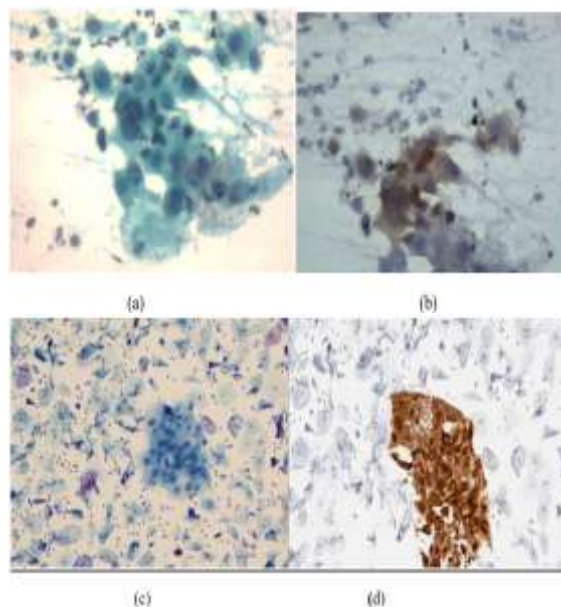


Figure 5: Distribution of study variables with P16 IHC

Figure 5 shows Distribution of study variables with P16 IHC: All nulliparous women (100%) were p16-negative. Among multiparous women with 1–3 pregnancies, 7% were p16-positive (93% negative), whereas 27% of grand multiparous women (>3 pregnancies) were p16-positive (73% negative). Higher parity correlated with increased p16 positivity, but the association was not statistically significant with p value=0.105 which is >0.05.

p16 positivity was observed in 11% of non-OCP users (89% negative) and 14% of OCP users (86% negative). This difference was not statistically significant p value=0.700 which is >0.05.

p16 positivity was present in 7% of VIA/VILI-negative cases (93% negative) compared to 25% of VIA/VILI-positive cases (75% negative). Although p16 expression was higher in the VIA/VILI-positive group, the difference was not statistically significant with p value=0.298 which is >0.05.



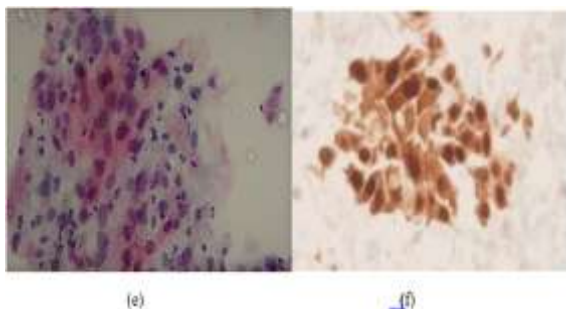


Figure 6: Cytomorphology and p16 IHC expression across Cervical lesions

Figure 6 shows Cytomorphology and p16 IHC expression across Cervical lesions.

a) Squamous metaplasia-pap cytology(40X), b) IHC p16 showing positivity in cell block, c) LSIL on pap cytology(10X), d) IHC p16 showing both cytoplasmic and nuclear positivity, e) HSIL- Pap cytology (40X), f) IHC p16 showing positive expression.

DISCUSSION

Most of the HPV infection are transient, which may regress spontaneously, but if it persists the risk of preneoplastic lesions as well as cervical carcinoma increases due to cell cycle dysregulation by incorporation of oncogenic viral proteins into the host genome.^[11,12]

Conventional Pap smear cytology is still one of the most efficient tests used for screening of cervical carcinoma in many countries because of its easy technique and cost effectiveness in screening large groups. The disadvantage of using Pap smear is that it has low sensitivity.^[13,14]

So, it was important to find out a better screening test for detection of High-risk HPV infection, thereby reducing the number of false positive cases as well as to bring down the number of unwanted colposcopies and biopsies.

P16INK4a has emerged as a surrogate marker of HPV, that can be used in both in cytology and histology sections. P16 expression reduces the interobserver variation significantly. Its pattern of expression also correlates with the degree of dysplasia.^[15]

Our study was done to find the expression of p16 in early cervical dysplasia cases and compared with the conventional pap smear cytology.^[16]

The overall age group of our study population ranges from 26 to 70 years with mean age of 47.1 years and median age of 47.5 years. This age group was similar to a study done by Chinmayee et al 2017, where most of the screened women were in the age group of 20 to 70 years.^[17]

Among this 63% of them were in reproductive age group and 37% were postmenopausal women. Most of our patients belong to upper lower socioeconomic status (63%), with 70% of them are multiparous, 25% were grand multiparous and 5% were nulliparous women.

Majority of our patients (57%) had abnormal white discharge as the chief complaints, followed by lower abdominal pain in 20 %, similar to study by sachan et al 2018 with 37% had white discharge followed by 25% with abdominal pain. Kamna gupta et al 2011, study also showed 48.2 % of early dysplasia cases had white discharge as major symptom.^[18]

Among the pap smear cytology report of 60 cases, 67% had inflammatory smear with no epithelial abnormality, 13% showed squamous metaplasia with inflammatory changes, 10% are considered unsatisfactory and ASCUS, LSIL and HSIL each contributed 3%. The observations are almost similar to Pankaj et al 2016 study with 78% had NILM with IS, 2 % with ASCUS, and LSIL – 1% and HSIL- 2 %.^[19]

P16 positive expression was seen in 12% of the study population. When comparing the pap smear cytology with p16 expression, 6 cases which showed unsatisfactory pap smear were all negative for p16 expression. Among 48 cases with no epithelial abnormality in pap smear 3 showed p16 positivity (SM+IS-2 cases and NILM-IS -1 case) and 4 dysplasia cases in pap smear (LSIL-2 and HSIL-2) also showed positivity in p16 expression. There was a significant association between pap smear and p16 expression. These findings were concordant with shidam et al 2011.^[20]

Among this those between age group of 20 to 40 years had normal pap smear and 100% negative expression with p16. In 41-60 years of age group 12% had dysplasia in pap cytology as well as positive expression in p16, and in >60 years group 25% had dysplasia and 37.5 % with positive expression. So, as the age advances the risk of dysplasia increases, with a significant p value for p16 expression. This observation coincides with sachan et al 2018 study done at gynaecology clinic, Department of Obstetric at King George Medical University Lucknow where 5.5% dysplasia is seen in more than 60 years.^[21]

Around 63% of our study population belong to upper lower socioeconomic status, 32 % belong to lower SEC and 5% of lower middle group according to modified kuppusamy scale. Among these 10.5 % of lower SEC and 33% of lower middle group had cervical dysplasia in pap smear. And 10.5 % of lower and upper lower and 33 % of lower middle group showed p16 expression. So, in our study there was a significant increase in dysplasia among lower middle group.

There was a higher incidence of dysplasia, 14% in cytology and 17% p16 positivity among women who were indulged in beedi rolling occupation for more than 5 years compared with those between 0- 5 years exposure (4.2%). As the years of exposure increases there is an increased incidence of dysplasia mainly due to mutagenicity caused by the tobacco dust. This is in concordance with study by joseph et al 2016.^[22] Most of our study population are multiparous (70%), among them 7% were positive for dysplasia in pap cytology and p16 expression, whereas among grand multiparas, 20 % had positive pap cytology and 27 %

positive p16 expression. So as parity of the women increases the frequency of dysplasia also increases which coincides with ashish et al 2015 study and sachan et al 2018, increased incidence of dysplasia occurs in parity >3.^[23]

In our study another screening test VIA-VILI was done in only 42 cases out of 60 cases. 17% of those who are VIA-VILI positive showed cervical dysplasia and 25 % positive in IHC .10 % of VIA-VILI negative cases also showed dysplasia in pap smear and 7 % positivity with IHC. Hence VIA-VILI positivity is a predictor of underlying dysplasia and hence such patients need further follow up.

Limitations

1. The study evaluated only 60 participants, which limits the statistical power and generalizability of the findings to broader populations.
2. Lack of Direct HPV DNA Testing: p16INK4a was evaluated as a surrogate marker without confirming actual high-risk HPV infection status through molecular HPV DNA or RNA testing.

CONCLUSION

In this study P16INK4a expression is significantly increased cervical dysplasia, which is an indirect marker of HPV infection. Hence p16 can be used as a screening tool for early identification of dysplasia in high-risk cases, thereby reducing the need for another repeated test. These patients can be followed up with biopsy so that progression to invasive carcinoma can be avoided.

In future the study can be conducted in larger population to confirm the above findings. These studies should facilitate the development of rational strategies for the prevention and treatment of cervical carcinoma.

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