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Original Research Article

Role of Proliferative Markers in the Differential Diagnosis of Cervical Cytology

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ABSTRACT

Cervical squamous intraepithelial lesions are premalignant lesions which are capable of progressing to invasive cervical cancer. Despite the advent of liquid based cytology and totally computerised analysis sand screening system, the diagnosis in cervical cytology is still subjected to high rates of discordance due to sampling errors, inter- and intra-observer variability as well as poor reproducibility. The **objective of the study** is to assess the role of proliferative markers i.e. Ki67 and PCNA in diagnosis of cervical squamous intraepithelial lesions.
Methodology- Out of this total 1006, 77 cervical smears were diagnosed as squamous intraepithelial lesions. All these were subjected to immunostaining by Ki67 and PCNA, 14 smears with diagnosis of NILM were also immunostained as control. The results showed clustering of labeling index of Ki67 and PCNA, ascending from NILM to ASCUS, ASCUS-H, LSIL, HSIL and finally carcinoma cervix.Ki67 and PCNA index were studied in all the immunostained slides and calculated to the nearest percent. Statistical analysis was done by student t test.
Result- In ascending order, comparison was done between NILM with ASCUS, ASCUS with LSIL, LSIL with ASC-H,ASC-H with HSIL and finally HSIL with CaCx, in both Ki67 and PCNA groups. p values were found significant in all the groups except between LSIL vs ASC-H.
Conclusion-The study showed stratification of lesions ascending from NILM,ASCUS, ASC-H, LSIL, HSIL, CaCx, in terms of Ki67 and PCNA labelling index. The study proved that these proliferative markers could be utilised as an adjunct to routine cervical smears analysis.

Keywords: Squamous intraepithelial lesions, cervical cancer, Proliferative markers, labeling index.

INTRODUCTION

In India, cervical cancer is the second most common cause of morbidity and high mortality associated with malignancy in women. [1] The squamo-columnar junction, the meeting place of squamous exocervix and the glandular endocervix where "fight of epithelia" takes place [2] is the site of origin for squamous intraepithelial lesion (SIL) including invasive squamous cell carcinoma. Cervical intraepithelial neoplasia (CIN) has three grades; CIN1 is equivalent to mild dysplasia, CIN2 moderate dysplasia and CIN 3 of severe dysplasia and carcinoma in situ. The 2014 Bethesda classification schema designed for cervical cytological specimens classified squamous intraepithelial lesion (SIL) into low and high grade and obviously invasive squamous cell

carcinoma. The LSIL (low grade squamous intraepithelial lesion) has CIN1 (as well as HPV-induced lesions that do not qualify as CIN),whereas HSIL (high grade squamous intraepithelial lesion) has CIN 2 and CIN 3.The Bethesda scheme has diagnosis of ASC-US, Atypical cells of undetermined significance and ASC-H, atypical squamous cells cannot exclude HSIL. [3-5]

Screening programs using Pap smears along with the advent of a universal reporting and classification system are highly successful. Microscopic analysis of conventional cervical smears or cell suspensions for liquid cytology has been proved gold standard for detecting abnormal cervical epithelial cells. Morphological assessment of cervical cytology
Can also be useful to determine the degree of dysplasia and the level of risk for developing cervical ca**.** [6] However, a number of cases have still been missed due to false negative results like sampling errors, inter- and intra-observer variability. [7]

Besides this, a large number of false positive results have been reported attributed to presence of inflammatory atypia, reserve cell hyperplasia or atypical immature metaplasia. So, beside conventional pap smear and colposcopy, newer methods of Liquid based cytology (LBC), Automated scanning devices, computer assisted microscopy, digital colposcopy with automated image analysis, human papilloma virus(HPV)testing, molecular markers and HPV vaccine have all been incorporated in cytological assessment of cervical smear. [3]

Liquid based cytology though becoming popular, it is very costly requiring expert team and a fully automated laboratory which are difficult to adopt in all centers, especially in a developing country like India. Inter- and intra-observer variability can pose a diagnostic dilemma in classification of cervical dysplastic lesions and immature squamous metaplasia on cytopathology. Moreover, the introduction of 'atypical squamous cells of undetermined significance' (ASCUS) and ASC-H, atypical squamous cells cannot exclude HSIL creates a limitations of morphologic interpretation. Sometimes specimens are encountered with cytologic features that lie between LSIL and HSIL; however, no new indeterminate cytology terminology has been proposed to avoid confusion due to poor reproducibility. [8] So, a grey zone persists still and that demands further search for reliable additional biomarker.

So, there is a constant need for additional sensitive and specific biomarkers which can improve standardization and quality control of cervical cancer screening programme. In this context, this study has been aimed at to discover the role of proliferation markers in increasing the diagnostic accuracy in equivocal cases on cervical cytopathology.

The practice of immunocytochemistry with conventional Pap smear can be helpful as an adjuvant with liquid based cytology or other investigations. Antigen Ki67 is a nuclear protein that is associated with cellular proliferation. It could be used as cellular marker for proliferation as well as degree of dysplasia. During interphase stage ,Ki67 antigen can be detected in cell nucleus whereas during mitosis it is relocated to surface of chromosomes.Ki67 is present in all active phases of cell cycle C1,S1,G2 mitosis but absent from resting cells G0. [9] Ki67 labeling index is correlated with clinical course of cancer, best examples are carcinoma of prostate, brain and breast. In cervical biopsies Ki67 is found to helpful in stratification of CIN and with HPV associated lesions. [2, 9, 10, 11] Moreover, Ki-67 staining is found to be advantageous over HPV testing specially for subclinical HPV infections which show negative staining. On the other hand, it is a low-cost laboratory technique. [12,13]

Proliferative cell nuclear antigen (PCNA) is a protein that acts as a processivity factor for DNA polymerase in eukaryotic cell. It is an example of DNA clamp. PCNA was originally identified as antigen expressed in nucleus of cells during

DNA synthesis phase of cell cycle. PCNA is important for both DNA synthesis and repair. PCNA is also a marker commonly used for immunohistochemical evaluation of proliferative activity. [4] The expectations of biomarker identification in cervical smear is to distinguish squamous intraepithelial lesion (SIL) from non SIL [2, 9,10] and also distinguishing HSIL from reactive epithelial changes. [4]

This study is expected to establish the role of immunological stains namely Ki67 and PCNA, the markers of proliferation. The prototype for this study is Goel et al, classic paper MIB1 and PCNA immunostaining was utilised as diagnostic adjunct to cervical pap smear. This study was conducted at Lucknow to determine role of MIB1 and PCNA as adjunct to pap smear for identification of ascending grades of cervical intraepithelial lesions.MIB1and PCNA labeling index was calculated .The highest proliferative index was found in carcinoma groups along with a significant positive correlation between ascending grades of squamous intraepithelial lesions (SIL) and labeling index for MIB1and PCNA. The study suggested these markers could be used as adjunct to cytomorphological interpretation of conventional pap smear. [14]

Aims and Objectives

The aim of the study is to assess the role of proliferative markers (Ki67 and PCNA) with the help of immunocytochemistry in stratifying the cervical squamous lesions (in ascending order) from routine conventional cervical Pap smear.

MATERIALS AND METHODS

This was a prospective and observational study done in the department of Pathology in a tertiary care hospital with cervical smear samples received from department of obstetrics and gynaecology over a period of two years. 1006 cases were included in this study of which 100 cases were selected for immunocytochemistry.

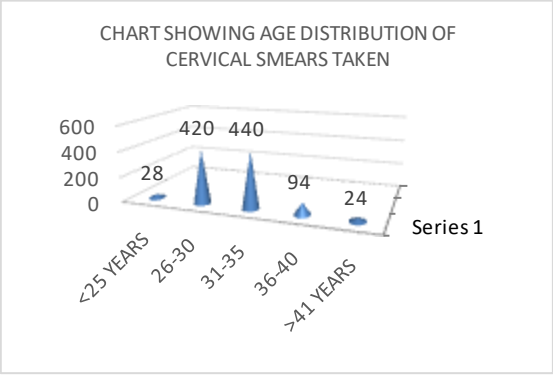
For the cytological examination of the exfoliative cervical smear, the pap smear were collected, fixed in alcohol, dried and transported to Pathology department. Here the slides were three in number for each patient. One slide was stained by papanicolaou stain and interpreted as per Bethesda system of reporting 2014.Another two slides was stained for immunocytochemistry by standard avidin-biotin technique. The smears were taken on poly-L-lysine coated slides used for immunocytochemical stain for PCNA and Ki67.

PCNA index study was done by using DAKO PCNA Kit manual. At least 1000 nuclei were counted in 1000x magnification and the results were expressed as a ratio of stained to total nuclei counted in percentage (PCNA labeling index i.e. L.I. %). 14 NILM cases to act as controls for the immunocytochemistry study by Ki67 and PCNA.

The results were analysed in Microsoft excel software, using the student t test after obtaining P values for both Ki67 and PCNA stained cases. Value of 0.05 was taken to be significant.

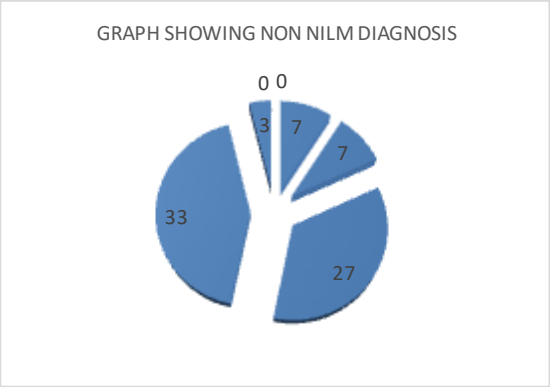
RESULTS AND ANALYSIS

A total of 1006 cases were assessed using the Bethesda system 2014. 100 cases selected from the 1006 cases of which 23 are NILM and rests are squamous abnormalities. So, 77 cases had squamous cell abnormality of which only three cases of cervical smear showed carcinoma cervix during the period of two years of the study.



Bar Diagram 1: Graph showing age distribution of cervical smears taken

A vast majority of patient were in the age group 26-35 years. The study showed sexually active women in age group less than 25 years were less forthcoming to come for the screening, there was also dearth of patients above age of 36 years



Bar Diagram 2, showing distribution of squamous cell abnormality (N=77)

TABLE 1, table showing results of immunopositivity with ki-67 (Ki67 index)

	<4%	5-9%	10-14%	15-20%	>20%	Total
NILM	23	2	1			26
LSIL		1	3	19	4	27
HSIL				4	29	33
ASCUS	1	5	1			7
ASC-H				5	2	7
						100

TABLE 2,table showing immunostaining with PCNA(PCNA index)

	<4%	5-9%	10-14%	15-20%	>20%	Total
NILM	21	1	1			23
LSIL		1	2	22	2	27
HSIL		1		5	27	33
ASCUS	2	4	1			7
ASC-H			1	5	1	7
CaCx					3	3
						100

The results were analysed in Microsoft excel software, using the student t test.

P values were obtained for both Ki67 and PCNA stained cases.

In both the groups first NILM results were compared with ASCUS cases,then ASCUS was compared with LSIL,then LSIL was compared with ASC-H,ASC-H was compared with HSIL,lastly HSIL was compared with carcinoma cervix.

Table 3. RESULTS OF Ki67 GROUP

NILM vs ASCUS	P VALUE-0.002672
ASCUS vs LSIL	0.00004027
LSIL vs ASC-H	0.77922
ASC-H vs HSIL	0.0084509
HSIL vs CaCx	0.0001642

Table 4. RESULTS OF PCNA GROUP

NILM vs ASCUS	P VALUE-0.008326
ASCUS vs LSIL	0.000000001615
LSIL vs ASC-H	0.457496
ASC-H vs HSIL	0.00242
HSIL vs CaCx	0.001354

The p values were significant in both groups (Ki67 and PCNA) for all squamous abnormalities, except for LSIL vs ASC-H in both the groups.

ILLUSTRATING PHOTOGRAPHS

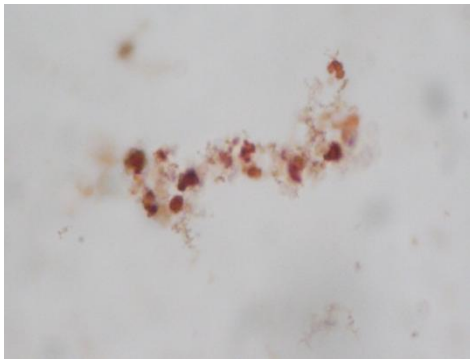


Figure 1.photomicrograph showing Ki67 index<10%, in a case of ASCUS(stained by monoclonal antibody against Ki67,x100)

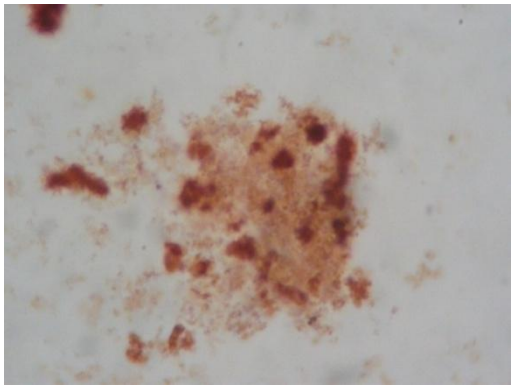


Figure 2.photomicrograph showing Ki67index10-20%in a case of LSIL, (stained by monoclonal antibody againstKi67x400)

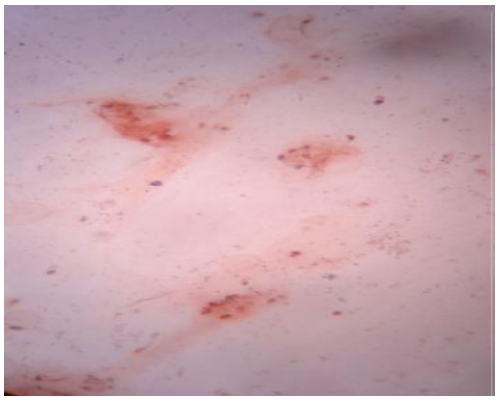


Figure 3.photomicrograph showing PCNA index <10%, in a case of ASCUS(stained by monoclonal antibody against PCNA,X100)

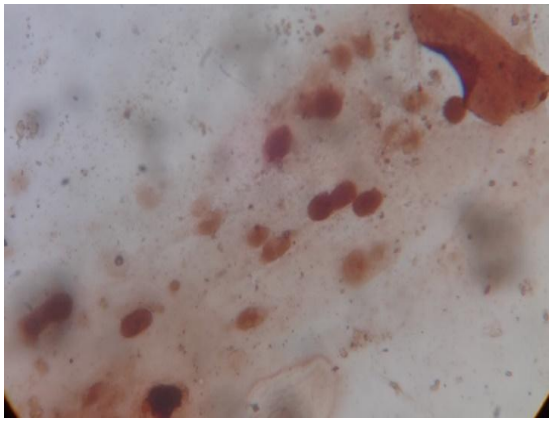


Figure 4.photomicrograph showing PCNA index,>20% in a case of HSIL,(stained by monoclonal antibody against PCNA,X400)

DISCUSSION

Cervical intraepithelial neoplasia (CIN) is a premalignant lesion characterized by cellular proliferation, abnormal maturation, and nucleo-cytoplasmic atypia. Cytological counterpart of these premalignant lesions is designed as lowgrade squamous intraepithelial lesions (LSIL), and highgrade squamous intraepithelial lesions (HSIL) as well as atypical squamous metaplactic lesions. All of them have capacity to regress to normal or progress to invasive cancer. Florid koilocytotic change and immature metaplastic squamous epithelium, nuclear atypia, basal cell hyperplasia, inflammatory changes, are all contribute to the diagnostic difficulty and poor reproducibility. Reactive/reparative epithelial changes and atrophy related changes are also well-recognized mimicar of HSIL. Moreover, morphological criteria existed at present do not predict about the further of progression these lesions. Here lies the utility of study of the proliferative activity of dysplastic and metaplastic cervical epithelial cells.

Out of the total of 1006 cervical smears taken during the period of September 2016 to September 2018 in our study, maximum clustering was found at age group of 25 to 35,with nearly equal distribution at age group 26-30 and 31-35 years respectively.[Bar diagram 1] Most of the data from Indian tertiary hospitals showed similar findings, since there were no concrete universal screening programme in India unlike the western countries, women below age of 25 were less forthcoming to approach the screening centers. [3,4,9,10]

0.65 % each of ASCUS and ASC-H, 2.6% OF LSIL and 3.1% of HSIL and three cases of carcinoma cervix were found out of the total1006 cases. [Bar diagram 2]

The expectations of biomarker identification in cervical smear were to distinguish squamous intraepithelial lesion (SIL) from non SIL.The prototype of similar study was done by GoelMM, Mehrotra et al [14] as afore said. RG Steinbeck, et al showed increasing histopathological atypia in cervical mucosa was correlated to an increase of PCNA followed by distinct aneuploidy and p53 overexpression. [15] However, we do not go for p53 immunostain in our study but PCNA immunostaining findings showed increased staining. Steinbeck R G et al, in their study ,studied proliferating cell nuclear antigen(PCNA), nuclear DNA content and mutant p53 overexpression in normal mucosa(n=10), in mild(n=16),moderate (n=9) and severe(n=17) atypical lesions as well as in squamous cell carcinoma(n=36) of the cervix uteri. The results showed that increasing histopathological atypia in cervical mucosa was correlated to an increase of PCNA followed by distinct aneuploidy and p53 overexpression. They contributed to a better understanding of the genesis of cervical carcinoma. [15]

NILM cases showed that 20 cases of Ki67 staining showed less than 4% staining, and 21 out of 23 cases of PCNA staining showed less than 4% staining characteristics. (Table 1 and 2) ASCUS showed positivity out of 7 cases in each Ki67 and PCNA group ,5 cases in 5-10% of Ki67 staining and 4 case in 5-10% in PCNA group (table1 and 2 and also to photomicrograph 1 and 3). Therefore a clustering was noted in 5-10% group for ASCUS. LSIL immunostaining pattern was found to have maximum clustering in 15-20% group in both the stains. (table1 and 2 and photomicrograph 2). The p values were

significant in both group s(Ki67 and PCNA) for all squamous abnormalities ,except for LSIL vs ASC-H in both the groups. . (Table3 and 4) .Surprisingly ASCUS and NILM showed statistically significant difference, both had <10% staining in both Ki67 and PCNA staining.

Mack T Ruffin, et al Surrogate endpoint markers for cervical cancer chemopreventive trial suggested that three types of markers might be necessary to describe malignant growth kinetics ; one measuring growth fraction(Ki67) and the second one evaluates cell cycle speed and third assesses S phase cell occurrence frequency(PCNA). [16]

Ki-67 are related to DNA replication and its positivity directly reflect active DNA replication. [5, 8, 17] Ki-67 immunopositivity could increase reproducibility and specificity of diagnosis. [18] However, Ki-67 alone cannot differentiate between dysplasia and immature squamous metaplasia as suggested by Hebbar A et al. The sensitivity and specificity with Ki-67 staining were found 90.5% and 87.5%, respectively. [19] Ikenberg *et al*. and Roelens *et al*. have studied the dual-staining of Pap cytology smears with p16/Ki-67 and found superior sensitivity over Pap cytology in detecting dysplasia suggesting a role in screening and triaging the ASCUS and LSIL cases on cervical cytology. [20,21] Michelle Follen Mitchell, et al [22] found PCNA superior to Ki67. We too got higher labeling index of PCNA compared to Ki67. I Busmanis, et al corroborated relationship between increased quantitative expression of Ki67 and tumour size in SCC in micro invasive and early stage disease. [23] Maeda MY, et al studied relevance of rates of PCNA, Ki67, p53 expression in cervical lesions. The study involved quantification of each marker in basal, intermediate and superficial cells. Ki67, PCNA positive cells were found to be increased in number with increased grades of cervical lesions. [21] Again these findings were in coherence with our findings**.** [24]

The use of immunocytochemistry with specific biomarkers of cell proliferation in conjunction with conventional Pap smear study could greatly improve the accuracy, precision, and sensitivity of cervical cancer screening programs. In a nutshell, we concluded that ki-67 and PCNA immunostaining might be of great useful in those cases, which were reported as low grade lesion but had a high proliferative index. This will place the lesions in higher grade, thus indicating the utility of proliferative markers in cervical ctology. So, a case of ACUS with high proliferative index should be kept for follow-up studies. These markers might be helpful particularly in developing countries with high disease burden like India where liquid based cytology and HPV DNA testing is still not included in routine cervical screening programme. The higher accuracy and reproducibility of the immunocytochemistry suggest the possibility of a more standardised and reproducible method of screening, producing a more accurate implementation of CIN-based management strategies.

CONCLUSION

Use of proliferative markers may be of greater importance in premalignant cervical lesions showing high proliferative index. This method is simple, cheap and cost effective and may be useful in resource limited developing countries where liquid based cytology and HPV DNA testing is still not included in routine cervical screening programme Although we do not included in our study ,cervical glandular lesions should also be addressed with proliferative marker status in future study. Proliferative biomarkers like ki-67 and PCNA could be used to stratify borderline and premalignant cervical squamous epithelial lesions, for assessment of risk of further progression, and of course to monitor treatment response.

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