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Original Research Article

IMMUNOHISTOCHEMICAL STUDY ON PTEN AND CYCLIN D1 IN NON-NEOPLASTIC AND NEOPLASTIC ENDOMETRIAL LESIONS

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Abstract

Introduction: Endometrial carcinoma (EC) is the most common gynaecological malignancy in developed countries and has been classified into two groups, type 1 and type 2. Type 1 or endometrioid endometrial carcinomas (EECA) accounts for 80% of EC and are thought to develop following a continuum of premalignant lesions ranging from endometrial hyperplasia without atypia (EH) and atypical hyperplasia (AH). PTEN (phosphatase and tensin homolog), a tumor suppressor gene is commonly inactivated in 83 % of endometrioid carcinoma and 55% of precancerous lesions. Cyclin D1, a cell cycle regulator is overexpressed in about 40% of endometrial carcinomas.

Aim: To study the expression of PTEN (Phosphatase and tensin homolog) and Cyclin D1 in non-neoplastic and neoplastic endometrial lesions by immunohistochemistry (IHC).

Methods: A 2 year cross-sectional study (September 2017 to August 2019) on 115 endometrial samples was done in the Department of Pathology, RIMS. Histomorphological features and IHC expression of PTEN and Cyclin D1 in the various endometrial lesions were studied and evaluated, data collected in IBM SPSS Statistics 21 was statistically analysed using Chi - square and Fisher's Exact test.

Results: Out of the 115 cases, 47(40.9%) were diagnosed as benign proliferative endometrium, 20(17.4%) benign secretory endometrium, 21(18.3%) hyperplasia without atypia, 15(13.0%) atypical hyperplasia and 12(10.4%) endometrial carcinoma with an age group spanning from 26-68 years (mean age = 46.4). Following IHC staining, 91.7%(11/12) and 83.3%(10/12) cases of EC and 80%(12/15) and 73.3%(11/15) cases of AH showed complete loss of PTEN expression and Cyclin D1 over expression, respectively when compared to other benign lesions and was statistically significant ($p < .001$).

Conclusion: Loss of PTEN and Cyclin D1 overexpression was seen in a significant number of EECA and AH, suggesting both as an early event in endometrial carcinogenesis. Therefore, we propose the use of PTEN and Cyclin D1 immunostaining as an adjunct to histopathological diagnosis as it may be informative in identification and further management of premalignant endometrial lesions that are likely to progress to carcinoma

Keywords: PTEN, Cyclin D1, endometrial hyperplasia, endometrial carcinoma, endometrioid endometrial carcinomas.

Introduction

Endometrial cancer is the most common gynaecological malignancy in developed countries and second most common in developing countries . The age standardized incidence rate (ASIR - 2019) of endometrial cancer in India is 2.3/100,000 women.[1,2] A dualistic model of endometrial tumorigenesis is currently recognized, broadly termed type 1 and type 2, based on a classification system hypothesized by Bokhman in 1983.[3] Type 1/Endometrioid carcinoma is the most common accounting for approximately 80 % of cases, it typically arises in a background of unopposed estrogen exposure and is thought to develop following a continuum of premalignant lesions ranging from endometrial hyperplasia without atypia, hyperplasia with atypia and well differentiated carcinoma. It is seen in 55-65 years age group and have a more favorable prognosis. Type II /Serous Carcinoma occurs in older women, usually arising in a background of atrophic endometrium and is characterized by high grade histology.[3] According to the recent 2014 WHO classification, endometrial hyperplasia is divided into two types a) Endometrial hyperplasia without atypia (EH) and b) Atypical endometrial hyperplasia/ Endometroid intraepithelial lesion (AH/EIN). Endometrial hyperplasia without atypia has 2-4 times the risk of transformation to endometrioid (type 1) carcinoma as compared to up to 45 times for atypical endometrial hyperplasia.[4] The pathogenesis of endometrial carcinoma and its precursor lesion is complex and involves many molecular disturbances. The most frequently altered gene in Type 1 endometrioid carcinoma is PTEN, reported to be altered in up to 83% of endometrioid carcinomas and 55%

of precancerous lesions, others include Cyclin D1, K-RAS, β-catenin, DNA mismatch repair genes, PIK3CA and PAX2. Type 2 endometrial cancers frequently show aneuploidy and p53 mutations or Her2/neu overexpression.[3,5]

PTEN, located at chromosome 10q23, encodes a protein with tyrosine kinase function and behaves as a tumour suppressor gene. It acts at the G1/S checkpoint of the cell cycle and enables apoptosis through an AKT-dependent mechanism. The protein has both lipid and protein phosphatase activity, with each serving different functions. The lipid phosphatase activity of PTEN causes cell cycle arrest at the G1/S checkpoint. Also, the up regulation of proapoptotic mechanisms involving AKT-dependent mechanisms is mediated through PTEN, as is the down regulation of anti-apoptotic mechanisms through Bcl-2.15 Mutation of PTEN increases the PI3KCA activation, resulting in phosphorylation of AKT.[6,7] The protein phosphatase activity of PTEN is involved in the inhibition of focal adhesion formation, cell spread, and migration, as well as the inhibition of growth factor-stimulated MAPK signalling. Thus, loss or altered PTEN expression results in aberrant cell growth and apoptotic escape.[8]

Cyclin D1 overexpression has been observed in 40% of endometrial carcinomas overexpress Cyclin D1 is a member of the Cyclin G1 family and controls the transition from G1 to S phase in the cell cycle. The Cyclin D1 protein is encoded by the binding of Cyclin D1 to the Cyclin dependent kinases 4 and 6 (CDK4/6) resulting in the formation of active complexes that phosphorylate the retinoblastoma tumour suppressor gene during the G1 phase. Multiple studies have shown Cyclin D1 overexpression as a potential biomarker for precancerous and cancerous endometrial lesions.[9]

This study was designed to evaluate the immunostaining pattern of PTEN and Cyclin D1 on various endometrial lesions and thereby recognize the subset of endometrial lesions that may be precancerous and explore the possibility of using PTEN and Cyclin D1 immunostaining as a new and effective diagnostic tool for screening of malignant and premalignant endometrial lesions.

Methods:

A cross sectional study spanning 2 years from September 2017 to August 2019 was conducted in the Histopathology section, Department of Pathology, RIMS. All endometrial curettage/biopsy and endometrial tissue from hysterectomy specimens received in the Department of Pathology, during the study period were included in the study. Inadequate endometrial tissue sample, products of conception, endometritis and with known case of malignancy in other systems were excluded from the study. All together 115 endometrial tissue samples were received. The tissues were fixed in formalin and taken up for routine histopathological studies and IHC studies for PTEN and Cyclin D1.

Monoclonal mouse Anti-Human PTEN, clone 6H2.1 (Master Diagnostica) and Monoclonal rabbit Anti-Human Cyclin D1, clone EP12 (Dako) were used in all immunohistochemical analysis. Endometrial stroma was taken as internal positive control for PTEN while basal cells of tonsillar epithelium was taken as positive control for Cyclin D1. Sections of 3-5 μm were cut on to poly L-lysine coated slides and were deparaffinized and rehydrated. Antigen retrieval was achieved by microwave method using Tris buffer. Slides were allowed to cool for 20 minutes and blocking reagent was applied and kept for 10 minutes. Tissues were covered with primary antibody and were incubated for 1 hour at room temperature in humidity chamber. Polymer HRP labelled secondary antibody detection kit was added on the sections and incubated in humidity chamber for 30 minutes. DAB chromogen was added on the section for 10 minutes and then washed with D/W. All slides were counterstained with Haematoxylin, dehydrated and mounted. Between each step, the slides were washed with phosphate buffer solution (PBS).

Immunoreactivity was regarded as positive when brown staining was localized in the nuclei or cytoplasm. The intensity of staining was graded as no nuclear staining (0), weak nuclear staining (1+), moderate nuclear staining (2+) and strong nuclear staining (3+). The extent of staining was estimated in percentage by counting at least 50 nuclei, calculating the ratio of reactive nuclei to total number of nuclei and multiplying it by 100. A score of 0 was given when < 10% cells were positive, 10 - 30% immunoreactive cells were scored as 1, 31 - 60% positive cells were scored as 2; and > 60% immunoreactive cells were scored as 3. Then the scores of intensity and extent were added, total score was given , 0-2 was considered as negative and 3-6 as positive score.[10]

Data collected was entered and analysed using IBM SPSS Statistics 21 for Windows. Descriptive statistics like mean, standard deviation, percentage and proportion were used in variables like age, histopathological and immunohistochemical findings. Statistical analysis was done using Chi - square and Fisher's exact test , p - value < 0.05 was considered as statistically significant at 95% confidence interval.

Ethical approval was obtained from the Research Ethics Board, RIMS, Imphal with reference number A/206/REB-Comm(SP)/RIMS/2015/307/50/2017, dated 26th March 2018. Informed written consent was taken from the participants for the study before recruitment. A code number was assigned and no names were taken to maintain confidentiality. Data collected were kept secured.

Results:

All together 115 histopathological specimens were analysed, the most common histopathological diagnosis was benign proliferative endometrium consisting of 47 (40.9%) cases, followed by endometrial hyperplasia without atypia 21 (18.3%), secretory endometrium 20 (17.4%) , atypical endometrial hyperplasia 15 (13.0%) and endometrial carcinomas 12 (10.4%) (Figure 2). Out of the 12 endometrial carcinomas, 11 (91.7%) was endometrioid adenocarcinoma and 1 (8.3%) was high grade serous carcinoma. The age range of the patients span from 26-68 years (Mean±SE, 46.05±8.87). Most number of cases i.e. 61 (53%) cases were found in the 41-50 years age group while the least number of cases i.e. 3 (2.6 %) cases were in the 21-30 years age group The maximum number of atypical hyperplasia were found in the age between 41-50 years accounting to 7 cases (58.3%). Endometrial carcinoma was most common in the age group 61-70 years accounting to 7 (58.3%) cases.

Table 1: shows the distribution of different endometrial lesions according to age groups.

Lesions	**Total number of cases**	**21-30 years**	**31- 40 years**	**41- 50 years**	**51-60 years**	**61-70 years**	**Mean ±SE**
Proliferative endometrium	47 (41%)	2 (4.3%)	14 (29.8%)	27 (57.4%)	4 (8.5%)	0 (0%)	42.68±6.301
Secretory endometrium	20 (17%)	1 (5.0%)	8 (40%)	11 (55.0%)	0 (0%)	0 (0%)	41.45±6.186
Hyperplasia without atypia	21 (18%)	0 (0%)	4 (19%)	14 (66.7%)	2 (9.5%)	1 (4.8%)	46.14±6.405
Atypical hyperplasia	15 (13%)	0 (0%)	2 (13.3%)	8 (53.3%)	2 (13.3%)	3 (20%)	50.67±8.723
Endometrial carcinoma	12 (11%)	0 (0%)	0 (0%)	1 (8.3%)	4 (33.3%)	7 (58.3%)	61.007±286
Total	115 (10%)	3 (2.6%)	28 (24.3%)	61 (53.0%)	12 (10.4%)	11 (9.6%)	46.05±8.876

Table 1. Distribution of cases according to age group

Table 2 shows the intensity and extent of immunoexpression of PTEN in proliferative, secretory, EH, AH and neoplastic endometrial samples. 80% of atypical endometrial hyperplasia and 91.7% of endometrial carcinoma showed loss of PTEN expression when compared to normal proliferative, secretory endometrium and hyperplasia without atypia which showed 100%, 90% and 95.2% PTEN positivity respectively as shown in table 3.

Table 2: Intensity and extent of PTEN immunoreactivity in normal ,hyperplastic endometrium and endometrial carcinoma

Lesion	**No. of cases**	**Intensity**				**Extent**			
		0	**1+**	**2+**	**3+**	**0**	**1+**	**2+**	**3+**
Proliferative	47	0 (0%)	4 (8.5%)	13 (27.7%)	30 (63.8%)	1 (2.1%)	2 (4.3%)	14 (29.8%)	30 (63.8%)
Secretory	20	0 (0%)	2 (10%)	7 (35.0%)	11 (55.0%)	2 (10%)	2 (10%)	6 (30%)	10 (50%)
Hyperplasia without atypia	21	1 (4.8%)	2 (9.5%)	8 (38.1%)	10 (47.6%)	2 (9.5%)	1 (4.8 %)	8 (38.1%)	10 (47.6%)
Atypical hyperplasia	15	9 (6%)	5 (33.3%)	1 (6.7%)	0 (0%)	12 (80%)	1 (6.7%)	2 (13.3%)	0 (0%)
Endometrial carcinoma	12	9 (75%)	3 (25 %)	0 (0%)	0 (0%)	10 (83.3%)	1 (8.3 %)	1 (8.3%)	0 (0%)
Total	115	19 (16.5.%)	16 (13.9%)	29 (25.2%)	51 (44.3.%)	2 (23.5%)	7 (6.15 %)	31 (27.0%)	50 (43.5%)

Table 3: Frequencies of cases showing immunoreactivity for PTEN

LESION	Total number of cases	PTEN	
		POSITIVE	NEGATIVE
Proliferative endometrium	47	47 (100%)	0 (0%)
Secretory endometrium	20	18 (90%)	2 (10.0%)
Hyperplasia without atypia	21	20 (95.2%)	1 (4.8%)
Atypical hyperplasia	15	3 (20.0%)	12 (80.0%)
Endometrial carcinoma	12	1 (8.3%)	11 (91.7%)
Total	115	89(77.4%)	26 (22.6%)

Table 4: Intensity and extent of Cyclin D1 immunoreactivity in normal, hyperplastic endometrium and endometrial carcinoma

Lesion	No. of cases	Intensity				Extent			
		0	1+	2+	3+	0	1+	2+	3+
Proliferative	47	19 (40.4%)	25 (53.2%)	2 (4.3%)	1 (2.1%)	32 (68.1%)	13 (27.7%)	1 (2.1%)	1 (2.1%)
Secretory	20	11 (55.0%)	6 (30.0%)	1 (5.0%)	2 (10.0%)	13 (65.0%)	3 (15.0%)	2 (10.0%)	2 (10.0%)
Hyperplasia without atypia	21	9 (42.9%)	8 (38.1%)	3 (14.3%)	1 (4.8%)	11 (52.4%)	4 (19.0%)	4 (19.0%)	2 (9.5%)
Atypical hyperplasia	15	0 (0.0%)	4 (26.7%)	5 (33.3%)	6 (40.0%)	1 (6.7%)	4 (26.7%)	5 (33.3%)	5 (33.3%)
Endometrial carcinoma	12	0 (0.0%)	2 (16.7%)	5 (41.7%)	5 (41.7%)	0 (0.0%)	2 (16.7%)	1 (8.3%)	9 (75.0%)
Total	115	39 (33.9%)	45 (39.1%)	16 (13.9%)	15 (13.0%)	57 (49.6%)	26 (22.6%)	13 (11.3%)	19 (16.5%)

Table 4 shows the extent and intensity of staining of Cyclin D1 in different endometrial samples. 83.3% of endometrial carcinoma and 73.3% atypical endometrial hyperplasia showed Cyclin D1 overexpression while normal proliferative, secretory endometrium and hyperplasia without atypia showed 8.5%, 20% and 47.6% Cyclin D1 positivity respectively, table 5.

Table 5: Frequencies of cases showing immunoreactivity for Cyclin D1

LESION	Total Number of Cases	CYCLIN D1	
		POSITIVE	NEGATIVE
Proliferative endometrium	47	4 (8.5%)	43 (91.5%)
Secretory endometrium	20	4 (20.0%)	16 (80.0%)
Hyperplasia without atypia	21	10 (47.6%)	11 (52.4%)
Atypical hyperplasia	15	11(73.3%)	4 (26.7%)
Endometrial carcinoma	12	10 (83.3%)	2 (16.7%)
Total	115	39 (33.9%)	76 (66.1%)

The sensitivity and specificity of the markers PTEN and Cyclin D1 were calculated and was found to be 85.2% and 96.6% respectively for PTEN while for Cyclin D1 it was 77.8% and 52.4% respectively. On comparing the various benign endometrial lesions with atypical hyperplasia and endometrial carcinoma for PTEN and Cyclin D1 in our study , it was found to be highly statistically significant ($p < .001$ for PTEN and Cyclin D1).

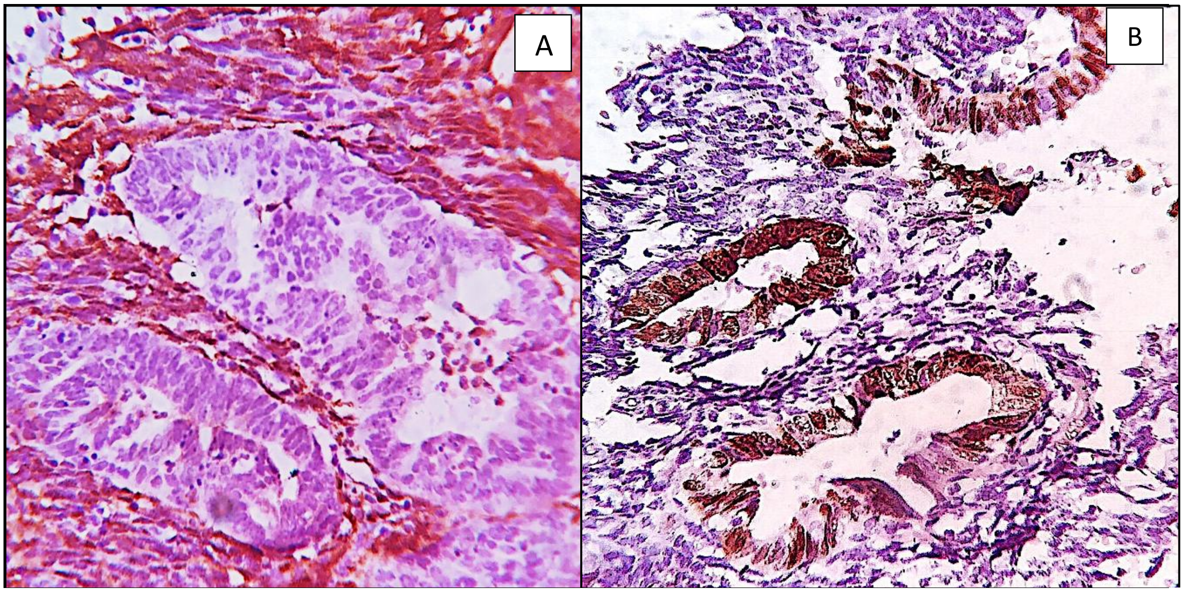

Figure 1: Photomicrograph of atypical hyperplasia, endometrium showing
A) PTEN negativity (40X, PTEN IHC stain, Master Diagnóstica)
B) Cyclin D1 positivity (40X, CyclinD1 IHC stain, Dako)

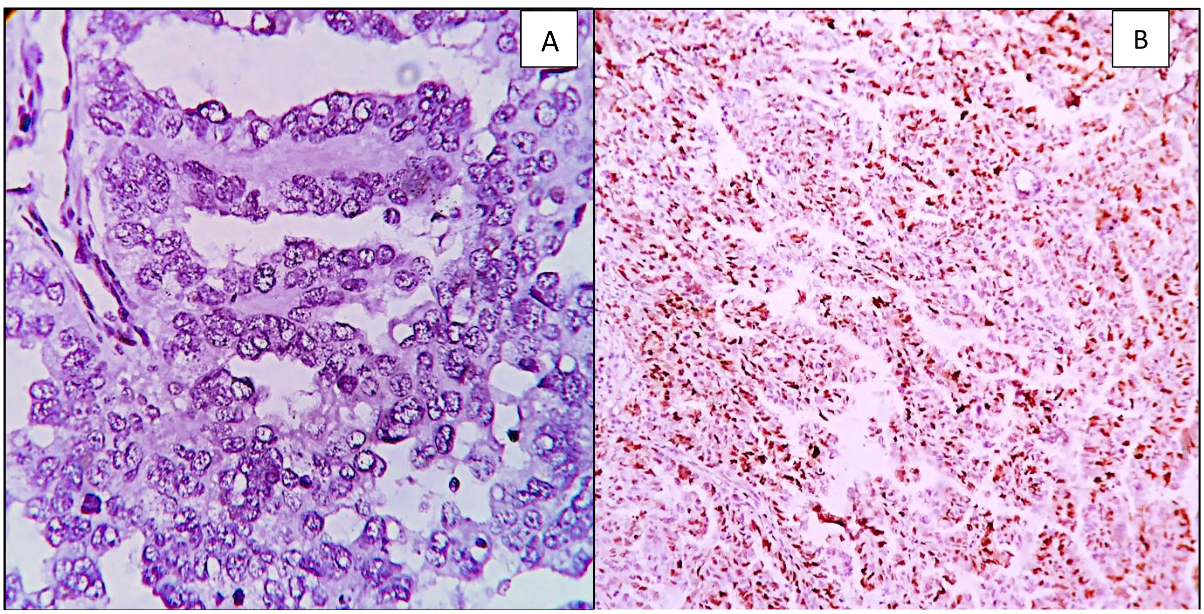

Figure 2: Photomicrograph of Endometrioid carcinoma showing
A) PTEN negativity (40X, PTEN IHC stain, Master Diagnóstica)
B) CyclinD1 positivity (10X, Cyclin D1 IHC stain, Dako)

Discussion:

In the present study endometrial lesions were mostly seen in age group of 41-50 years similar to Shawana et al[10] and Suri et al[11] with age group of 41-50 years and 41-45 years respectively. Endometrial carcinoma was seen in the age group of 61-70 years old with a mean age of 61.00 years which was comparable to Suri et al[11] with age group of 61-65 years old.

PTEN positivity was found in all 47 (100%) cases of proliferative endometrium, 18 (90%) cases in secretory endometrium, which was similar to several studies in which PTEN positivity was seen in 100% of the cases of proliferative and secretory endometrium.[10,12,13,14,15,16] However , in the study done by Tantbirojn et al[17], 100% positivity was seen in proliferative endometrium but only 60% in secretory endometrium.

In hyperplasia without atypia, 20 (95.2%) cases showed PTEN positivity which was similar other studies which showed 100% PTEN positivity.[12,13,15] But other studies reported PTEN positivity in 76%[17], 89%[14], 85.7%[10] and 84.6%[16] of the hyperplasia without atypia cases.

In atypical hyperplasia, 12 (80.0%) cases showed loss of PTEN which was similar to other studies, where there were 100%[16] , 66.6%[10] and 60%[17] loss of PTEN. However , in other studies the loss of PTEN in atypical hyperplasia was 25%[12], 33%[13], 50%[14] and 37%[15] which was much lower compared to our study. This may be attributed to different manufactures of the antibody and methods of antigen retrieval during immunohistochemical staining, our study used antibodies from Master Diagnostica company.

Eleven (91.7%) cases of endometrial carcinoma showed loss of PTEN which is little higher compared to other studies where the loss of PTEN was 60%[17], 52%[12], 70%[14], 61%[15], 61%[10] and 71.4%[16] . This may also be attributed to our smaller sample size of endometrial carcinoma (n=11) and the occurrence of mostly endometrioid type of endometrial carcinoma (91.7%).

Cyclin D1 were positive In 4 (8.5%) cases of proliferative endometrium, 43 (91.5%) cases were negative. In secretory endometrium , 4 (20.0%) cases were positive for Cyclin D1 and 16 cases (80.0%) were negative. However, in the studies done by Suri et al[18] and Thukral et al[16], Cyclin D1 was positive in 30% and 40% of proliferative and secretory endometrium respectively which is much higher compared to our study.

Ten (47.6%) cases of hyperplasia without atypia were positive for Cyclin D1, this finding is similar other studies where Cyclin D1 was positive in 45%[18], 50%[10], 50%[16] and 57%[19] . In contrast, it was much lower in the studies done by Liang et al[20] and Bookya et al[21] where Cyclin D1 was positive in only 30% and 18% respectively.

In atypical hyperplasia, 11 (73.3%) cases were positive for Cyclin D1 which corroborates with other studies.[10,16,19] In the studies of Liang et al[20]and Bookya et al[21] , Cyclin D1 was positive in 49% and 45% of atypical hyperplasia respectively. However, in the study by Suri et al[18] all the cases of atypical hyperplasia , 100% showed Cyclin D1 positivity.

In our study, 10 (83.3%) cases of Endometrial carcinoma were positive for Cyclin D1 which is comparable to the studies done by Suri et al[18] and Thukral et al[16] where 85.71% and 85% each were Cyclin D1 positive. However , in other studies Cyclin D1 was positive in 68%[19], 46.1%[22], 67%[20], 50%[21] and 48%[10] of Endometrial carcinoma which is little lower compared to our study.

The sensitivity and specificity of PTEN for atypical hyperplasia and Endometrial Carcinoma were 85.2% and 96.6% while for Cyclin D1 it was 77.8% and 52.4% respectively in our study. While comparing the various benign endometrial lesions with atypical hyperplasia and endometrial carcinoma using immunoreactivity for PTEN and Cyclin D1 in our study , it was found to be highly statistically significant ($p < .001$, PTEN and $p < .001$, Cyclin D1). Statistical significance was also observed between hyperplasia without atypia and atypical hyperplasia using PTEN ($p = 0.043$) which is comparable to other studies that showed similar observations.[10,15,17] However, there was no statistical significance in immunoreactivity between hyperplasia without atypia and atypical hyperplasia using Cyclin D1 ($p = 0.123$). No statistical significance was also seen between atypical endometrial hyperplasia and endometrial carcinoma for PTEN and Cyclin D1 ($p = 0.60$ and $p = 0.66$) which was also similar to the study done by Sithara et al[15].

Conclusion:

In our study, there was progressively diminishing PTEN expression and increase in Cyclin D1 overexpression from normal proliferative endometrium to hyperplasia without atypia to atypical hyperplasia and endometrial carcinoma (endometrioid type). Thus, it appears that PTEN inactivation and Cyclin D1 overexpression might initiate in precancers from a normal background state, and additional damage accumulates in the transition from premalignant to malignant disease. From the present study, it can be concluded that loss of PTEN expression and overexpression of Cyclin D1 as a potential marker for predicting the subsequent risk of endometrial carcinoma. And, therefore the combined use of histological features and immunohistochemical markers of PTEN and Cyclin D1 in endometrial hyperplasia and endometrial carcinoma may be of great diagnostic utility.

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