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Immunohistochemical Study of p16INK4A, MIB–1 and CK17 in Pre–neoplastic and Neoplastic Epithelial Lesions of Cervix

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Abstract

Background: Cervical intraepithelial neoplasia (CIN) II and CIN III have a high progression rate to invasive squamous cell carcinoma (SCC). Histopathological assessment is known to have intra and inter–observer diagnostic discrepancies even among two panels of pathologist. Subsequently, to improve on the accuracy of histopathological examination, various IHC biomarkers have been evaluated in the biopsy of cervix.

Aim: The present study was undertaken to evaluate the immunoexpression and interrelationship of p16INK4A, MIB–1 and CK17 in histopathologically diagnosed cases of CIN and invasive cervical carcinoma (ICC) which could aid in differentiating CIN and ICC from benign cervical lesions.

Materials and Methods: This study included 120 cases of cervical lesions; out of which 20 cases were each of negative for malignancy/dysplasia (NED), CIN I and CIN III, 10 cases of CIN II and 50 cases of ICC. A technique of manual tissue microarray was employed for the study of immunohistochemical markers such as p16INK4A, CK17 and MIB–1 in all cases. Results were subjected to statistical analysis.

Results: The difference in p16 immunoexpression between NED (0/20, 0%) and CIN+ICC (97/100, 97%) cases was statistically highly significant. ($p<0.01$) The sensitivity, specificity, positive and negative predictive value and diagnostic accuracy of p16 immunoexpression in comparison to histopathological diagnosis was 97%, 100%, 100%, 86.96% and 97.5% respectively. The overall agreement of p16 staining with histopathological diagnosis was 97.5% ($\kappa=0.9151$ i.e. very good)

The difference in MIB–1 immunoexpression between CIN–I (6/20, 30%) and CIN II+III (30/30, 100%), CIN (36/50,72%) and ICC (50/50, 100%) cases was statistically highly significant. ($p<0.01$) The difference in MIB–1 immunoexpression between NED (0/20, 0%) and CIN+IC (86/100, 86%) cases was statistically highly significant. ($p<0.01$) The sensitivity, specificity, positive and negative predictive value and diagnostic accuracy of MIB–1 immunoexpression in comparison to histopathological diagnosis was 86%, 100%, 100%, 58.82% and 88.33% respectively. The overall agreement of MIB–1 staining with H&E diagnosis was 88.33%. ($\kappa=0.6719$ i.e. good)

The difference in CK17 immunoexpression between CIN–I (11/20, 55%) and CIN–II+III (26/30, 86.67%) cases was statistically significant. ($p=0.030$) The difference in CK17 immunoexpression between CIN (37/50, 74%) and ICC (46/50, 92%) cases was statistically significant. ($p=0.033$) The difference in CK17 immunoexpression between NED (0/20, 0%) and CIN+ICC (83/100, 83%) cases was statistically highly significant. ($p<0.01$) The sensitivity, specificity, positive and negative predictive value and diagnostic accuracy of CK 17 immunoexpression in comparison to histopathological diagnosis was 82%, 100%, 100%, 52.63% and 85% respectively. The overall agreement of CK 17 staining with histopathological diagnosis was 85% ($\kappa=0.6029$ i.e. moderate)

The agreement between p16 and MIB–1 immunostaining was 89.16%. ($\kappa= 0.7$ i.e., good) The agreement between CK17 and MIB–1 immunostaining was 86.6%. ($\kappa= 0.683$ i.e., good) The agreement between p16 and CK17 immunostaining was 84.16%. ($\kappa= 0.5908$ i.e., moderate)

Conclusion: The findings of the present study indicate that the IHC report of p16, MIB–1 and CK–17 in CIN and ICC cases if included in each histopathology report could aid in accurate diagnosis which could facilitate in better patient management.

Keywords: p16, MIB–1, CK–17, cervical carcinoma, Ki67

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Introduction

Cervical cancer continues to be a major public health problem in developing countries of the world and ranked at fourth position among various cancers in women. It is the second most common cancer in India with the age–standardized rate of 22 per 100,000 females and mortality rate of 20.7%.[1]

Cervical carcinoma screening has been performed by the conventional pap smears, which has substantially reduced the incidence of cervical neoplasia, especially in developed countries.[2] However, a substantial number of cases of cervical cancer have still been missed due to subjective test criteria.[3]

The association of cervical cancer with high risk human papilloma virus (HR–HPV) infection such as HPV–16/18 has long been established. Several Indian studies performing HPV detection tests in cervical samples showed that about 5.0% of women in the general population are estimated to harbor cervical HPV–16/18 infection at a given time and 82.7% of invasive cervical cancers are attributed to HPV–16/18.[4] In a study by Catherine et al which included 27,172 married middle aged women from rural Maharashtra, HPV DNA status for HR HPV types was assessed using the second–generation hybrid–capture II assay, the prevalence of HPV infection was found to be 10.3%.[5] In our institute, selected women with abnormal pap smear and normal pap smear with suspicious unhealthy cervix after application of 5% acetic acid and/or of Lugol's iodine, underwent HPV DNA polymerase chain reaction (PCR) testing with subtyping. HPV prevalence was found to be 10% and prevalence of serotype 16 was 50% and that of serotype 18, 31 and 66 was 16.66% each.[6] Studies have also shown that HR HPV DNA presence is elevated in low grade cervical lesions indicating active viral multiplication in host cell.[1,7] They further document that anti–HPV protein expression is detectable in initial transient cervical lesions but not in advanced lesions such as invasive SCC where p16 is over–expressed.[7] Viral persistence is necessary to bring about a genomic integration of these viral genes to cause morphological phenotypic change in the squamous cell and subsequently development of high grade dysplasia in them.[8]

The protein p16 INK4A (synonym for p16) serves as a surrogate marker for the oncogenic activities of HPV in replication competent cells of cervical epithelia. p16 is a tumor suppressor protein and cyclin dependent kinase (CDK) 4 and 6 inhibitor. The phosphorylation of retinoblastoma protein (RBp) is a molecular "ON–OFF" switch for the cell cycle. In the hypophosphorylated form, RBp binds to transcription factors such as E2F responsible for cell cycle progression. p16 inhibits the CDK and thereby prevents the phosphorylation of RB, keeping it in the hypophosphorylated form, i.e. its active form. However, in HPV infection, the viral gene E7 binds and inactivates RBp which results in accumulation of E2F and increased p16 levels through negative feedback regulation. Because this protein is not expressed in the normal cervical epithelium, p16 overexpression allows to specifically identify dysplastic lesions and reduce interobserver disagreement occurring in cervical biopsy diagnosis.[7]

MIB–1 (Molecular Immunology Borstel), is a proliferative marker. In 1990, it was demonstrated that the MIB–1 antibody detects Ki–67 antigen in the G1, S, G2 and M phase, but it is absent in the G0 phase. Thus, few studies have highlighted the usefulness of MIB–1 in CIN grading.[3,7]

Cytokeratin (CK) 17 is a marker for endocervical reserve stem cells which gives rise to metaplasia and expression of CK17 that decreases and disappears as the metaplastic epithelium matures.[9] One study mentions CK17 as a good marker of malignant transformation, with increasing in its expression according to the severity of cervical lesions.[10] Thus, p16, MIB–1 and CK17 markers in tissue sections have the potential that can be utilized to aid in the histopathological diagnosis of pre–neoplastic and neoplastic lesions of the cervix.[9,10]

The present study was undertaken to evaluate the immunoexpression and interrelationship of p16INK4A, CK17 and MIB–1 in histopathologically diagnosed cases of CIN and invasive cervical carcinoma (ICC) which could aid in differentiating CIN and ICC from benign cervical lesions.

Materials and Methods

This cross–sectional study was conducted in a tertiary care hospital. Ethical clearance was obtained from institute's ethical committee. This study included 120 cases of cervical lesions; out of which 20 cases were each of non–specific chronic cervicitis (NED), CIN I and CIN III and 10 cases of CIN II and 50 cases of ICC. These cases were diagnosed and/or operated upon (biopsy and/or hysterectomy) during the period from August 2012 to June 2015. The available data such as age, menopausal status, presenting symptoms and type of specimen for all the patients were collected from the records of histopathology section of the department of pathology. Cases in which records/slides/blocks were not available were excluded from the study.

All routinely processed paraffin embedded tissue blocks and Hematoxylin and Eosin (H&E) stained slides of these cases were retrieved. The slides were reviewed

by two experienced histopathologists. The cases were classified according to histomorphological subtype and pathological staging was done as per guidelines of World Health Organization.[11] FIGO and TNM staging were done in cases received as surgical specimen.[11]

We selected the representative slide and paraffin block for immunohistochemistry (IHC) staining. A technique of tissue microarray array (TMA) was employed along with IHC staining of entire section in some cases.[12] The monoclonal antibodies to p16 (clone 5A8A4, Thermo Scientific), Ki–67 (clone MIB–1, Dako) and CK17 (clone E3, Dako) were used. Appropriate positive controls were taken for the IHC stains as per literature.[10] Negative control (without adding primary antibody) was included in all batches. Antigen retrieval was done using Citrate Buffer Antigen Retrieval Protocol. Pressure cooker was used as heating source.

The sections stained with monoclonal antibodies using IHC staining procedure were examined alongside H&E stained specimens, to identify the precise locations of the lesions. p16 immunoreactivity was evaluated taking into account the percentage positivity of tumor cells. Positivity was seen as a brown reaction product staining the nucleus or cytoplasm or both. Scoring of percentage positive tumor cells was carried out as follows: 0% staining as no score, 0–5% as 1+, >5–25% as 2+ and >25% as 3+. Less than or equal to 5% staining was considered negative and more than 5% staining was considered. Intensity of immunostaining was taken as 1+ (weak), 2+ (variable) and 3+ (strong). Intensity of p16 staining was graded into focal and diffuse depending upon the distribution of positive cells. IHC score was obtained as a product of percentage positive tumor cells (0–3) and staining intensity score (0–3) thus achieving a maximum of 9.[8] For MIB–1, immunopositivity was considered when there was strong nuclear staining. Because basal staining is a normal finding, the slides were first assessed for basal staining (lower one–third versus suprabasal). Staining in the upper two–thirds of the epithelium was considered positive. The MIB–1 Labeling index (MLI) is defined as the percentage of MIB–1 positive cells in a total of 1,000 dysplastic cells counted. MLI was calculated for each case by evaluating the percentage positive nuclei. MLI of >10% was considered positive.[9,13]

CK17 immunostaining was considered positive when cytoplasmic staining involved all squamous cell layers. Focal staining or completely unstained cell layers was considered as negative. In case positive, whether the positivity was focal or diffuse was also mentioned.[9]

The Primer of Biostatistics 7.0 program was used for statistical analysis. Quantitative data was presented with the help of mean. Qualitative data was presented with the help of frequency and percentage table. Sensitivity, specificity, predictive values and accuracy of biomarkers (p16, CK17 and MIB–1) were calculated. The results were considered to be statistically significant when the p value was < 0.05 and highly statistically significant when p value was < 0.01.

Results

A total of 120 cases were included in the present study. Out of which, 91 cases were received as cervical biopsies (79 punch and 12 cone amputated) and 29 cases were received as surgical specimens (trans abdominal hysterectomy). Out of 120 cases, squamous cell carcinoma(SCC) were maximum (n=49), of which 21 cases were of large cell keratinizing subtype(LCKSCC), 22 cases were of large cell nonkeratinizing subtype (LCNKSCC), 5 cases were of papillary squamo–transitional subtype (PSTC) and one of micro–invasive subtype. There was one case of small cell carcinoma. All cases of SCC and one case of small cell carcinoma are considered together as invasive cervical carcinoma (ICC) in further discussion.

The youngest and oldest patient of CIN was 25 and 82 years of age respectively with a mean age of 48.32±13.87years. The youngest and oldest patient of ICC was 30 and 80 years of age respectively with a mean age of 55.82±11.87years. Out of the 120 cases studied, 84(70%) of them were postmenopausal. The most common presenting symptom in cases of CIN and ICC was excessive vaginal bleeding (18/50, 36%) followed by postmenopausal bleeding (16/50, 32%). Clinical staging was available in 39 cases. As most of the specimen received were biopsies, pathological staging was not possible in them. In 10 biopsy cases, the stages were obtained from the record. 48.7% (19/39) of the cases presented at an advanced stage of the disease (IIIB).

Percentage of p16 positive cells in CIN I was 1+ in 10% (n=2) of cases, while it was 2+ in 90% (n=18) cases. All 10 (100%) cases of CIN II were of 3+. In CIN III, 18 (90%) cases showed 3+, while one (5%) case each showed 2+ and no staining. All 50 (100%) cases of ICC showed 3+ staining. Out of 20, 95% (19) of NED cases were negative and only 1 case (5%) showed 1+ positivity. Considering >5% cut of value for p16 expression, p16 showed 0%, 90% (18/20), 96.67% (29/30) and 100% (50/50) immunopositivity in NED, CIN I, II+III and ICC cases respectively. Thus we can conclude that the percentage of p16 positivity of cells statistically significantly increased from CIN I to CIN II+III to ICC. (χ^2= 102.143; $p<0.01$) The mean percentage of p16 positive cells statistically significantly increased with increasing severity of the lesion i.e. from NED to CIN I to CIN III to ICC. (F=227.02 (ANOVA); $p<0.01$) (Table 1)

In CIN–I, 6 cases each showed 1+ and 2+ while 8 cases showed 3+ staining intensity. In CIN II, only 1 case showed 1+, 4 cases showed 2+ while 5 cases showed 3+ staining intensity. In CIN III, one case each showed 0 and 1+, 11 cases showed 2+, while 7 cases showed 3+ intensity. In IC, 3 cases showed 1+, 15 cases 2+ while 32 cases showed 3+ intensity. CNSS cases showed almost nil (5%) intensity. Thus, we can conclude that maximum cases of CIN–I, II and ICC showed 3+ and that of CIN III showed +2 intensity. This was statistically highly significant. (χ^2=122.96; p<0.01)

In CIN I and CIN II, number of cases showing focal positivity were 6/20 (30%) and 2/10 (20%) respectively, while those with diffuse positivity were 14/20 (70%) and 8/10 (80%) respectively. In CIN–III and IC, all cases (100%) showed diffuse positivity. Thus, the number of cases showing diffuse p16 positivity was more than those showing focal positivity in all CIN and IC cases. With increasing severity of lesion from CIN I to III to ICC, percentage of cases showing diffuse p16 positivity statistically significantly increased. (χ^2= 20.915; p<0.01) Percentage of cases showing p16 IHC Score was found to increase from 0 to 9 in CIN I, II and ICC cases and that of CIN III showed a maximum score of 6. Maximum p16 score of 9 was seen in 64% of ICC cases. (Table 2) (Figure 1a, d)

The difference in p16 immunoexpression between NED (0/20, 0%) and CIN+ICC (97/100, 97%) cases was statistically highly significant. (χ^2=95.053; p<0.01) The difference in p16 immunoexpression between CIN–I (18/20, 90%) and CIN II+III (29/30, 97.5%) cases was statistically insignificant. (χ^2=0.133; p=0.715) The difference in p16 immunoexpression between CIN (47/50, 95%) and ICC (50/50, 100%) cases was statistically insignificant. (χ^2= 1.901; p=0.168)

4/29 cases belonging to stage I, 2 each showed p16 score of 6 and 9. 9/29 cases belonging to stage II, 2 cases showed p16 score of 2 and 7 cases showed score of 9. 16/29 cases belonging to stage III, 3, 4 and 9 cases showed p16 score of 3, 6 and 9 p16 score respectively. No cases belonged to stage IV in the present study. There was no association between number of cases showing p16 immunoexpression in ICC with stage of the disease. (χ^2=3.838; p=0.428)

The sensitivity, specificity, positive and negative predictive value and diagnostic accuracy of p16 immunoexpression in comparison to histopathological diagnosis was 97%, 100%, 100%, 86.96% and 97.5% respectively. The overall agreement of p16 staining with histopathological diagnosis was 97.5% (κ=0.9151 i.e. very good)

MLI was 0% in all NED cases. CIN I, II, III and ICC showed MLI of 30% (6/20) and 100% each respectively. Thus, with increasing severity of lesions from CIN I to III to ICC percentage of cases showing MIB–1 positivity statistically significantly increased. (χ^2=99.316; p<0.01) The mean MLI statistically significantly increased with increasing severity of the lesions. (F=227.02 (ANOVA); p<0.01) (Table 3) (Figure 1c, e)

The difference in MIB–1 immunoexpression between CIN–I (6/20, 30%) and CIN II+III (30/30, 100%) cases was statistically highly significant. (χ^2=59.432; p<0.01) The difference in MIB–1 immunoexpression between CIN (36/50, 72%) and ICC (50/50, 100%) cases was statistically highly significant. (χ^2=14.037; p<0.01) The difference in MIB–1 immunoexpression between NED (0/20, 0%) and CIN+IC (86/100, 86%) cases was statistically highly significant. (χ^2=56.544; p<0.01)

The sensitivity, specificity, positive and negative predictive value and diagnostic accuracy of MIB–1 immunoexpression in comparison to histopathological diagnosis was 86%, 100%, 100%, 58.82% and 88.33% respectively. The overall agreement of MIB–1 staining with H&E diagnosis was 88.33%. (κ=0.6719 i.e. good)

CK17 was positive in 55% (11/20), 80% (8/10), 90% (18/20) and 92% (46/50) cases of CIN I, II, III and ICC respectively. All NED showed 100% (202/20) CK17

Histopathological Diagnosis	No. of cases (120)	p16 immunoexpression	
		Mean ± S.D.	Range
NED	20	0.05 ± 0.22	0–1
CIN–I	20	8.25±1.83	5–12
CIN–II	10	40.7±16.74	27–80
CIN–III	20	43.45±17.38	0–70
ICC	50	82.88±0.22	50–98
CIN=cervical intraepithelial neoplasia, ICC=invasive cervical carcinoma			

Table 1: p16 IHC positivity in NED, CIN and ICC cases

p16 score	Score=0	Score=1	Score=2	Score=3	Score=4	Score=6	Score=9
CIN–I (n=20)	0	1(5%)	5(5%)	1(5%)	6(30%)	7(35%)	0
CIN–II (n=10)	0	0	0	1(10%)	0	4(40%)	5(50%)
CIN–III (n=20)	0	1(5%)	1(5%)	0	0	11(55%)	7(35%)
ICC (n=50)	0	0	0	3(6%)	0	15(30%)	32(64%)

CIN=cervical intraepithelial neoplasia, ICC=invasive cervical carcinoma

Table 2: p16 IHC score in normal, CIN and ICC cases

Histopathological Diagnosis	No. of cases (n=120)	MIB–1 Labelling index	
		Mean ± S.D.	Range
NED	20	1.7±0.73	1–3
CIN–I	20	8.95±4.09	3–17
CIN–II	10	15.4±2.98	12–20
CIN–III	20	31.95±6.54	19–45
ICC	50	51.04±15.78	20–85

CIN=cervical intraepithelial neoplasia, ICC=invasive cervical carcinoma

Table 3: MIB–1 expression in NED, CIN and ICC cases

P16 expression	MIB–1 expression	
	positive	negative
positive	85	12
negative	1	22

Table 4: Co–relation between p16 and MIB–1 IHC marker

P16 expression	CK17 expression	
	Positive	negative
positive	80	17
negative	2	21

Table 5: Co–relation between p16 and CK17 IHC marker

CK17 expression	MIB–1 expression	
	Positive	negative
positive	76	6
negative	10	28

Table 6: Co–relation between CK17 and MIB–1 IHC marker

negativity. Thus percentage of cases showing CK17 immunoexpression statistically significantly increased with increasing severity of the lesion from CIN I to CIN III to ICC. ($X^2 = 63.59$; $p < 0.01$) (Figure 1b, f)

The difference in CK17 immunoexpression between CIN–I (11/20, 55%) and CIN–II+III (26/30, 86.67%) cases was statistically significant. ($\chi^2=4.717$; $p=0.030$) The difference in CK17 immunoexpression between CIN (37/50, 74%) and ICC (46/50, 92%) cases was statistically significant. ($\chi^2= 4.536$; $p=0.033$) The difference in CK17 immunoexpression between NED (0/20, 0%) and CIN+ICC (83/100, 83%) cases was statistically highly significant. ($\chi^2=50.016$; $p<0.01$)

In CIN I and CIN II, number of cases showing CK 17 focal positivity was 9/11 (82%) and 5/8 (62.5%) respectively which was more than those showing diffuse positivity. While, in CIN III and IC, number of cases showing diffuse positivity was 13/18 (72.2%) and 37/46 (80.4%) which was more than those showing focal positivity. Thus, with increasing severity of lesions from CIN I to III to ICC percentage of cases showing diffuse CK17 positivity statistically significantly increased. ($\chi^2 = 19.96$; $p<0.01$) 4/5 (80%) cases of stage I, 11/13 (84.6%) cases of Stage II and all 18/18 (100%) cases of Stage III showed positive CK17 immunostaining. Thus, with increasing stage, number of cases showing CK17 positivity also increased. No case of ICC belonged to Stage IV in present study. However, this was not statistically significant. ($\chi^2 = 3.373$; $p=0.185$)

The sensitivity, specificity, positive and negative predictive value and diagnostic accuracy of CK 17 immunoexpression in comparison to histopathological diagnosis was 82%, 100%, 100%, 52.63% and 85% respectively. The overall agreement of CK 17 staining with histopathological diagnosis was 85% ($\kappa=0.6029$ i.e. moderate)

The agreement between p16 and MIB–1 immunostaining was 89.16%. ($\kappa= 0.7$ i.e. good) (Table 4) The agreement between p16 and CK17 immunostaining was 84.16%. ($\kappa= 0.5908$ i.e. moderate) (Table 5) The agreement between CK17 and MIB–1 immunostaining was 86.6%. ($\kappa= 0.683$ i.e. good) (Table 6)

Authors	Clone	p16 Expression				
		Normal (%)	CIN I (%)	CIN II (%)	CIN III (%)	ICC (%)
Klaes R et al.[14] 2001	DCS-50.1/H4-NA29, 375P, ZJ11, JC8, G175-405	ND	15/17 (88)	10/10 (100)	43/43 (100)	–
Keating JT et. al.[15] 2001	NM	3/24 (12.5)	21/24 (87.5)	34/37 (91.8)	–	ND
Agoff SN et al.[16] 2003	E6H4	19/133 (11)	6/7 (86)	–	9/11 (82)	32/44 (72)
Gupta R et. al.[8]	6H12	2/20 (10)	10/20 (50)	12/20 (60)	14/20 (70)	19/20 (95)
Lesnikova I et al.[17] 2009	JC8	ND	180/249 (72.3)	212/233 (91)	178/181 (98.3)	131/133 (98.5)
Izadi–Mood Net al.[18] 2012	F12	ND	9/11 (82)	–	10/11 (91)	18/20 (90)
Sari Aslani et al.[18]2013	N1633	12	2/4 (50)	5/5 (100)	14/14 (100)	–
Hebbar A et. al.[19] 2017	G175-405	ND	5/10 (50)	7/10 (70)	9/10 (90)	6/6 (100)
Present study, 2017	5A8A4	0	18/20 (90)	10/10 (100)	19/20 (90)	50/50 (100)

NED= nonspecific chronic cervicitis, CIN=cervical intraepithelial neoplasia, ICC=invasive cervical carcinoma, ND=not done, NM=not mentioned

Table 7: Summary of Recent studies on p16 expression in NED, CIN and ICC

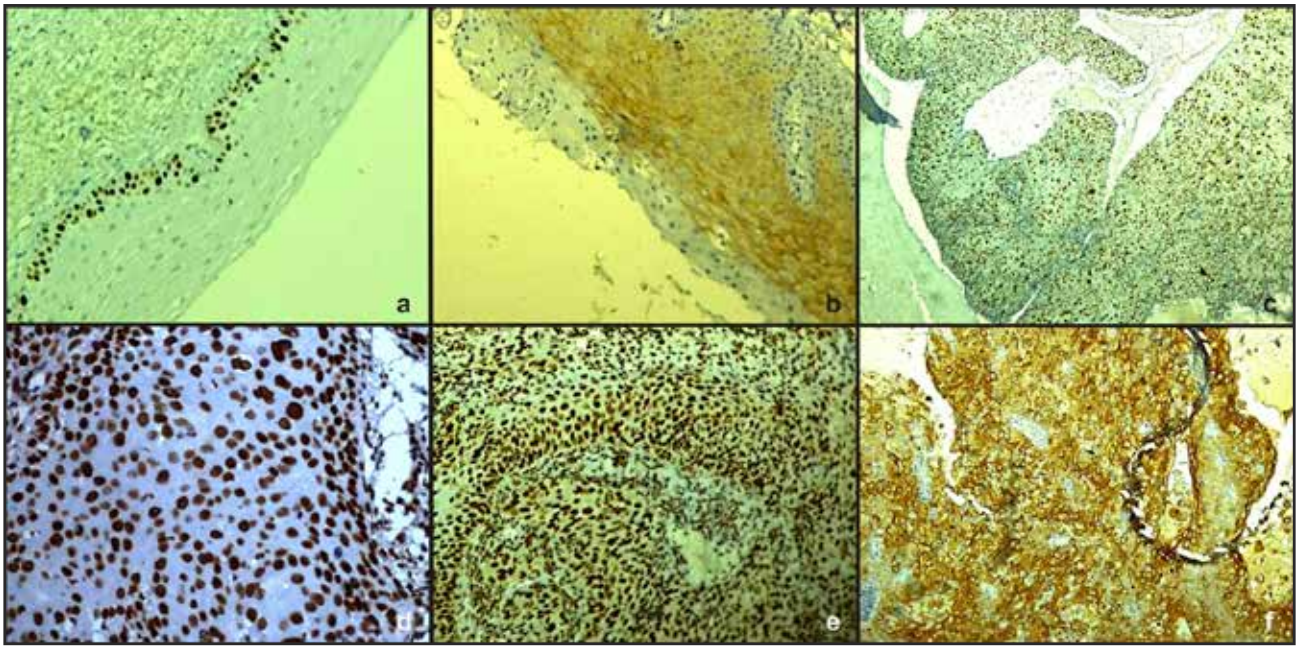

Figure 1: Photomicrograph of a case of a) CIN I showing immunostained positive reaction for p16, b) CIN II staining positively for CK17, c) CIN III showing MIB–1 positivity, SCC showing d) p16 score of +3, e) MIB–1 positivity and f) CK17 positivity (x400)

Two cases diagnosed as CIN I histopathologically when stained with biomarkers showed absence of p16 immunoexpression however were CK17 positive and MIB–1 negative. These were reclassified as NED with atypical immature squamous metaplasia.

Discussion

Cervical intraepithelial neoplasia (CIN) is a precursor to invasive SCC. It is classified as CIN I, II and III. Cervical intraepithelial neoplasia II and III have a high progression rate to an invasive SCC.[11] Discordance on histopathological diagnosis of cervical cancer precursor lesions have been documented in several studies, suggesting a need to identify biological markers that could help the pathologist make an accurate diagnosis.[3,9] IHC markers that have shown a potential in this direction are p16, MIB–1 and CK–17.[9]

IHC study of p16 revealed that there was a significant overexpression in different groups as we moved from NED to CIN I to CIN II+III to ICC. This was found to be a statistically significant finding on making a comparison between NED versus (v/s) CIN+ICC group ($p < 0.05$). However, on making an intergroup comparison such as CIN I v/s CINII+III group and CIN v/s ICC group, this was found to be statistically insignificant ($P > 0.05$). Our study was broadly in agreement with the results of the previous publications with values ranging from 50 to 100% for CIN and ICC cases and that of 1.8% to 12.5% in NED cases. (Table 7)

A few variations in p16 expression in some of the studies in various grades of CIN and ICC can be attributed to the different clones of antibody used in different studies.[8] Most of the studies have assessed p16 up regulation in HPV 16 and 18 subtypes. One study mentions the possibility of prevalence of HPV subtypes other than 16, 18 causing these variations in p16 expression which needs further evaluation.[8] In our institute, HPV prevalence was found to be 10% and prevalence of serotype 16 was 50% and that of serotype 18, 31 and 66 was 16.66% each.[6] Present study included 49 cases of SCC and one case of small cell carcinoma. p16 overexpression has been documented in cervical small cell carcinoma in a study done by Masumoto et al as also seen in this case.[20] In a study by Gupta et al ANOVA was applied for comparison of the mean p16 immunoreactivity scores in each category of NED, CIN and SCC. It was distinctly noticed that p16 was markedly up regulated in the higher grades of intraepithelial neoplasia. They further state that as one progressed from CIN II/III to SCC the difference in p16 expression was statistically significant in comparison to normal cervix with p values being 0.021, 0.008 and 0.000 respectively. However, the increased expression in cases of CIN1 in comparison to normal epithelium was not found to be statistically significant (p value=0.627).[8] In present study, the mean percentage of p16 positive cells increased highly statistically significantly with increasing severity of the lesion i.e. from NED to CIN I to III to ICC. ($F=227.02$ (ANOVA); $p<0.01$) In this study, maximum cases of CIN–I, II and ICC showed 3+ intensity whereas that of CIN III showed +2 intensity. NED showed almost nil intensity (1/20, 5%). A study by C. Queiroz et al showed the increasing reaction intensity of p16 from normal cases (only one case was weakly positive) to invasive carcinoma, which showed moderate to strong intensity similar to ours.[21] A study by Izadi–Mood et al verified a direct relationship between lesion severity and reaction intensity among p16 positive cases. They found that the frequency of positive cells and the reaction intensity were statistically significantly different when compared among different histological groups. They further document that logistic regression model when applied in their study showed that the reaction intensity was superior to any other analyzed parameter, thus being the best indicator of the expression of p16.[18] Comparison of p16 cellular reaction pattern of CIN–I in various studies with present study showed that majority of cases showed focal or diffuse positivity and that of CIN II+III showed mainly diffuse positivity.[22,23] There were also a significant number of CIN–I cases showing no reaction in studies conducted by Lakshmi et al and Galgano et al, though in our study this was not found.[22,23] The overall agreement of p16 marker with H&E diagnosis was 78% in study done by Hebbar et al[19] while study by Aslani SF et al[9] showed overall agreement as 96.1%, as compared to our study showing it as 97.5% ($\kappa=0.9151$ i.e. very good).

Our cases showed statistically significant increase in the MIB–1 expression in relation to increase in the severity of the lesions. Our results showed MIB–1 positivity in 72% of CIN and 100% of ICC cases. In the present study, mean MLI increased highly statistically significantly with increasing severity of the lesions. ($F=227.02$ (ANOVA); $p<0.01$) These findings are similar to the other studies such as Looi ML et al[24] and Carreras R et al.[25] The difference in MIB–1 immunoexpression amongst CIN I and CIN II+III group and NED and CIN+ICC group were statistically significant similar to a study by Zhong et al.[26] Thus MLI may be useful in distinguishing the different grades of dysplasia. The overall agreement of MIB–1 and H&E diagnosis was about 88%, similar to studies done by Hebbar et al[19] and Aslani SF et al.[9]

In our study, CK17 positivity in terms of percentage and intensity statistically significantly increased from NED to CIN–I to III to ICC. None of the NED cases in present study showed CK17 positivity. The difference in CK17

immunoexpression was statistically significant amongst CIN I and CIN II+III group and NED and CIN+ICC group. Our study was broadly similar with results of Chaloob et al[10] and Ikeda et al[27] The overall agreement of CK17 positivity with H&E diagnosis was 46.7% in study done by Aslani SF et. al.[9], while our study showed the overall agreement as 85% though the clone used in both studies was same. This study showed that with increasing stage, number of cases showing CK17 positivity also increased though statistically insignificant as shown by studies such as Chaloob et al[10] and Carrilho et al[28]. Chaloob et al found absence of CK17 correlation with HPV (16+18) in CIN and ICC cases and concluded that CK17 is a marker of cervical dysplastic cells and not of cervical infection by HPV.[10]

Correlation between p16 and MIB–1 IHC markers in present study showed overall agreement of 89.16% similar to that of study done by Hebbar A et al[19] and Aslani SF et al[9]. There was no study documented in literature for correlation between p16 and CK17 IHC markers as well as CK17 and MIB–1 IHC markers to compare with the present study.

Supplementary use of p16, CK17 and MIB–1 staining significantly improves the accuracy of grading CIN lesions by a single pathologist, equivalent to an expert consensus diagnosis. According to a study, almost two–thirds of atypical immature squamous metaplasia (AISM) cases could be re–classified as benign based on negative p16 staining. Another one–third could be re–classified as HSIL if showed both MIB–1 and p16 staining. Another study showed a strong uniform cytoplasmic CK17 positivity of the proliferating cells together with p16 negativity in ISM lesions. The lesions featuring both metaplastic changes and atypia with staining of both p16 and CK17 are classified as high–grade dysplasia.[9] In the present study, two cases diagnosed as CIN I histopathologically when stained with biomarkers showed absence of p16 immunoexpression however were CK17 positive and MIB–1 negative. These were reclassified as NED with ISM.

The following conclusions were drawn from the above study.

- p16, MIB–1 and CK17 expression was observed in CIN I, II, III and ICC. No case of NED showed significant p16, MIB–1 and CK17 positivity.
- The mean percentage of p16 and MIB–1 positive cells statistically significantly increased with increasing severity of the lesion i.e. from NED to CIN I to CIN III to ICC. ($p<0.01$) CK17 positivity in terms of percentage and intensity statistically significantly increased from NED to CIN I to III to ICC. ($p<0.01$)
- The difference in p16, MIB–1 and CK17 immunoexpression between NED and CIN+ICC cases was statistically highly significant. ($p<0.01$).
- The difference in MIB–1 and CK17 immunoexpression amongst CIN I v/s CIN II+III and CIN v/s ICC group was statistically significant. The difference in p16 immunoexpression between CIN I v/s CIN II+III ($p=0.715$) and CIN v/s ICC ($p=0.168$) group was statistically insignificant.
- The sensitivity, specificity, positive and negative predictive value and diagnostic accuracy of p16 immunoexpression in comparison to histopathological diagnosis was 97%, 100%, 100%, 86.96% and 97.5% respectively. ($\kappa=0.9151$ i.e. very good)
- The specificity and positive predictive value of MIB–1 and CK 17 in comparison to histopathological diagnosis was 100%
- The agreement between p16 and Ki–67 immunostaining was 89.16%. ($\kappa= 0.7$ i.e. good) followed by agreement between CK17 and Ki–67 immunostaining which was 86.6%. ($\kappa= 0.683$ i.e. good) and that between p16 and CK17 immunostaining was 84.16%. ($\kappa= 0.5908$ i.e. moderate)

Conclusion

Thus, panel of IHC markers consisting of p16, MIB–1 and CK17 could be routinely incorporated into the surgical pathology report to help pathologist to differentiate between benign and neoplastic cervical lesions. MIB–1 and CK 17 could aid in differentiating amongst CIN I and CIN II+III group.

Conflict of Interest: Nil.

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