



Presence and Positivity of High Risk HPV with Increase in the Severity of Cytological Abnormalities Detected on Pap Smear: A Study of 40 Cases

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Introduction

Cervical cancer ranked second among most commonly diagnosed cancer and in less developed countries it is third leading cause of cancer related death among females^[1,2,3] It is estimated that each year, 527,000 new case occur and 275, 000 deaths. Globally, 15% of all cancer in females is cervical cancer.^[2,4] In India about 20% of all cancer related deaths is due to cervical cancer in women and is the number one cause of death in middle aged Indian women.^[5]

Cancer cervix is a multifactorial disease. Human Papilloma virus (HPV) infection is the most important risk factor.^[6] It has been shown recently that cervical cancer is strongly associated with the presence of high risk or oncogenic Human Papilloma virus (HPV) types (up to 100%).^[7,8] The HPV virus is belongs to the family *Papovaviridae*, genus *papillomavirus*.^[9,10] having double stranded, circular DNA. The HPV subtypes which specifically affect the anogenital tract are 16, 18, 31, 33, 35, 39, 45,51, 52, 56, 58, 66 and 69.^[11] It is important to understand genomic organization of the virus to understand the oncogenic process induced by HPV that leads to development of cervical dysplasia. Significant

regions include the early (E), the late (L),^[12] and the long control region (LCR).^[13] E6 binds with p53 tumor suppressor gene, causes its degeneration while E7 binds retinoblastoma gene products.^[14] thus, inactivation of p53 and pRB leads to cell cycle progression^[15] and immortalization of normal cervical cells, so it is the expression of viral oncogenes E6 /E7 which is prerequisite for progression toward malignancy and maintenance of the cancerous phenotype^[16,17].As the severity of the lesion increases levels of E6/E7 also rises.^[18,19] E6 and E7 transcripts could be useful as markers of disease progression.^[18]

Thus, the objective of the study is to evaluate the expression of E6/E7 mRNA of HPV types 16, 18, 31, 33, 35, 39 ,45 , 51, 52, 56 , 58 , 59 , 68 and 82 (which together accounts for 95% of cervical cancer)^[4] in cervical samples using flow cytometry and its correlation with epithelial cell abnormalities detected by pap smear.

Materials and Methods

This study was conducted in Department of Pathology, Mahatma Gandhi Medical College and Maharaja Yeshwant Rao Hospital, Indore,

Madhya Pradesh, India. It is a prospective study. The study duration was one year from July 2017 to June 2018. Sample size for the study was of 40 cases.

Material Required

1. Sure Path vials.
2. Cytobrush with detachable head
3. Vortex
4. normal glass slides
5. Centrifuge machine
6. Pap stain.
7. Micro centrifuge
8. Micropipettes
9. Water bath ($65\pm 1^\circ\text{C}$)
10. Flow cytometer with a 488 nm laser
11. Data acquisition and analysis software
12. Buffer A (Fixation Buffer)
13. Buffer B (Hybridization Buffer)
14. HPV E6/E7 Probe Cocktail
15. HPV E6/E7 Positive Control Cells
16. HPV E6/E7 Negative Control Cells
17. 4°C refrigerator and -20°C freezer
18. 1.5ml polypropylene micro centrifuge tubes
19. 15ml polypropylene conical tubes
20. Disposable DNase-free pipet tips
21. Formaldehyde (37% by weight, methanol stabilized)

Method

After obtaining informed consent 40 women attending Gynaecology OPD were randomly selected on the basis of inclusion criteria. A detailed history was taken.

Results & Observation

1. Analysis of pap smear

Pap smear	No of. cases	%
Total smear	40	100
Inflammatory smear	25	62.5
Epithelial cell abnormality	15	37.5
Atypical epithelial cells Of -Atypical squamous cells undetermined significance [ASCUS]	7	17.5
Atypical squamous cells Cannot exclude HSIL (ASC-H)	2	5
Low grade squamous intraepithelial lesion (HPV/mild dysplasia /CIN I [LSIL])	4	10
Malignancy	2	5

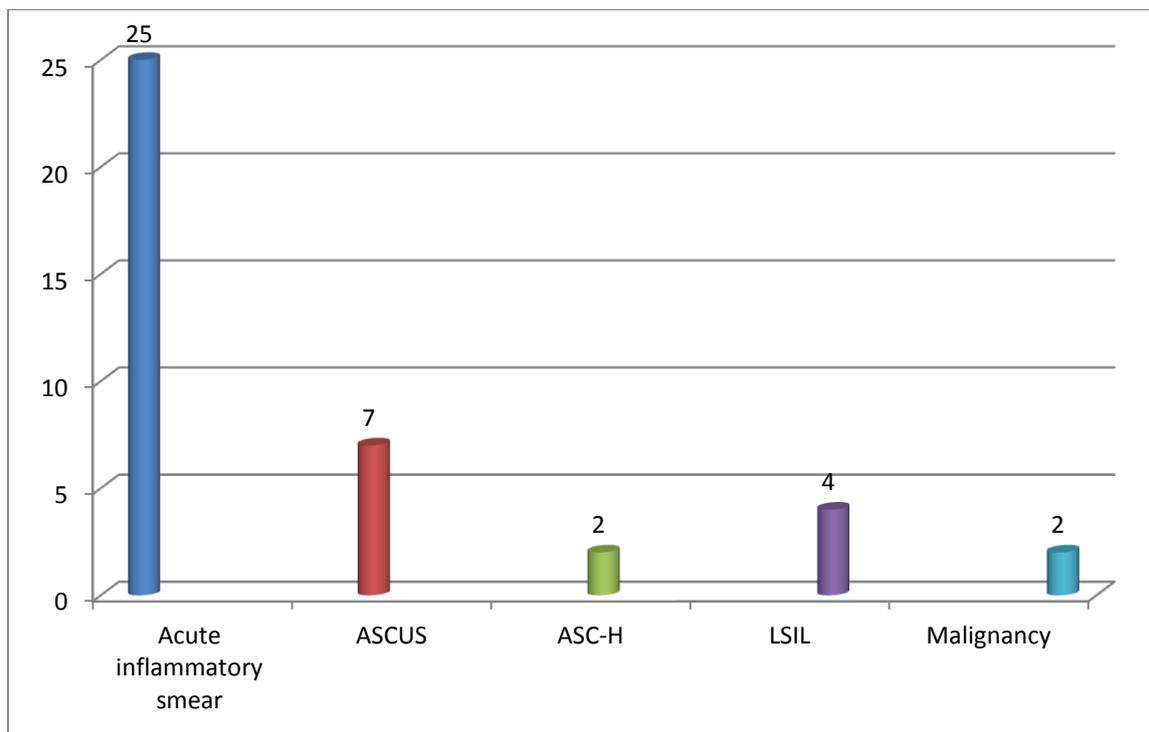
All 40 selected women were examined per vaginally and by speculum after acquiring a detailed history and verbal consent from them. The woman was placed in dorsal lithotomy position. After proper positioning of the woman, cervix was viewed by introducing Sims' vaginal speculum and anterior vaginal retractor and external os was identified. Pap smears were made by introducing cervical brush/ cytobrush with a detachable head were inserted into the external os and rotating it through 360 degrees 8-10 times in clockwise direction near the squamo-columnar junction. The cellular material thus obtained was quickly, but gently smeared on a clean glass slide. The glass slide was then immediately put into the Coplin jar containing 95% ethanol which acted as a fixative. The prepared smears were then stained according to Papanicolaou's technique.

The white head of the cervical brush was detached and put into the Sure Path preservative vial. Vial was then shaken well and stored at room temperature till the samples were processed for the run in flow cytometer. (Figure 1)

The cytological interpretation of the smears was made according to the new Bethesda system. Data collected for age, socioeconomic status, parity, clinical features and examination, results on conventional pap smear and flow cytometric analysis of HPV mRNA E6/E7 was organized, interpreted, compared and analysis on appropriate statistical software.

Table No. 1 shows that in the present study, 40 pap smears were analyzed showing the distribution pattern of various condition. Out of

which acute inflammatory smear is 25 (62.5%) and epithelial cell abnormality comprises 15 cases (37.5%).

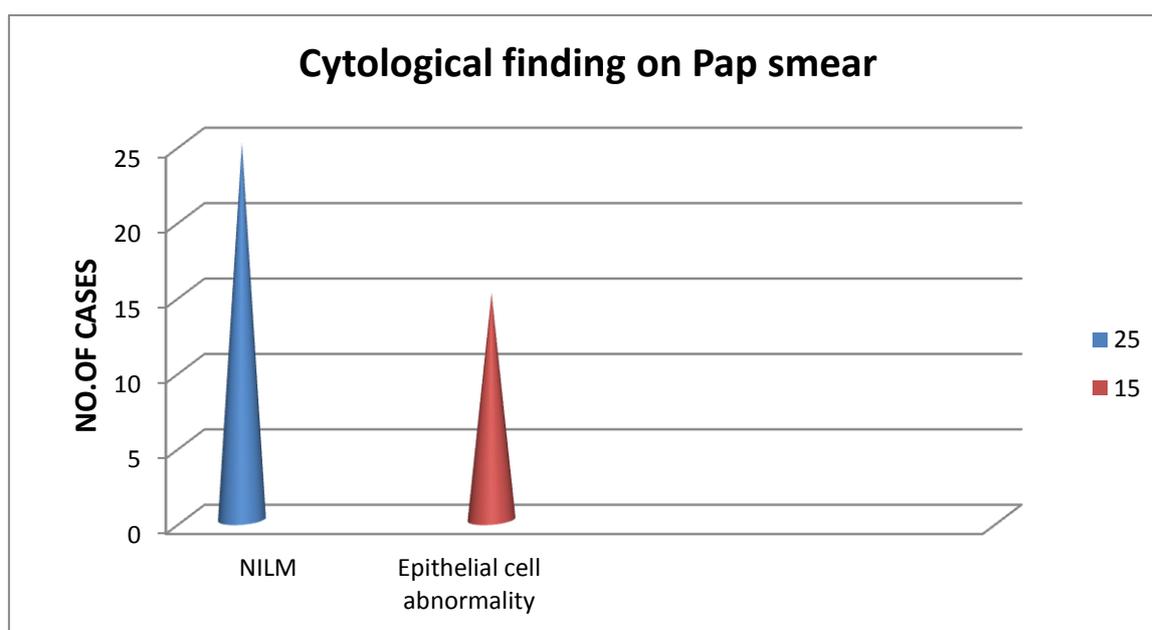


Graph 1- Analysis of pap smear

Table 2 Distribution of Negative for intraepithelial lesion or malignancy and Epithelial cell abnormality

Cytological findings	No. of cases	Percentage%
Negative for intraepithelial lesion or malignancy	25	62.5
Epithelial cell abnormality	15	37.5
Total	40	100.00

Table 2 shows in the present study epithelial cell abnormality is 37.5%.



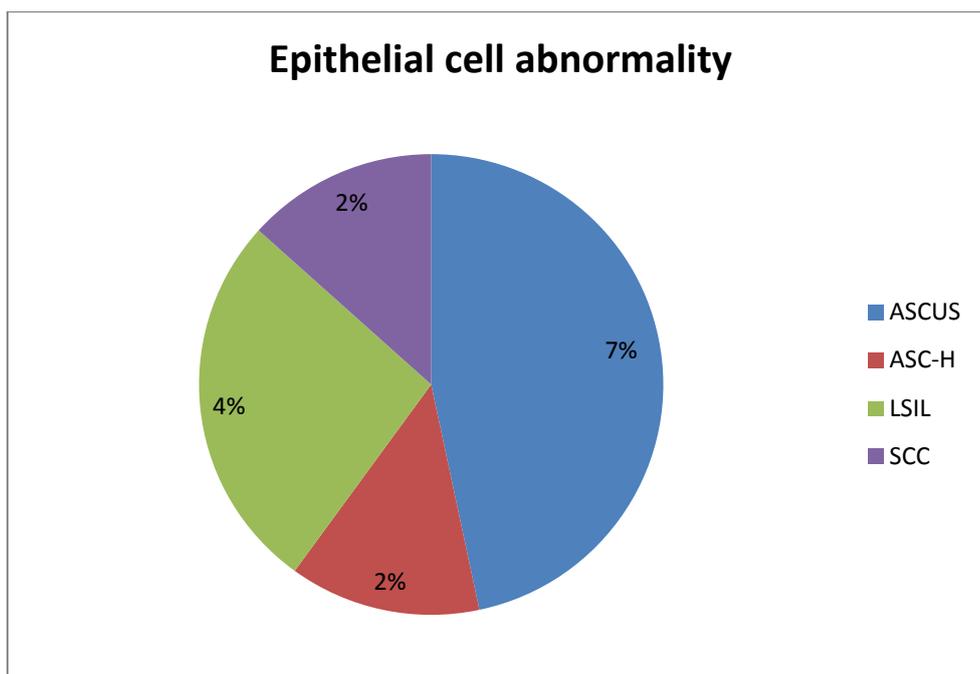
Graph 2 Distribution of NILM and Epithelial cell abnormality.

3. Distribution of epithelial cell abnormality

Epithelial cell abnormality	No. of cases N= 15	Percentage %
ASC-US	07	46.66
ASC-H	02	13.33
LSIL	04	26.66
SCC	02	13.33

Table No 3 shows out of 15 cases of epithelial cell abnormality, 7 cases show Atypical squamous cells of uncertain significance, 2 cases show Atypical squamous cells of uncertain significance

cannot exclude HSIL, 4 cases shows low grade squamous intraepithelial lesions. A total of 2 cases show invasive carcinoma cervix.



Graph 3 Distribution of epithelial cells abnormalities

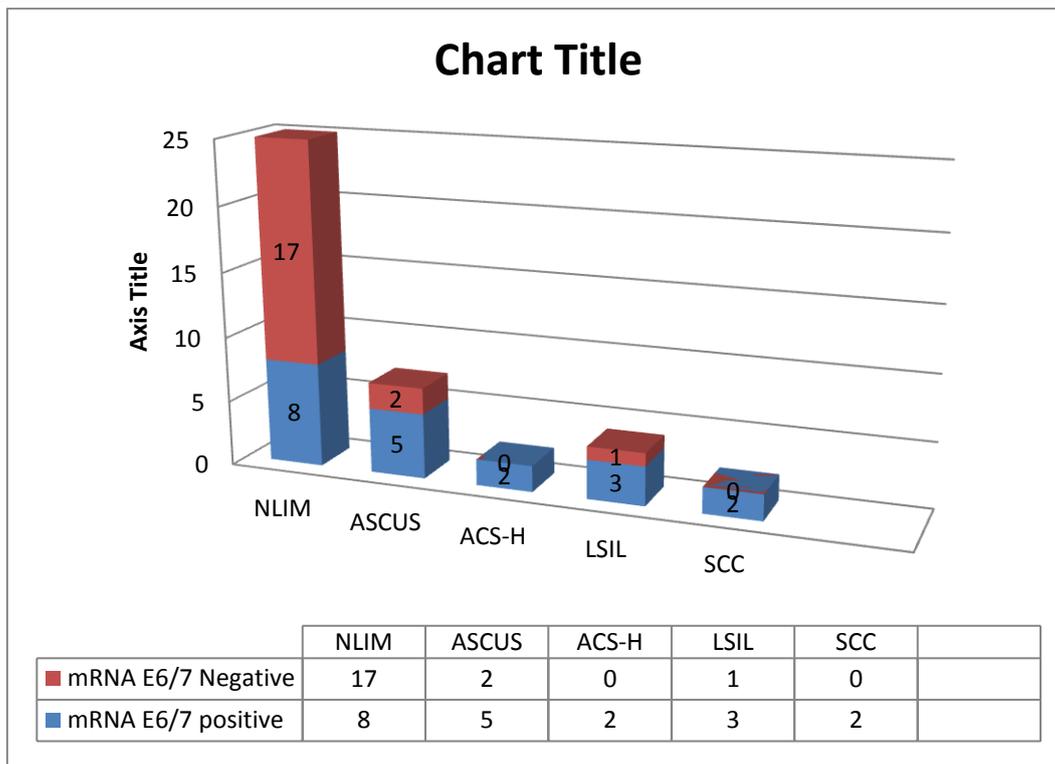
4. Percentage positivity of HPV mRNA E6/E7 compared with severity of cytological diagnosis

Cytological diagnosis	No. of cases by pap smear	No. of cases positive for HPV mRNA E6/E7 (%)
Negative for intraepithelial lesion or malignancy	25	08 (32%)
ASCUS	07	05 (71%)
LSIL	04	03 (75%)
ASC-H	02	02 (100%)
SCC	02	02 (100%)

Table no. 4 shows positivity of HPV mRNA E6/E7 increases with the severity of cytological evaluation. The positive rate of HPV mRNA E6/E7 rise from 32% for those who were negative for intraepithelial lesion or malignancy, 71% for

ASCUS and 75% for LSIL to 100 % for carcinoma cervix.

It is also noted that HPV mRNA E6/E7 test detect extra 8 cases (32%) which were labeled as negative for intraepithelial lesion or malignancy.



Graph 4 showing HPV mRNA E6/E7 positivity distribution in relation with cytological diagnosis on pap smear.

Figure 1 Flow Cytometry Set Up In Our Department



Figure 2 ASCUS on pap smear (low power)

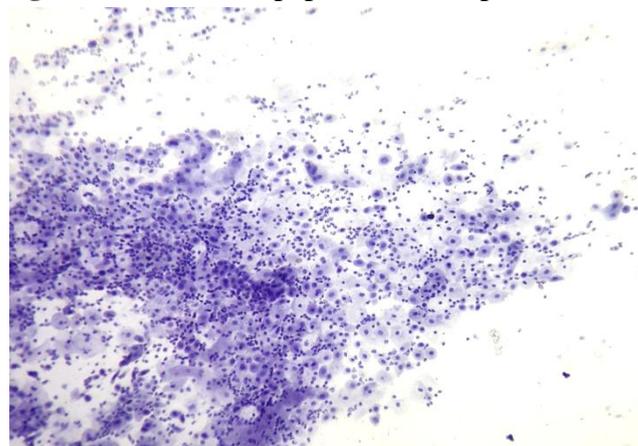


Figure 3 ASCUS on pap smear (high power)

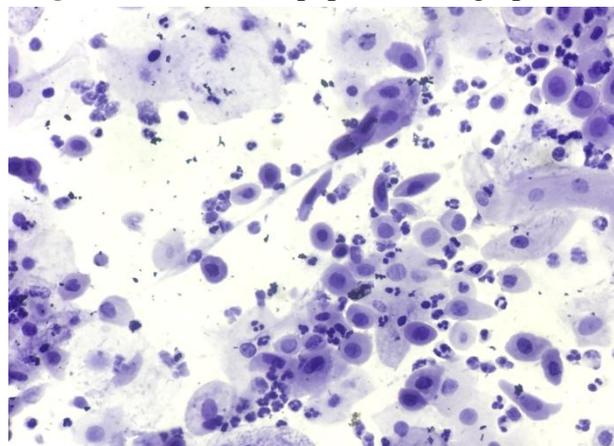


Figure 4 LSIL on pap smear (low power)

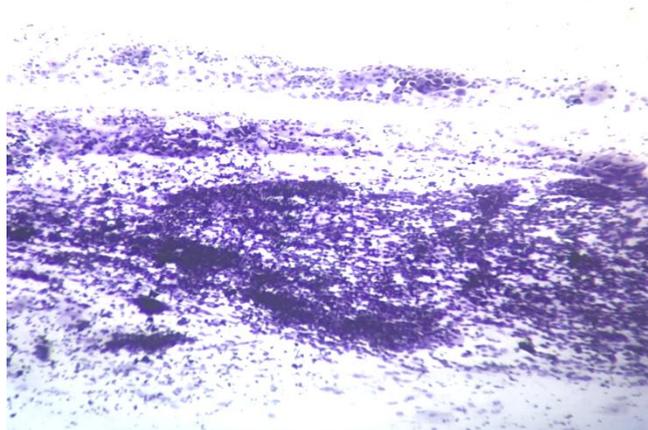


Figure 5. LSIL ON pap smear (high power)

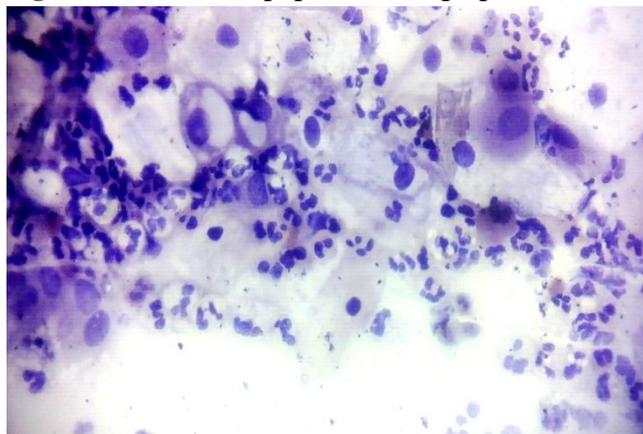


Figure 6 ASC-H on pap smear (low power)

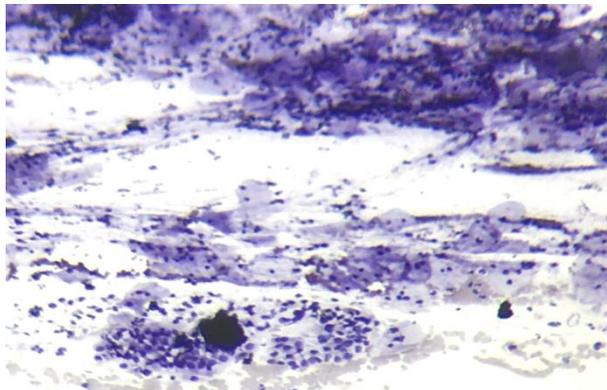


Figure 7 ASC-H on pap smear (high power)

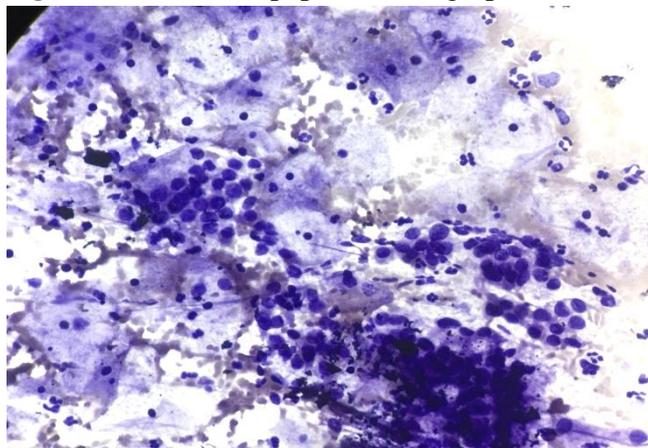


Figure 8 SCC on pap smear (low power)

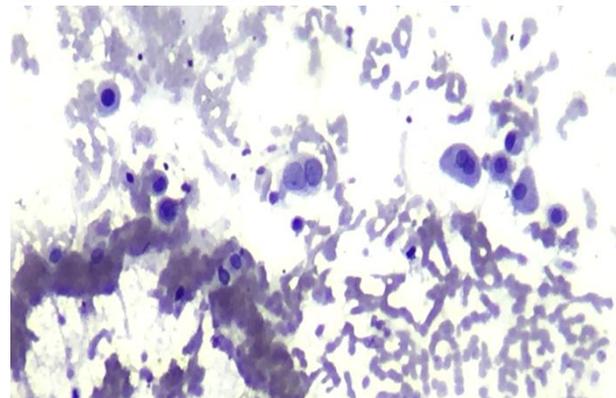
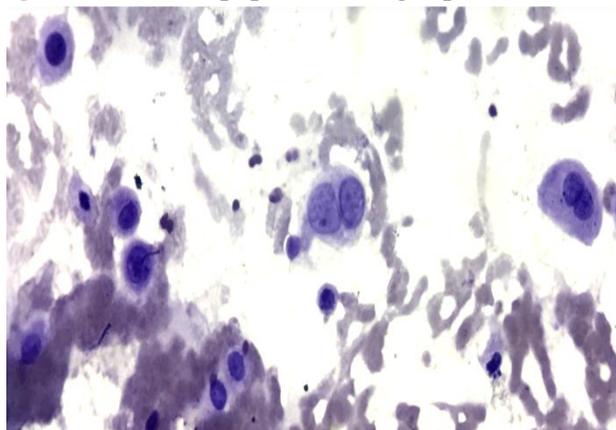


Figure 9 SCC on pap smear (high power)



Discussion

The present study comprised of taking cervical samples of 40 symptomatic women attending the outpatient department of Obstretic & Gynaecology and these cervical samples were processed for flow cytometric analysis of HPV E6/E7 mRNA detection and results were compared with pap smear to screen patients who are at risk for developing cervical cancer.

Our study revealed ASCUS (17.5%) to be the most common epithelial cell abnormality. Similar results were obtained in other studies which also concluded that ASCUS to be the most common epithelial cell abnormality^[20,21]. ASCUS progresses to LSIL, HSIL and SCC. AGUS progresses to adenocarcinoma.^[22,23]

Fusté P^[24] (2008) and Insinga RP^[25] (2008) found that HPV is associated with more than 99% of all cervical cancer cases while in our study 100% cases with cervical carcinoma show hpv e6/e7 rmna positivity which is in favour with the result of our study. HPV causes almost 100% of cases of

cervical cancer and limitation of study methodologies is most important reason behind an underestimation of HPV prevalence in cervical cancer.^[26]

In present study e6/e7 test is positive in 80 % of women with ASCUS, ASC-H, LSIL or greater while it is 32% positive in women with negative cytology. Results are consistent with study conducted by Jefferson Elias Cordeiro Valença in 2016 in which 83.8% of women with ASCH, HSIL, or greater is positive while, 57.9% of women presenting a negative cytology show E6/E7 test positive. cases which shows negative cytology and positive e6/7 test may be due to false positive cytology or it may be due to non evidence of morphological changes with viral integration into host genome^[27,28] According to Li and Kristensen,^[29] it could be the latent infection which presented with positive e6/7 test and negative cytology. Negative E6/E7 tests observed in women could be due to the presence of other types of HPV, not screened in this study.^[29]

mRNA positivity increased with severity of cytological abnormality and ranged from 32% (8/25) IN normal cytology to 71% (5/7) in ASCUS , 75% (¾) in LSIL to 100 % (2/2) in invasive cervical carcinoma. Similar was found in study conducted by Rijkaart^[30] et al in 2012 where it is 32% (64/202) in normal cytology to 47%(41/88) in BMD and 68% (58/85) in _BMD. This increase was statistically significant (P _ 0.01) as found in their study. Similarly^[31] Tong-Yu Liua in 2013 The positive rate of mRNA rose from 29.0% (9/31) for those who were negative for intraepithelial lesion or malignancy, 21.5% (31/144) for atypical squamous cells of unknown significance, and 57.6% (19/33)for low-grade squamous intraepithelial lesion, to 59.8% (76/127) for more severe than high-grade squamous intraepithelial lesion..also stated by Cattani ^[32] et al in 2009 E6 and E7 transcripts were detected in 20 of 80 patients with normal cytology findings (25%) by the mRNA test. The proportion of patients with detectable transcripts increased progressively with the grade of the lesions

observed, rising from 25% for patients with ASCUS (5/20 patients) to 50% for those with LSILs (23/46 patients) and 96% for those with HSILs or ASC-H (24/25 patients). All cases Pap smear positive for SCC were positive for HPV RNA.

VARNAI^[33] et al in 2007 found that Expression of HPV E6/E7 mRNA was detected in 58% while in OUR STUDY we found it to be 50% (20/40). While Tong-Yu Liua^[31] in 2013 found that A total of 135 (40.3%) patients were positive for HPV E6/E7mRNA.

In present study 80 % (12/15) of patients with abnormal cytology show hpv e6/e7 mrna positivity while study conducted by Ozer Birge^[34] in 2018 found that 55.3% of patients with abnormal cytology show HPV E6/E7mRNA positivity. Pap smear has been used for the detection of preinvasive lesions. However, false negative values of 20-50% of a pap smear lead the researchers to find supplementary methods to increase the accuracy^[35]. In recent years, screening for HPV virus has become the interest of investigations. It is only the absence or presence of virus detected by HPV DNA; however, the potential of progression to invasive carcinoma and the probability of concomitant pre-invasive lesion cannot be predicted by the viral DNA test. Furthermore, lesions that will regress spontaneously are diagnosed and 'over-treatment' is made. By this way, the detection of E6/E7 mRNA proteins of human Papilloma virus seems to be more reasonable, as these are responsible for oncogenic transformation ^[36,37].

Prevalence of HPV in our study is 50% (20/40) which is in consistent with study done by rashmirani senapati in 2017 where they found prevalence to be 60.33%. while the prevalence in case of invasive cervical cancer is 100% while rashmirani senapati ^[38] found it to be 93.80%.

Conclusion

From our study we came to conclusion that as severity of cytological abnormality increases percentage positivity of HPV mRNA E6/7 also

increases. As per review of literature as HPV is most important risk factor for cervical carcinogenesis and its detection at molecular levels will help in early preventive measures rather than to wait for cytomorphological abnormality to appear.

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