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ORIGINAL ARTICLE:

Immunohistochemical Evaluation of Preneoplastic and Neoplastic Lesions of Cervix using p16INK4A and MIB-1

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Abstract:

Introduction: Cervical cancer is fourth most common cancer in women and account for about 5% of cancer deaths in females' worldwide. In India, cervical cancer ranks the first most frequent cancer among females between 15 and 44 years of age. About 99% cervical cancersare linked with Human papilloma virus (HPV).

Aim: To evaluate the immunohistochemical expression of p16/INK4A and MIB-1 in preneoplastic and neoplastic lesions of cervix.

Material and Methods:

The cross sectional study was conducted on 63 cases in department of pathology of our institute over a period of two year from Dec 2018 to Dec 2020. The specimens were processed and three sections of 2-3 micron thickness were made and one stained with H&E stain, examined and 63 cases were selected. Other two sections were used for immunohistochemical staining

Result: In the present study, out of 63 cases, maximum 40 (63.49%) cases were observed in 31-50 years age group. In this study on P16INK4a immunostaining, maximum cases (8/15) of CIN-I and (9/15) of CIN-II histology were found grade-2 and grade-3 positivity respectively. Maximum (2/3) cases of CIN-II and all cases of squamous cell carcinoma histology showed grade-3 positivity. In this study onMIB-1 (Ki67) immunostaining, Maximum (12/15) cases of CIN-I were found grade-1 positive. All cases of CIN-II, III and carcinoma cervixes were positive.

Conclusion: We concluded that p16 INK4A and MIB-1 over expression was observed in CIN I/LSIL, CIN II/HSIL, CIN III/HSIL. Significant up regulation of P16 INK4A and MIB-1 was observed in carcinoma cervix.

Key words: CIN, Ki67, SCC, Molecular Immunology Borstel

Introduction:

Cancer of Cervix is fourth most common cancer in women and one of the top three cancers occurring in women less than 45 year age worldwide, with about 570000 new cases and 311000

deaths occur in 2018 worldwide.[1] Current estimates indicate that 97000 women are diagnosed with cervical cancer and 60000 die from this disease in India alone in 2018.[2]Almost all cervical squamous cell cancer cases are linked with Human papilloma virus (HPV).[1]P16/INK4A is a tumor-suppressor protein controlling the G1 checkpoint, and that genetic and epigenetic abnormalities in genes can lead to both escape from senescence and cancer formation. The p16/INK4A locus is deleted in a wide spectrum of tumors including melanoma, pancreatic adenocarcinoma, glioblastoma, certain leukemias, non-small cell lung cancer, cervical cancer, and bladder carcinoma.[3]MIB-1 (Molecular Immunology Borstel) is a proliferative marker also known as Ki-67. The expression of Ki-67 antigen is limited to cells in phase G1, S and G2 with the highest levels, present in M phase. Ki-67 is more likely to be expressed in aneuploid tumors compared to diploid tumors, and it is associated with a high mitotic count and high histology grade.[4]

Material & Method:

The cross sectional study was conducted on 63 cases selected from the cases received in pathology department of our Medical College in North India over a period of two year from Dec 2018to Dec 2020. All case received in our department during the study period and fulfilling the inclusion criteria was included in study as sample size, in which cases CIN-1 (n=15), CIN-II (n=15), CIN-III (n=3), carcinoma cervix (n=15) were included in the study and 15 case with normal cervical epithelium (n=15) were included as a control. Inadequate biopsies were excluded from the study.

Methodology: All cervical biopsy and hysterectomy specimen received in histology laboratory were fixed in 10% formalin saline. The tissue was processed and the paraffin embedded block was made. Thin sections of 2-3 micron thickness were cut by microtome from paraffin blocks and stained with routine H and E staining and examined under the microscope. Other section was taken on polylysine coated slide for immunohistochemical staining.

Immunohistochemical Staining: Two to three micrometer thin sections were cut and place on slide. Section were deparaffinized and rehydrated through graded alcohol followed by distilled water. Antigen retrieval was carried out at 120^0C in citrate buffer (Ph=6.0) for 15 min. Endogenous peroxidase activity was then treated with blocking antibody provided in the kit. P16 immunostaining was performed using the mouse monoclonal antibody; MIB-1 immunostaining was performed using the mouse antihuman clone ki-67. The above-mentioned primary antibody were applied for 90 min followed by enzymeconjugate (streptavidin horse radish peroxidase) for 45 min. Colour development was accomplished with concentrated diaminobenzidine solution diluted to 50-times and sections were counterstained with 10% hematoxylin. A histological section of squamous cell carcinoma cervix was used as positive control.

Interpretation of immunostaining: After staining the slides, slides are viewed under ordinary light microscope, grading is done as follows:

For p16 immunopositivity consider when there will be diffuse strong nuclear & cytoplasmic staining, heterogeneous or focal moderate nuclear staining also consider positive. Week cytoplasmic staining considered negative. Grading perform for each case by the number of

positive cells in different epithelial clusters as Grade 0,1,2 and 3 based on the number of positive cells 0%, 1-10%, 10-50% and > 50% respectively.[5]

For MIB-1 Immunopositivity consider, if strong nuclear staining is seen as basal layer, normal finding. The slides first assess for basal staining (lower one-third versus suprabasal). Staining in the upper two-third of the epithelium consider positive. Labeling indices calculate for each case by evaluating the percentage of positive nuclei. The cases were divided into three groups I,II and III according to 0-10%, 10-20% and > 20% positive nuclei respectively.[6]

Results:

The present study was carried out in department of pathology to evaluate the preneoplastic and neoplastic lesions of cervix using p16/INK4A and MIB-1 by immunohistochemistry.

The present study was conducted on 63 cases of cervical lesion;15 (23.80%) were of normal histology [Figure 1a], 15 (23.80%) cases were of CIN-1 (mild dysplasia) [Figure 2a], 15 cases (23.80%) were of CIN-2 (moderate dysplasia) [Figure 3a], 03 (04.76%) cases were of CIN-3 (severe dysplasia) [Figure 4a] and 15 (23.80%) cases were squamous cell carcinoma of cervix [Figure 5a].

In this study, out of total 63 cases, 42 (66.67%) cases were cervical biopsies and 21 (33.33%) cases were hysterectomy specimen. Out of 63 cases, maximum 40 (63.49%) cases were observed in 31-50 years age group followed by 51-60 years age group 13 (20.63%) cases[Table-1]. Out of 63 cases, 48 cases (76.19%) belonged to rural areas, whereas 15 cases (23.81%) belonged to urban areas. Out of 63 cases, 55 (87.30%) cases belonged to Hindu community, whereas eight (12.70%) cases belonged to Muslim community, while no single case was found in other religion. The patients presented with complaint of discharge per vaginum, Irregular bleeding per vaginum, post-menopausal bleeding, low backache, post coital bleeding, urinary retention[Table-2].

P16INK4a immunostaining:In this study, all15 cases with normal histology were found negative [Figure 1b]. Out of 15 cases with CIN-I histology, maximum cases (eight cases) were grade-2 positive followed by grade-3 positivity in five cases and two cases with grade-1 positivity [Figure 2b]. Out of 15 cases of CIN-II histology, maximum cases (9 cases) were found grade-3 positive followed by grade-2 positivity in five cases and one case with grade-1 positivity [Figure 3b]. Out of 3 cases of CIN-III histology, maximum (two) cases were found grade-3 positive and one case showed grade-2 positivity [Figure 4b]. All 15 cases of squamous cell carcinoma histology showed grade-3 positivity [Figure 5b] [Table-3].

MIB-1 (Ki67) immunostaining: In this study, all 15 cases of normal cervical histology were negative [Figure 1c]. Out of 15 cases, maximum cases (12 cases) were found grade-1 positive followed by two cases showed grade-2 positivity[Figure 2c] and one case was negative. Out of 15 cases of CIN-II, all were positive, in which eight were grade-1 positive and seven were grade-2 positive [Figure 3c]. All three cases of CIN-III histology were showed grade-2 positivity [Figure 4c]. Out of 15 cases, all were positive, in which maximum cases (9 cases) were found grade-3 positive [Figure 5c] [Table-4].

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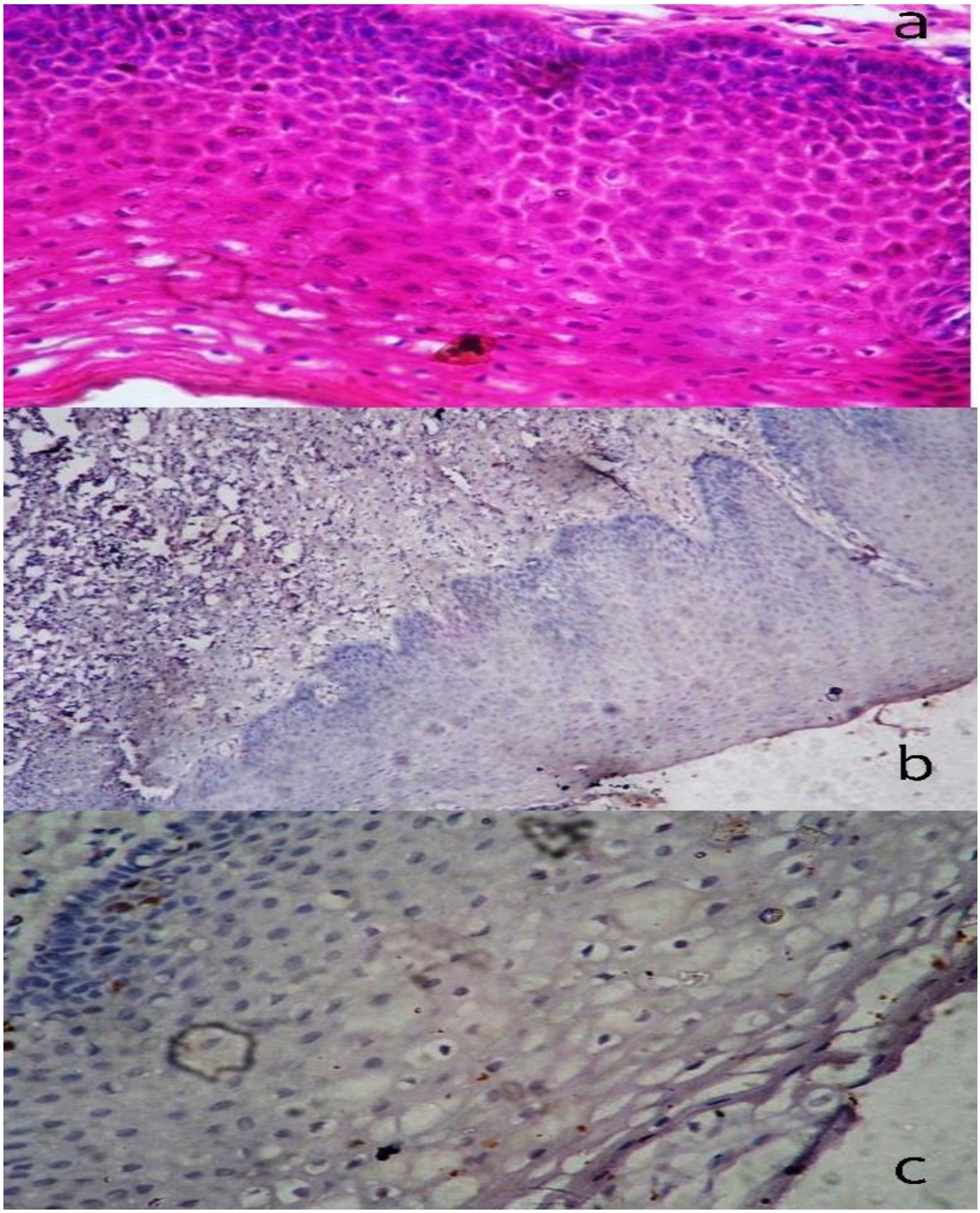

Fig-1: (a) Showing normal squamous epithelium of cervix (H&E, 40x), (b) Showing p16 negative immunostining in normal cervical epithelium (IHC, 40X), (c) Showing MIB-1 negative immunostining in normal cervical epithelium except mild normal positivity in basal layer (IHC, 40X).

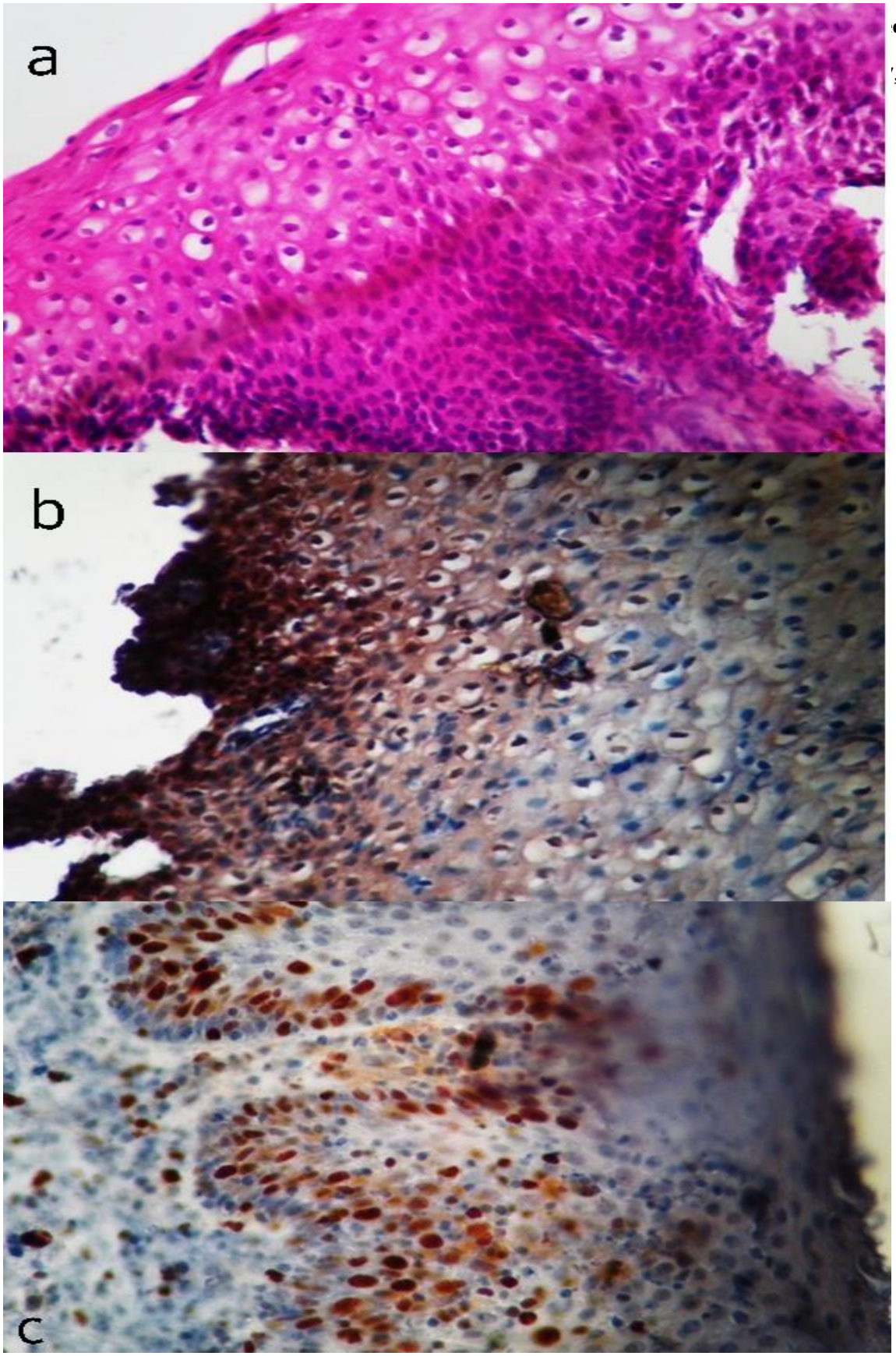

Fig-2: (a) Showing CIN-I histomorphological feature (H&E, 40X), (b) Showing nuclear and cytoplasmic p16 immunohistochemical staining in CIN-1 (IHC, 40X), (c) Showing nuclear MIB-1 immunohistochemical staining in CIN-I (IHC, 40X).

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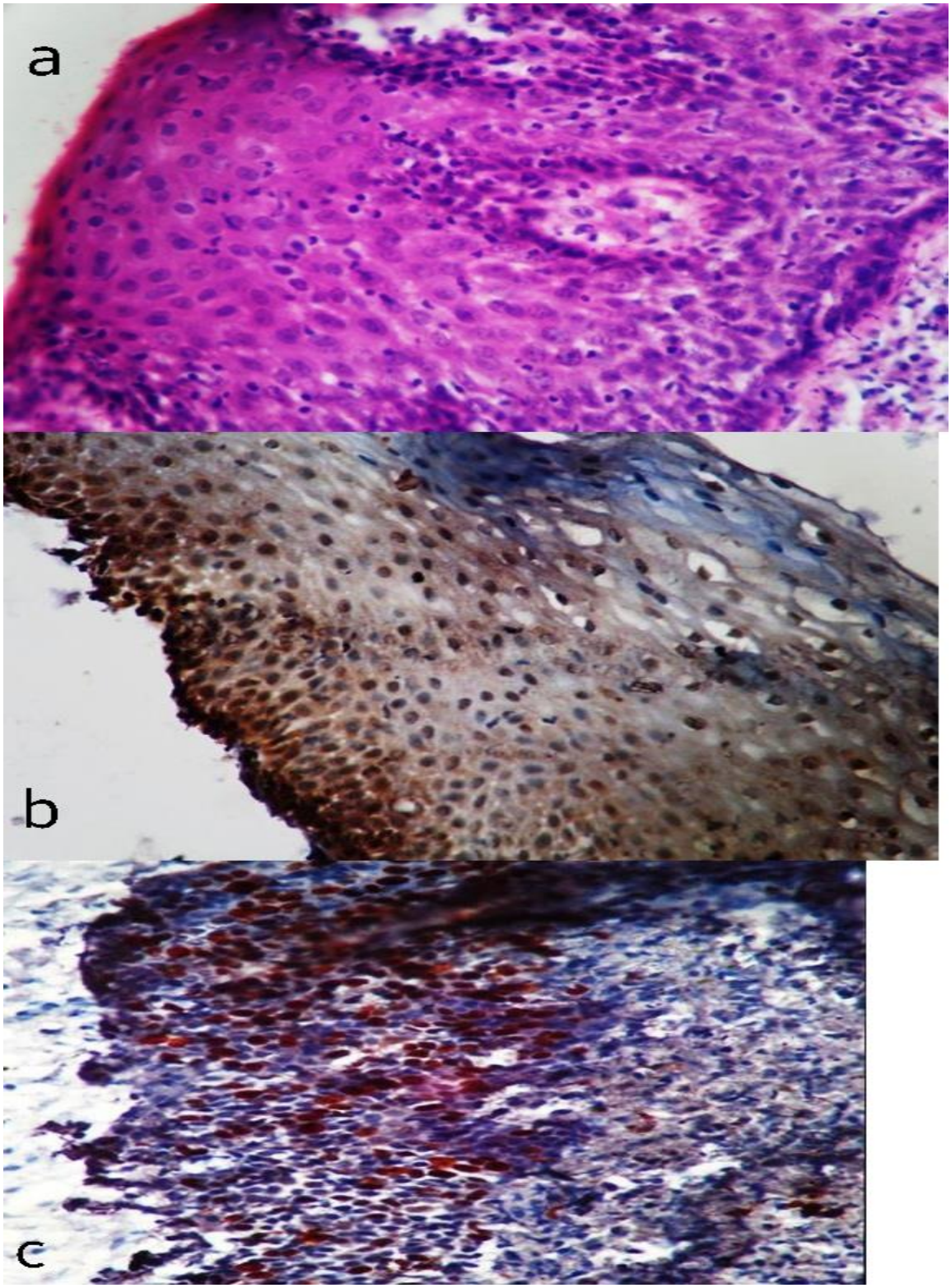

fig-3: (a) Showing CIN-II histomorphological feature (H&E, 40X), (b) Showing nuclear and cytoplasmic p16 immunohistochemical staining in CIN-II (IHC, 40X), (c) Showing nuclear MIB-1 immunohistochemical staining in CIN-II (IHC, 40X)

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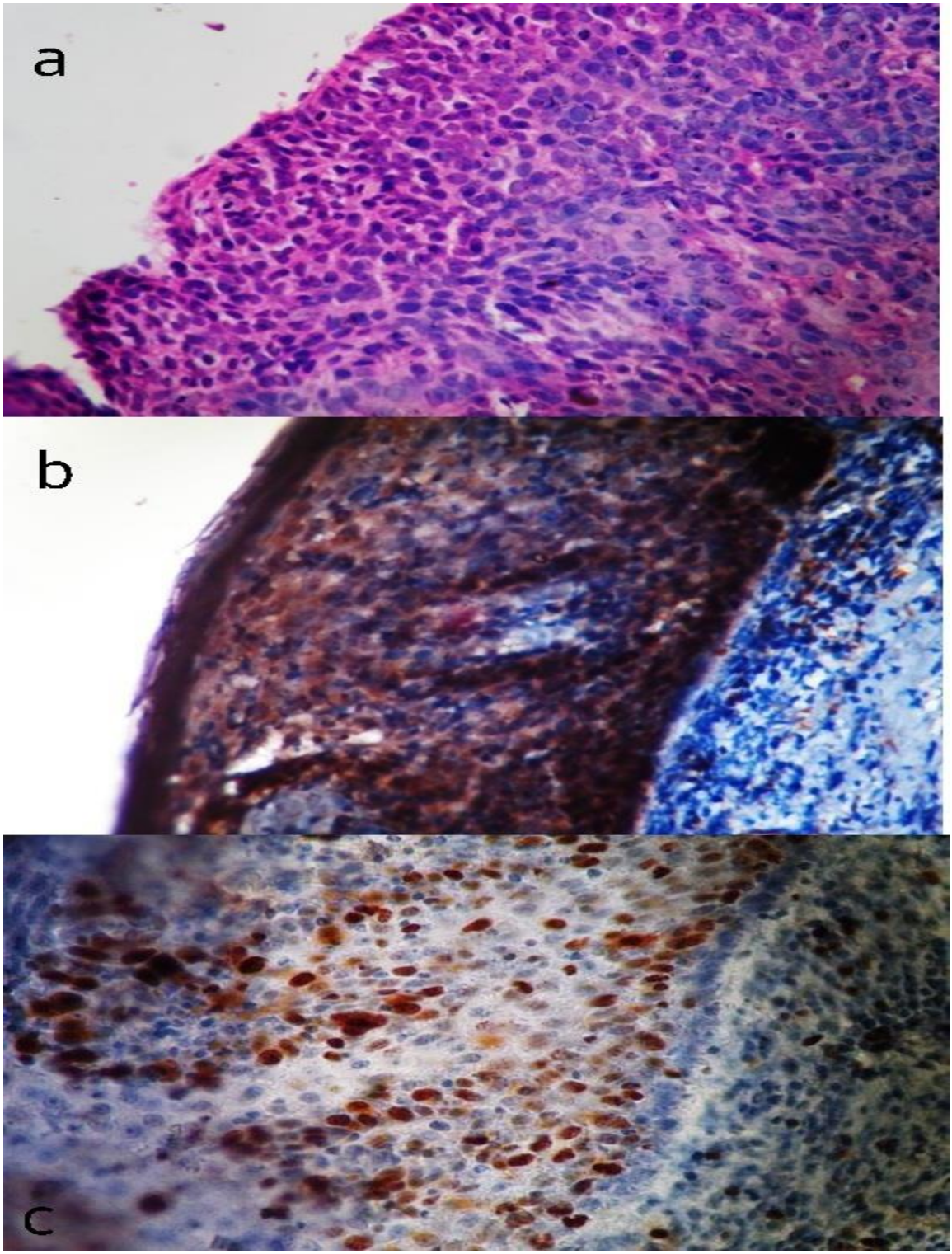

Fig-4: (a) Showing CIN-III histomorphological feature (H&E, 40X), (b) Showing nuclear and cytoplasmic p16 immunohistochemical staining in CIN-II (IHC, 40X), (c) Showing nuclear MIB-1 immunohistochemical staining in CIN-III (IHC, 40X)

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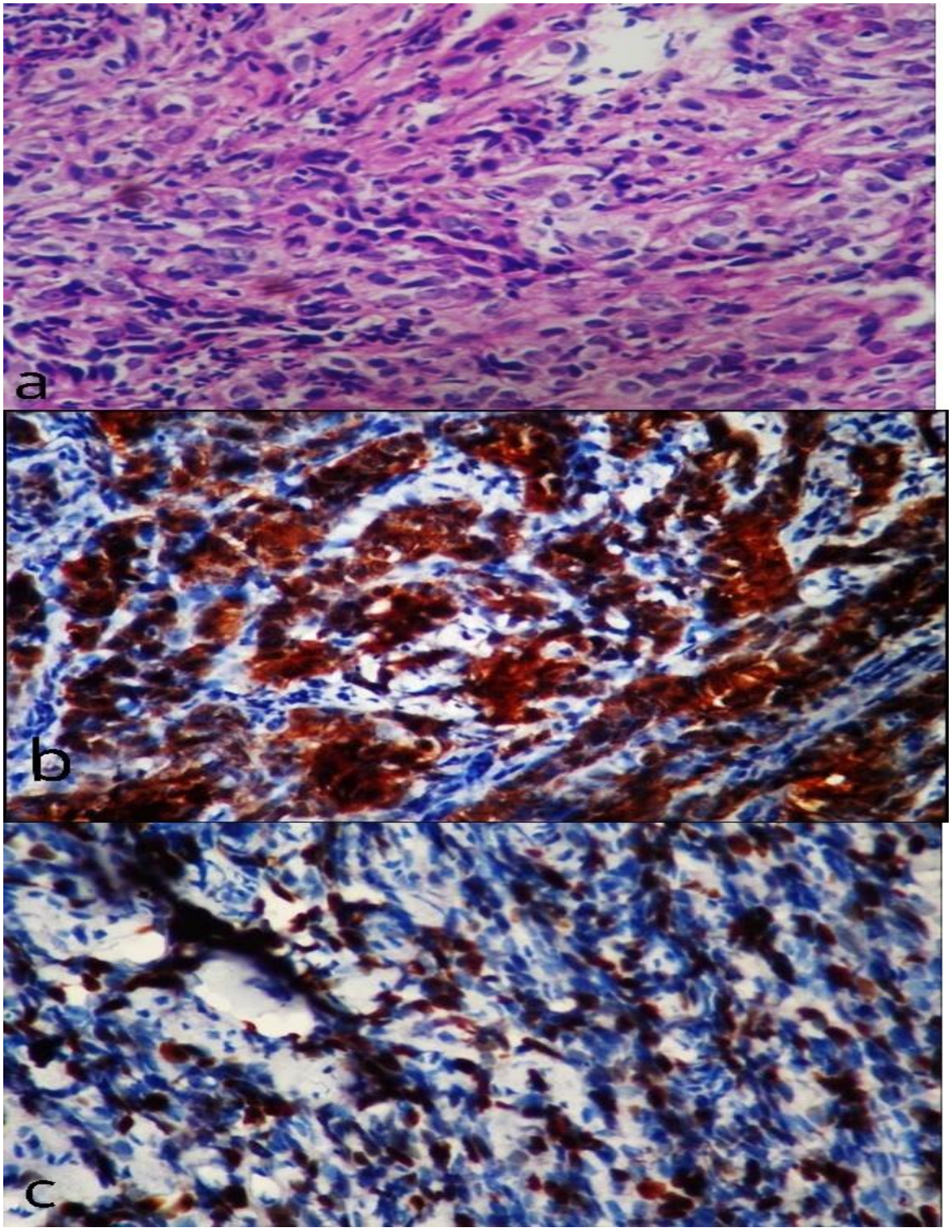

Fig-5: (a) Showing squamous cell carcinoma histomorphological feature (H&E, 40X), (b) Showing nuclear and cytoplasmic p16 immunohistochemical staining in squamous cell carcinoma (IHC, 40X), (c) Showing nuclear MIB-1 immunohistochemical staining in squamous cell carcinoma (IHC, 40X).

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Tables:

TABLE-1: ShowingAge wise distribution of cases studied

S. No.	Age group(in years)	No. of cases	Percentage
1	21-30	02	03.17
2	31-40	22	34.93
3	41-50	18	28.57
4	51-60	13	20.63
5	61 and above	08	12.70

TABLE-2: Showingdistribution presenting symptoms of patients

S. No.	Presenting symptom	No. of cases	Percentage
1	Discharge per vaginum	31	49.20
2	Irregular bleeding P/V	14	22.23
3	Post-menopausal bleeding	05	07.94
4	Low backache	06	09.52
5	Post coital bleeding	06	09.52
6	Urinary retention	01	01.59

Table 3: Results of Immunohistochemical Analysis of p16/INK4a

S. No.	Group	No. of Cases	Grade			
			0	1	2	3
1.	Normal	15	15	0	0	0
2.	CIN- I	15	0	2	8	5

3.	CIN-II	15	0	1	5	9
4.	CIN-III	3	0	0	1	2
5.	SCC	15	0	0	0	15

Table 4: Results of Immunohistochemical Analysis of MIB-I

S. No.	Group	No. of Cases	Grade			
			0	1	2	3
1.	Normal	15	15	0	0	0
2.	CIN-I	15	1	12	2	0
3.	CIN-II	15	0	8	7	0
4.	CIN-III	3	0	0	3	0
5.	Ca cervix	15	0	0	6	9

Discussion:

Histological interpretation of cervical biopsy/hysterectomy specimen was mainstay of histological evaluation of the human cervix. A wide array of potential biomarker has been evaluated for the diagnostic usefulness of cervical cancer and its precursors.

The early diagnosis of cervical cancer is central to papanicolaou (Pap) smear screening programmes and histologic interpretation of biopsy specimen by the pathologist, which has significantly reduced the mortality of cervical cancers, however, the Pap test is not very accurate due to subjective test criteria. HPV is known to be a major causative agent in cervical neoplasia and invasive cervical carcinoma.[1, 8] Many different HPV types associated with cervical neoplasia have been discovered and divided into high and low risk categories based on their

association with invasive cervical carcinoma.[9] Central role in carcinogenesis of cervix is of HPV, esp. types 16 and 18.Two markers that have showed a potential in this direction are p16/INK4A and MIB-1.[10]

P16, cyclin-dependent kinase (CDK) inhibitor, is the product of INK4A gene on 9 chromosome and bind specifically to CDK4/6 complex and control the cell cycle at G1-S transition ultimately results in inhibition of cell cycle progression. P16 inhibits the CDKs that phosphorylate the Rb protein and control the p-Rb mediated G1-S phase transition of the cell cycle.[11]Expression of E6 and E7 oncoproteins during the transformation phase in the life cycle of HPV virus causes the inhibition of p53 and Rb tumor suppressor gene respectively. The inactivation of pRb by E7 causes the p16/INK4A overexpression by negative feedback mechanism and ultimately p16 overproduction.[12, 13]

MIB-1 (Molecular Immunology Borstel) also known as Ki67 is a well-known cell proliferative marker, MIB-1 antibody detects Ki-67 antigen. Ki-67 is one of the most widely studied proliferating cell antigens. The expression of Ki-67 antigen is limited to cells in phase G1, S and G2 with the highest levels present in M phase and highlight the cells with active DNA replication.[10]Von Hoven *et al*[14]suggested it is a sensitive biological indicator of progression in CIN lesions.Therefore this antibody may be a useful marker of proliferation in dysplastic lesions.

The present study was therefore undertaken to find out the immunohistochemical staining of these two markers in cervical intraepithelial lesions to assess the use these markers as a diagnostic adjunct to diagnose preneoplastic and neoplastic lesions of cervix.

In present study, the maximum number of cases affected was found 4^{th} to 6^{th} decade of life. These finding was concordant with Devi*et al*[2], Adetona*et al*[13]. However, SHI*et al*[15]found peak incidence in the 3^{rd} and 4^{th} decade of life and Kalyani*et al*[7] found maximum number of cases between 5^{th} to 7^{th} decade.

In the present study the patients were mostly Hindus and only few were Muslim with a ratio of 7:1 found concordant with Wynder*et al*.[16]

In this study cases from rural areas predominated over urban population with a ratio of 3.2:1. This can be explained on the basis of the fact that maximum number of patients reported belonged to rural areas however Shah *et al*[17] found that the risk of development of cancer of the cervix varies with life style of an individual, social custom and geographical distribution.

As regards of the presenting symptoms discharge per vaginum has been the most common complaint followed by irregular bleeding per vaginum, which was found concordant with Devi *et al*[2], Kalyani *et al*[7]and Gray *et al*[18].

In this study all cases of carcinoma were found squamous cell carcinoma. The was found concordant with SHI*et al*[15],Lesnikova *et al*[19]and somewhat concordant with the findings of Devi *et al*[2], Vedula*et al*[12],Izadi-Mood *et al*[11],and Gray *et al*[18] as they reported that majority of invasive cervical carcinoma are squamous cell carcinoma. However, Kalyani *et al*[7] studied squamous cell carcinoma cases only.

P16 expression in cervical lesions:

In the present study, p16/INK4A immunohistochemistryrevealed that there was a significant over expression and upregulation in different groups from normal cervical epithelium to dysplasia of varying degree to carcinoma with increased p16 positivity. The result was found concordant with, Devi *et al*[2], Vedula *et al*[12], Shi*et al*[15], Adetona*et al*[13], Izadi-Mood *et al*[11], Lesnikova *et al*[19], Sano *et al*[20] and Klaes *et al*[21]. While Kalyani*et al*[7] studied only squamous cell carcinoma cases and found block positivity in 89.3% cases and ambiguous positivity in 6.6% cases. However, Klaes *et al*[21] also found detectable expression of p16 in normal cervical epithelium, inflammatory lesions and CIN I lesions associated with low risk of HPV types. P16 positivity were found in all (15/15, 100%) cases of invasive carcinoma cervix and positivity increased with increasing severity of CINs. Similar result was found by Murphy *et al*.[5]

In this study, we observed diffuse nuclear and cytoplasmic staining in all cases. P16 basically is a nuclear protein hence immunohistochemistry should show nuclear staining. However in dysplasia both nuclear and cytoplasmic staining with p16 is observed possibly because of post transcriptional modification or overproduction of p16 protein forcing its transfer into the cytoplasm.[5]

In this study, we found only cytoplasmic staining in two cases of CIN-I/ LSIL. Similar findings have been reported by Volgareva *et al*[22] in their study.

P16 is a negative regulator of normal proliferation, working through a negative feedback loop to down regulate CDK4. This function is bypassed by HPV-7 causing p16 up regulation in proliferating cells. The detection of elevated levels of p16 is a clear indicator of abnormal proliferation. It can thus optimize inter-observer agreement in the diagnosis of cervical intraepithelial neoplasm.

P16 may be rarely negative in cervical dyskaryosis that may have important implications for the use of p16 staining as a standalone test and support the use of combination of markers of cervical dyskaryosis.[5]However in our study we did not find any dysplasia negative for p16 in tissue biopsies.

In this study, p16 over-expression was found restricted to CIN I,II,III and carcinoma cervix. No positive staining was observed in the adjacent normal cervical epithelia. The p16/INK4A positivity increased in the following order CIN-I, CIN-II, CIN-III and carcinoma cervix. Therefore, p16 immunostaining allowed precise identification of even small CIN or cervical cancer lesions in biopsy sections and helped to reduce interobserver variation and also reduce false positive and false negative interpretation and thereby significantly improve cervical cancer and precancerous lesion detection.

MIB-I expression in cervical lesions:

In this study MIB-I positivity was found in all cases of CIN II (15/15, 100%), CIN III (3/3, 100%), carcinoma cervix (15/15, 100%) and degree of positivity increase from normal to carcinoma group via the varying degrees of CIN, labeling index of positively stained nuclei increased with the severity of CIN to carcinoma group. Similar results were found by, Devi*et*

al[2], Shi*et al*[15], Adetona*et al*[13] Goel *et al*[6]in review literature. Proliferative index was significantly increased in the carcinoma group in comparison with dysplasia.

MIB-I immunohistochemistry revealed that there was a significant over expression of MIB-1 in different groups and as we move from normal cervical epithelia to varying severity of CINs to carcinoma, the MIB-1 positivity increased. Therefore, this antibody may be a useful marker of proliferative activity of premalignant and malignant lesions of cervix.

Correlation between grades of p16 and MIB-1 among cervical neoplasia showed an increasing p16 expression with consistently increasing MIB-1 LI in the groups of increasing severity of cervical neoplasia.

Conclusion:

P16/ INK4A and MIB-1 immunohistochemistry was performed on 63 cases. We observed that p16/ INK4A and MIB-1 over expression were observed in CIN I/LSIL, CIN II/HSIL, CIN III/HSIL.Significant up regulation of p16/ INK4A and MIB-1 was observed in carcinoma cervix.With increasing severity of CINs/SILs, the p16/INK4A and MIB-1 expression increased progressively. No positive staining of p16 and MIB-1 was observed in normal cervical epithelium.Correlation between grades of p16 and that of MIB-1 among cervical neoplasia showed an increasing p16 expression with consistently increasing MIB-1 LI in the groups of increasing severity. To conclude p16/INK4A and MIB-1 expression showed a definite increasing pattern with increasing CIN grades and malignancy. They may be used as biomarkers, as an adjunct to definitively diagnosed preneoplastic and neoplastic lesions of cervix.

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