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Research Article

Role of apoptotic index, mitotic index and MIB-1 antibody expression as biomarkers in preneoplastic and neoplastic lesions of uterine cervix

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ABSTRACT

Background: In India, cervical cancer ranks as the 2nd most frequent cancer among women. This study was carried out to study the role of apoptotic index (AI), mitotic index (MI) and MIB-1 labelling index (LI) as biomarkers in preneoplastic and neoplastic lesions of uterine cervix.
Methods: Total of 90 cases was studied, over a period of two years, in the Department of Pathology as well as Obstetrics and Gynaecology, LLRM Medical College and associated SVBP Hospital, Meerut, India. Apoptotic Index and Mitotic Index were calculated on H & E stained sections in all the 90 cases while ki-67 immunostaining was done only on 44.44% (40/90) cases.
Results: Apoptotic Index, Mitotic Index and MIB-1 expression were found to increase as the grade of lesion increased from cervical dysplasia to invasive cervical carcinoma. In CIN-I, MIB-1 positive cells were confined to lower one-third of ectocervix and full thickness was involved in CIN-III.
Conclusion: We found proliferative indices (Mitotic index and MIB-1 labelling index) and Apoptotic index useful in determining the grading of dysplasia and cervical carcinoma.

Key words: Cervical dysplasia, Cervical carcinoma, Apoptotic index, Mitotic index, MIB-1 Labelling index

INTRODUCTION

According to GLOBOCAN 2012, cervical cancer is the world's 4th most common cancer among women.[1] In India, it ranks as the 2nd most frequent cancer among women.[2]

Pap smear has been the standard screening test all over the world. It has low sensitivity but high specificity. The histological diagnosis of cervical biopsies that is often considered as gold standard can be significantly hampered by intra and inter-observer variability.[3,4] It is difficult to distinguish CINs reliably from non-neoplastic lesions and CIN I from CIN II/III, resulting in either over-treatment or under-treatment.[5,6]

Therefore additional diagnostic and prognostic markers for detection of cervical precursor lesions and cervical carcinoma are needed, which could save the patient from unnecessary surgical intervention and high screening costs.

Counting of mitotic figures is the oldest way of assessing proliferation. Ever since the introduction of microscopes made the recognition of mitotic figures possible, counting mitotic index has been applied as a diagnostic tool, especially in tumour pathology. Even though many other ways of assessing proliferation have become available, the ease with which mitoses can be recognized without special equipment apart from a decent microscope and a well stained H and E slide has led to the increasing popularity of this way of counting of mitotic figures up to the present.[7]

Ki-67 is a proliferation marker known as predictive factor for tumor development. It is a nuclear antigen, a protein encoded by ki-67 on 10q25. It is expressed during all active phases of the cell cycle (G1, S, G2, M), except G0, thus being present only in dividing cells and absent in resting cells.[8,9] Ki-67 is detected by monoclonal antibody MIB-1. Therefore this antibody can be a useful marker of proliferation in dysplastic lesions and, in addition, can be of prognostic value.[10]

Along with cell proliferation, cell death is another phenomenon which is responsible for control of cell number in normal and neoplastic tissue. Apoptosis is genetically controlled death which enables the elimination of the cells that have been damaged.[11] As apoptotic tumor cells can be morphologically identified and counted by light microscopy, there has been interest in the application of the apoptotic index in malignant growths as a putative prognostic marker.[12]

The present study is conducted to evaluate the role of MIB-1 antibody expression, apoptotic index and mitotic index, in tissue sections, as biomarkers in pre-neoplastic and neoplastic lesions of uterine cervix.

METHODS

The present study is a retrospective as well as a prospective study done on a total of 90 cases, over a period of two years, in the Department of Pathology, in collaboration with the Department of Obstetrics and Gynaecology, LLRM Medical College and associated SVBP Hospital, Meerut, India. Clinical history and examination findings of the patients were collected in all the cases. All specimens were processed and routinely stained with H and E stain while IHC was done in 44.44% (40/90) cases. Tissue sections with inadequate study material and with extensive necrosis and haemorrhage were excluded from the study.

Immunostaining method for MIB-1

44.44% (40/90) cases were subjected to IHC staining for Ki-67. Four micrometer thin sections were taken. After antigen retrieval, endogenous peroxide activity was blocked with 3% hydrogen peroxide. MIB-1 immunostaining was performed using Monoclonal Mouse Anti-Human Ki-67 Antigen Clone MIB-1 antibody (Dako Denmark A/S, Glostrup, Denmark).

Positive control for MIB-1: A histological section of known case of squamous cell carcinoma cervix was used as positive control with each batch of staining.

Negative control for MIB-1: For negative control, 1% non-immune serum was used in place of primary antibody, with rest of the steps being the same as for the positive control.

Interpretation

A detailed histopathological examination of H and E stained slides was carried out. Cases were divided into two broad categories: cervical dysplasia and invasive squamous cell carcinoma (SCC) cervix. Cervical dysplasia cases were again sub-divided into CIN I, II and III. Cases of SCC cervix were sub-divided into well differentiated, moderately differentiated and poorly differentiated SCC.

Calculation of apoptotic index

The H and E stained sections were examined using a 40 X objective. From each section, four areas devoid of any preservation or fixation artefact were selected. In each section, 1000 tumor cells were evaluated for the presence of apoptotic cells and apoptotic bodies. The apoptotic cells were recognized by certain well defined features like cell shrinkage, condensation and deep eosinophilia of cytoplasm and pyknotic, round to crescentric or irregular nucleus.

Apoptotic bodies which typically appear as tiny round and pyknotic nuclear fragments were seen scattered amongst the tumor cells and occasionally forming small clusters were also identified (Figure 1).

Apoptotic index (AI) was calculated as the number of apoptotic cells and apoptotic bodies expressed as a percentage of total number of tumor cells counted in each case.[13]

$$AI = \frac{\text{Number of apoptotic tumor cells per section}}{\text{Total number of tumor cells per section}} \times 100$$

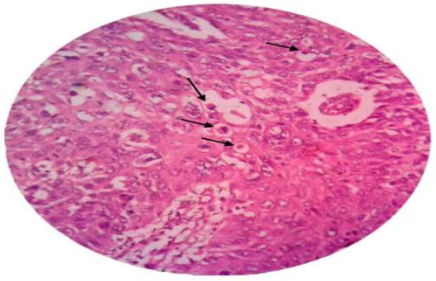

Figure 1: Photomicrograph showing numerous apoptotic bodies in carcinoma cervix (H and E, 400X)

Apoptosis was high in areas of necrosis and therefore these areas as well as areas with intense inflammatory infiltrate were excluded from the evaluation. Apoptotic cells in the stroma around the tumor were also disregarded.

Calculation of mitotic index

Mitotic index (MI) was calculated in the similar manner as apoptotic index in H and E stained sections using 40 X objective, evaluating 1000 tumor cells for the presence of mitoses. Mitoses can be recognised by the presence of hairy extensions when focusing up and down, while the nuclear envelope is absent and cytoplasm is basophilic rather than eosinophilic (Figure 2).[7]

Mitotic index was calculated as the number of mitoses expressed as a percentage of total number of tumor cells counted in each case.[14]

$$MI=\frac{\text{Number of mitoses per section}}{\text{Total number of tumor cells per section}}\times 100$$

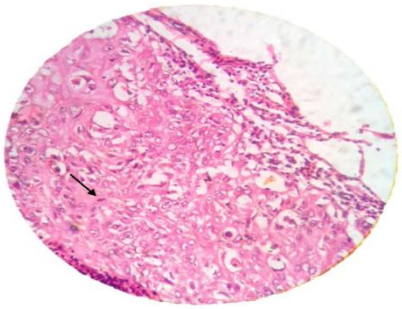

Figure 2: Photomicrograph showing mitosis in carcinoma Cervix (H & E, 400X)

Calculation of MIB-1 labelling index

The slides immunostained for Ki-67 antigen were evaluated to calculate MIB-1 Labelling Index (LI). LI was calculated by the number of positively stained cells (nuclear staining) per 100 cervical epithelial cells in different areas under X400 magnification and the mean is calculated (Figure 3).

Positive nuclei were expressed as the percentage of total nuclei counted.[15] MIB-1 Labelling Index was calculated as follows:

$$LI=\frac{\text{Number of cells showing positive staining}}{\text{Total number of cells}}\times 100$$

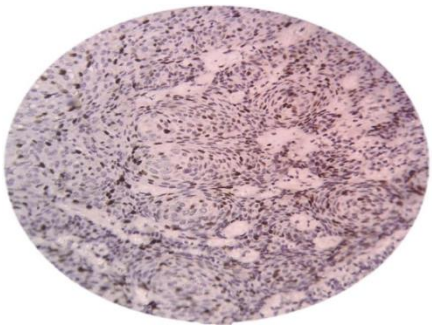

Figure 3: Ki-67 Immuno-expression in squamous cell carcinoma cervix (400X).

Grading of Ki-67 expression

The sections stained for Ki-67 proliferation (revealed as nuclear staining) were evaluated to calculate LI and graded using scores from 1 to 3.[16]

'+++'- High proliferation- >50 % positive cells
'++'- Moderate proliferation- 30 % - 50 % positive cells
'+'- Low proliferation- 10 % - 30 % positive cells

RESULTS

The women in present study belonged to 20 years to more than 70 years of age. Maximum cases of cervical dysplasia were seen in 31-40 years of age group (17/28 cases, 60.71%). Mean age of dysplasia was 42.5 years. Majority of women with cervical carcinoma were between 51-60 years of age (21/62 cases, 33.87%), mean age being 49.4 years. Cervical dysplasia was found to be more common in pre-menopausal women (21/28 cases, 75 %), whereas cervical carcinoma in postmenopausal women (34/62 cases, 54.84%).

Out of total 90 cases, 31.11% (28 cases) were diagnosed as cervical intraepithelial neoplasia (CIN) and 68.89% (62 cases) were as invasive cervical carcinoma. The cases with CIN were further subdivided into CIN I, CIN II (11.11%, 10 cases each) and CIN III (8.89%, 8 cases), depending on the thickness of the ectocervical epithelium involved by dysplastic cells. The cases with invasive carcinoma were subdivided into well differentiated (WD-SCC, 27.78%, 25 cases), moderately differentiated (MD-SCC, 36.67%, 33 cases) and poorly differentiated (PD-SCC, 4.44%, 4 cases) (Table 1).

Table 1: Distribution of total cases according to histopathological diagnosis.

Histopathological diagnosis	Number of cases	%
Cervical intra-epithelial neoplasia (CIN)	**28**	**31.11**
CIN I	10	11.11
CIN II	10	11.11
CIN III	08	8.89
Invasive cervical carcinoma	**62**	**68.89**
WD-SCC	25	27.78
MD- SCC	33	36.67
PD-SCC	04	4.44
Total	**90**	**100**

WD-SCC=well differentiated squamous cell carcinoma; MD-SCC=moderately differentiated squamous cell carcinoma; PD-SCC=poorly differentiated squamous cell carcinoma

Apoptotic index (AI) and mitotic index (MI) were calculated in H and E stained sections of total 90 cases of cervical dysplasia and invasive cervical carcinoma. The mean AI and MI increased progressively with increasing

grades of cervical dysplasia i.e. from CIN I (AI=0.15%±0.143, MI=0.20%±0.188) to CIN II (AI=0.30% ± 0.236, MI=0.35%±0.184) to CIN III (AI=0.58% ±0.323, MI=0.55%±0.151). The difference between CIN I and CIN II were not found to be significant but that between CIN II and CIN III was found significant.

Mean AI and MI also increased as the lesion changed from dysplasia (AI=0.329%±0.2904, MI=0.35%±0.2219) to carcinoma (AI=1.226%±0.532, MI=1.08%±0.2162). The difference was found to be statistically extremely significant (p<0.0001) (Table 2).

Table 2: Apoptotic index and mitotic index in different grades of cervical dysplasia and carcinoma cervix.

Category	Number of cases and %		Mean AI (%)± SD	Mean MI (%)± SD
CIN-I	10	11.11%	0.15±0.143	0.20±0.188
CIN-II	10	11.11%	0.30±0.236	0.35±0.184
CIN-III	08	8.89%	0.58±0.323	0.55±0.151
WD-SCC	25	27.78%	0.98±0.481	0.94±0.189
MD-SCC	33	36.67%	1.32±0.401	1.17±0.151
PD-SCC	04	4.44%	1.98±0.918	1.28±0.350
Total	**N= 90**	**100%**		

When comparison was made between different sub-groups of SCC, an increase in both AI and MI was observed from WD-SCC (AI=0.98%±0.481, MI=0.94%±0.189) to MD-SCC (AI=1.32%±0.401, MI=1.17%±0.151) to PD-SCC (AI=1.98%±0.918, MI=1.28%±0.350). The difference was found to be statistically significant for AI. The difference between MI of WD-SCC and MD-SCC was found to be statistically extremely significant (p<0.0001) and that between MD-SCC and PD-SCC was not found statistically significant (Table 2).

Table 3: MIB-1 labeling index in different grades of cervical dysplasia and carcinoma cervix.

Category	Number of cases and %		Mean LI (%) ± SD	Range (Min – Max)
CIN-I	08	20.0	8.38±3.204	4-15
CIN-II	05	12.5	15.6±4.722	10-21
CIN-III	06	15.0	29.8±8.635	20-42
WD-SCC	11	27.5	51.6±12.428	40-70
MD-SCC	07	17.5	64.9±13.359	50-80
PD-SCC	03	7.5	83.7±4.041	79-86
Total	N=40	100%		

Table 4: Ki-67 expression in different grades of cervical dysplasia and carcinoma cervix.

	Ki-67 expression			
Category	**Number of cases**	**Low**	**Moderate**	**High**
CIN-I	08	08	-	-
CIN-II	05	05	-	-
CIN-III	06	02	04	-
WD-SCC	11	-	06	05
MD-SCC	07	-	02	05
PD-SCC	03	-	-	03
Total	**40 (100%)**	**15 (37.5%)**	**12 (30%)**	**13 (32.5%)**

MIB-1 Labelling Index (LI) was calculated in immunostained sections of total 40 cases (44.44%) of cervical dysplasia and invasive cervical carcinoma. MIB-1 positive cells were seen confined to lower one-third of

ectocervix in CIN-1 and full thickness was involved by MIB-1 positive cells in CIN-III. Increasing MIB-1 positivity was observed while moving through different grades of CINs. Mean LI was 8.38%±3.204 in CIN-I, 15.6%±4.722 in CIN-II and 29.8%±8.635 in CIN-III. The difference was found to be statistically very significant. Mean value of MIB-1 Labelling index also increased as the nature of the lesion changed from dysplasia to SCC.

Mean LI of CIN group was 17.05%±10.870 and of carcinoma group was 60.62%±16.209 (p<0.0001). Mean LI was 51.6%±12.428 in WD-SCC, 64.9%±13.359 in MD-SCC and 83.7%±4.041 in PD-SCC, showing an increasing trend from WD-SCC to PD-SCC. The difference was statistically significant between WD-SCC and MD-SCC as well as between MD-SCC and PD-SCC (Table 3).

Ki-67 expression was low to moderate in cervical dysplasia while moderate to high in invasive carcinoma. Out of total 40 cases, 37.5% (15 cases) showed low, 30% (12 cases) showed moderate and 32.5% (13 cases) showed high Ki-67 expression (Table 4).

DISCUSSION

Biological behavior of the tumor depends not only on its proliferative activity, as measured by mitotic index (MI) and MIB-1 labeling index (LI), but also on the number of cells dying by apoptosis. Apoptosis plays a role in eliminating the cells with defective repair process and has been recognized as a physiological mechanism for controlling cell numbers.[17] The mitotic index and MIB-1 expression could be used as a marker for dividing cells.[18]

In present study, the mean apoptotic index (AI) increased progressively with increasing grades of cervical dysplasia, similar to the studies done by Nam JH et al, Sagol O et al, Mysorekar VV et al, Gupta K et al and Bhardwaj S et al (14) (Table 5).[13,14,19-21]

Table 5: Comparison of mean apoptotic and mitotic index in different grades of dysplasia with other studies.

Study	CIN - I		CIN - II		CIN - III	
	AI	MI	AI	MI	AI	MI
Mysorekar VV et al[13]	2.13%±1.06	1.70%±0.25	2.60%±1.61	1.96% ±0.91	3.15%±1.62	2.82% ±1.24
Gupta K et al[21]	0.106%±0.084	-	0.24%±0.106	-	0.34%±0.18	-
Bhardwaj S et al[14]	0.62%±0.6	0.009%±0.08	1.0%±0.44	0.15% ±0.05	1.0%±0.06	0.25% ±0.05
Present study	0.15%± 0.143	0.20% ±0.188	0.30% ±0.236	0.35%±0.184	0.58%±0.32	0.55% ±0.15

The difference in apoptotic values between CIN I and CIN II were not found to be significant in present study but that between CIN II and CIN III was found significant (p< 0.05). Sagol O et al did not find any statistically significant difference between CIN I and CIN II as well as between CIN II and CIN III groups but Mysorekar VV et al (13) reported that the difference was statistically significant (p<0.01) between mild and moderate dysplasia as well as between moderate and severe dysplasia.[20,13] In the study of Gupta K et al, mean AI values between CIN II and CIN III were not found to be statistically significant but that between CIN I and CIN II; CIN I and CIN III were statistically significant.[21]

Table 6: Comparison of MIB-1 Labelling Index in different grades of dysplasia and carcinoma cervix, with other studies.

Study	CIN - I	CIN - II	CIN - III	Invasive carcinoma
Payne et al[25]	22.1%±12.4	22.9%±8.6	28.6%±15.0	-
Bulten et al[26]	16%	25%	39%	-
Carreras et al[31]	25%	_	68%	65.5%
Natalia et al[32]	35.6%	51.9%	40.9 %	57.8%
Gupta et al[21]	5.54%±2.185	18.9%±2.491	42.5%±7.937	50.754%±12.625
Sansanwal et al[23]	38%	-	60%	-
Present study	8.38%±3.204	15.6%±4.722	29.8%±8.635	60.62%±16.209

AI increased significantly as the lesion changed from dysplasia to SCC. Similar finding was reported by Nam JH et al, Sagol O et al, Dey P et al and Mysorekar VV et al.[13,19-22] An increase in AI was also observed from WD-SCC to MD-SCC to PD-SCC. The difference was found to be statistically significant, similar to results of Nam JH et al and Gupta K et al.[19,21]

In present study, mean MI showed progressive increase as the grade of the lesion increased from CIN-I to CIN-II to CIN-III. The difference was not found significant between CIN-I and CIN-II but it was significant between CIN-II and CIN-III. Similar findings were also reported by Sagol O et al, Dey P et al, Mysorekar VV et al Sand Bhardwaj S et al (Table 5).[13,14,22] Sagol O et al and Dey P et al did not find any statistically significant difference between CIN-I and CIN-II as well as between CIN-II and CIN-III.[20,22] As compared to present study, the mean MI of CIN-I, CIN-II and CIN-III were much higher in the study done by Mysorekar VV et al.[13] The difference was statistically significant ($p<0.01$) between different grades of CIN in their study.

It was also observed in present study that mean MI increased as the nature of lesion changed from dysplasia to carcinoma. Sagol O et al also reported that the SCC cervix group showed significantly higher mitotic cell counts when compared with pre-neoplastic lesions. Dey P et al, Mysorekar VV et al and Bhardwaj S et al also reported significant difference in mean MI with increasing grades of lesion from dysplasia to carcinoma.[13,14,20,22]

In present study, mean MI was found to increase from WD-SCC to MD-SCC to PD-SCC. The difference between WD-SCC and MD-SCC was found to be extremely significant ($p<0.0001$) while between MD-SCC and PD-SCC it was not found significant. Sansanwal P et al also reported that average MI was highest in PD-SCC (62/50 hpf), in MD-SCC it was 54/50 hpf and least in WD-SCC (42/50 hpf). In contrast, Bhardwaj S et al found no statistically significant difference between the MI of well differentiated and less differentiated carcinomas of cervix.[14]

In the present study, it was observed that MIB-1 positive cells were confined to lower one-third of ectocervix in CIN-1 and full thickness in CIN-III. This was in concordance with the study conducted by McCluggage WG et al, Payne S et al and Sansanwal P et al.[23-25]

Payne P et al observed that with increasing grade of CIN, the growth compartment stretched more superficially so that in CIN III almost the full thickness of epithelium was cycling, as shown by Ki-67 immuno-positive cells.[25]In present study, we observed increasing MIB-1 positivity while moving through different severity of CINs, difference being statistically very significant. This is in concordance with the study of Payne P et al, Bulten J et al, ter Harmsel B et al, Nam JH et al, Conesa-Zamora P et al, Gupta K et al and Sansanwal P et al(Table 6).[23-29] We also observed that the mean value of MIB-1 Labelling index increased significantly as the nature of the lesion changed from dysplasia to SCC. Similar results were observed by Nam JH et al, Mehrotra A et al, Srivastava S et al and Gupta K et al.[19,15,29,21]

We also found an increasing trend in MIB-1 Labelling index (LI) from WD-SCC to PD-SCC. The difference was statistically significant between WD-SCC and MD-SCC as well as between MD-SCC and PD-SCC. The studies conducted by Nam JH et al, Pahuja S et al and Sansanwal P et al also showed an increase in LI from WD-SCC to PD-SCC, similar to present study.[19,23,25-27] In contrast, Gupta K et al found the mean LI of MD-SCC to be less than WD-SCC, while it was maximum in PD-SCC like our study.[21]

CONCLUSION

Apoptotic cells and mitoses can be readily demonstrated on routine H and E stained sections. Therefore, these indices are the simplest techniques that can be employed in any laboratory.

Apoptotic index and mitotic index increased with increasing grades of dysplasia, from dysplasia to SCC cervix as well as from well differentiated to poorly differentiated SCC. MIB-1 positive cells were found to be confined to lower one-third of ectocervix in CIN-I and full thickness in CIN-III, hence confirming the histopathological diagnosis.

Ki-67 antigen, as expressed by MIB-1 labelling index, was found to increase with increasing severity of CINs, from CIN to SCC and also from well differentiated to poorly differentiated SCC. So, we found that proliferative indices (Mitotic index and MIB-1 labelling index) and apoptotic index are useful in determination of grading of dysplasia and carcinoma. Hence study concluded that proliferative activity of cervical lesion as determined by mitotic index and MIB-1 antibody expression are reliable indicators of its malignant potential and together with apoptotic count gives an idea about the net growth of tumor.

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