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STUDY OF EXPRESSION OF BCL-2 AND Ki-67 IN BIOPSY SPECIMENS OF NORMAL ENDOMETRIUM AND OTHER ENDOMETRIAL LESIONS - AN OBSERVATIONAL STUDY

RUNNING TITLE: Expression of Bcl-2 and Ki-67 in endometrial lesions

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ABSTRACT

Background: Endometrial cancer is one of the common gynecologic cancer and accounts for nearly 5% of cancer cases and more than 2% of deaths due to cancer in women worldwide.Bcl-2 is an anti-apoptotic gene involved in the regulation of apoptosis and Ki-67 is a recognized indicator of cell mitotic activity. Increased expression of both indicates tumorigenesis. Analyzing the expression of these markers in cyclical endometrium, endometrial hyperplasia and in endometrial carcinoma will help in the development of new treatment modalities specific against these markers. **Aim and objectives:** To evaluate the expression of Bcl-2 and Ki-67 in endometrial lesions and to compare the pattern of expression of Bcl-2 and Ki-67 in different endometrial lesions and in cyclical endometrium. **Methods:** Hospital based observational, cross- sectional study was conducted in 57 patient of different cyclical endometrium and endometrium lesions. The selected tissue blocks were processed for hematoxylin and eosin stain and IHC staining for Bcl-2 and Ki-67. **Results:** In this study, 11 cases of proliferative endometrium and 9 cases of secretory endometrium were studied. Mean Bcl-2 score was 7.6 in proliferative phase and 1.3 in secretory phase whereas mean Ki-67 score was 7.27 in proliferative phase and 2.4 in secretory phase. In this study 10 cases of Disordered proliferative phase showed mean score 11.2 of Bcl-2 that was slightly higher than hyperplasia which has Bcl-2 score of 6.2. Of the total 12 cases of hyperplasia 9 cases were of simple hyperplasia and 3 were of atypical hyperplasia. In simple hyperplasia mean score was 6.2 for Bcl2 and 4.8 for Ki-67. In atypical hyperplasia mean score was 4 for Bcl-2 and 2.6 for Ki- 67. **Conclusions:** Bcl-2 and Ki-67 have been found to be reliable markers to indicate the progression of the disease and might be a novel indication for treatment and follow-up.

Keywords: Endometrial lesions, Bcl-2, Ki-67, cyclical endometrium

Abbreviations:

DPP: Disordred proliferative phase/pattern, EC:Endometrial carcinoma

IHC: Immunohistochemistry, EIN: Endometrial Intraepithelial Neoplasia

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EH: Endometrial hyperplasia

Introduction

Endometrial carcinoma is one of the common gynecologic malignancy that accounts for nearly 5% of cancer cases and more than 2% of deaths due to cancer in women worldwide.1Incidence of endometrial cancer and mortality rates have increased in recent years2-4 . Risk factors of endometrial cancer are obesity, ageing, early menarche, late menopause, nulliparity, and postmenopausal estrogen therapy.5

An excess of estrogen relative to progestin, can induce exaggerated endometrial proliferation (hyperplasia), which is a precursor lesion of endometrial carcinoma.6 The incidence of EH is roughly three times higher than EC and certain atypical forms of EH are considered to represent direct precursor lesions to endometrioid EC.7 Bcl-2 is an anti-apoptotic gene involved in the regulation of apoptosis and Ki-67 is a recognized indicator of cell mitotic activity. Increased expression of both indicates tumorigenesis9-14. Thus the pattern of expression in hyperplastic and premalignant states of endometrium helps us to study the progression of these conditions to frank malignancy. Analyzing the expression of these markers in cyclical endometrium, endometrial hyperplasia and endometrial carcinoma will enable in identifying the specificity and sensitivity of these markers in determining the malignant potential15 as well as in the treatment modalities specific against these markers. In this article we will study whether anti-apoptotic and cellular proliferation has role in endometrial neoplasia and if yes, what is their expression profile in various proliferative, premalignant and malignant lesions of endometrium.

Aim and Objectives

Aim:

To evaluate the expression of Bcl2 and Ki-67 in endometrial lesions

Objectives

1. To observe the expression of Bcl-2 and Ki-67 in cyclical endometrium.
2. To compare the pattern of expression of Bcl-2 and Ki-67 in endometrial lesions with that of cyclical endometrium.

Material and Methods

Study setting: This study was conducted in Department of Pathology, Dr. S.N. Medical College, Jodhpur after ethical clearance from the institutional ethics committee. The relevant material was obtained from the records of histopathological section of department of pathology of Dr. S.N. Medical College, Jodhpur. The selected tissue blocks were processed for hematoxylin and eosin stain and IHC staining for Bcl-2 and Ki-67.

Study Design: Observational study

Study participants: All the cases which fulfilled inclusion criteria, were included in the study.

Inclusion criteria:

All endometrial biopsies and hysterectomy specimens received by the department of pathology.

Exclusion criteria

1. Specimen not received in formalin.
2. Blocks with inadequate material.

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Sample selection

Biopsy specimen received between July 2020 to December 2022 in the Department of Pathology of our institute were selected.

Sample size:

Sample size was calculated using the below formula for estimation of single sample mean-

$$N=\frac{Z^2_{1-\alpha/2}\sigma^2}{E^2}$$

Where,

$Z^2_{1-\alpha}$=Standard normal deviate for assumed confidence level (taken as 1.96 for 95% confidence interval)

σ =expected standard deviation of set up error (taken as 2.7 considering this as minimum mean positivity of bcl2 in secretory phase as per reference article)

E=Relative error /relative precision (taken as 10%)

Sample size was calculated to be minimum 28 subjects (cyclical endometrium) and 28 subjects (endometrium neoplastic lesions).

Methodology

Paraffin block of cases that fulfilled the inclusion criteria were selected. Patient name, age, registration number, type of biopsy specimen and its gross feature were noted from histopathology records. Blocks were cut serially at 3 to 5 micron thickness using rotatory microtome to prepare slides. Slides were stained with routine hematoxylin and eosin stain and then mounted with DPX to review, after noting the diagnosis by microscopic details, sections were taken for IHC staining by automated method on Leica BOND RX auto stainer. Golden brown colour membrane and cytoplasmic staining were taken as a positive reaction.

Interpretation

Bcl-2 positivity was indicated by cytoplasmic positivity in glandular and stromal cells. Placenta was used as a control for Bcl-2 cytoplasmic grading, in which the syncytiotrophoblast cells stain for grade 4 positivity[51]. Ki67 positivity was indicated by nuclear positivity in glandular cells. The mean percentage of positive glandular cells for both Bcl-2 and Ki-67in the functional layer of endometrium were determined by counting 1000 cells in 10 randomly selected high power fields. There is no standard grading system for the Bcl-2 antigen. This grading is based on the journals.[47,51,52]

Table 1-Positivity for both Bcl-2 and Ki67 were scored as

Grade 1	**< 25%**
Grade 2	**25-50%**
Grade 3	**51-75%**
Grade 4	**76-100%**

Table 2-Immunostaining intensity were scored as

Grade 1	**Mild**
Grade 2	**Moderate**

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Grade 3	**Strong**
Grade 4	**Very strong**

Weighted score = positivity × intensity.

Bcl-2 stained uniformly all glandular epithelial cells so number of cells showing positivity wese always kept as grade 4. In Ki67 only cells of very strong intensity were counted as positive so intensity was always kept grade 4. So 4 was kept constant. Bcl-2 was graded, mainly based on the intensity and Ki67 was graded, mainly based on positivity and both are multiplied by 4. Thus maximum score was 16, and both Bcl-2 and Ki67 were given a score out of 16. The correlation between Bcl-2/Ki67 and various clinicopathological parameters were analysed and strength of association was calculated using Pearson Chi square test. P values less than 0.05 was considered as significant.

Observations and Results

Table 3: Age group distribution of the study cases

Age (yrs)	No. of patients	Percentage(%)
18-40	12	21.05
41-50	21	36.84
≥51	24	42.11
Total	57	100.00

The age of the patients studied were divided into three groups mainly reproductive, perimenopausal and post menopausal.

Table 4: Distribution of endometrial lesions on histopathology

Endometrial lesions	No. of patients	Percentage(%)
Proliferative phase	11	19.30
Secretory phase	9	15.79
Disordered proliferative phase	10	17.54
Simple hyperplasia	9	15.79
Atypical hyperplasia	3	5.26
EIN	3	5.26
Carcinoma in situ	1	1.75
Carcinoma	11	19.30
Total	57	100.00

A total of 57 cases were studied for Bcl2 and Ki67 expression which included 19.30% of proliferative endometrium,15.79% of secretory endometrium,10% of DPP,9% of simple hyperplasia,5.26% of atypical hyperplasia,5.26 %of EIN, 1.75% of carcinoma insitu,11% of carcinoma.

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Table 5: Expression of Bcl-2 in various endometrial lesions

Endometrial lesions	No. of cases	BCL2 score					
		Negative	Score 4	Score 8	Score 12	Score 16	Mean score
Proliferative phase	11	1	3	4	2	1	7.6
Secretory phase	9	6	3	0	0	0	1.3
Disordered proliferative phase	10	0	0	5	2	3	11.2
Simple hyperplasia	9	2	0	3	2	2	8.8
Atypical hyperplasia	3	2	0	0	1	0	4
EIN	3	2	0	1	0	0	2.6
Carcinoma insitu	1	0	0	0	1	0	12
Carcinoma	11	0	0	0	10	1	12.36
Total	57	13	6	13	18	7	59.86

Bcl-2 showed maximum expression in carcinoma and carcinoma in situ, DPP showed higher expression than hyperplasia .Secretory phase showed decreased expression compared to all lesions.

Table 6: Expression of ki-67 in various endometrial lesions

Endometrial lesions	No. of cases	Ki-67 score					
		Negative	Score 4	Score 8	Score 12	Score 16	Mean score
Proliferative phase	11	1	4	2	4	0	7.27
Secretory phase	9	5	3	1	0	0	2.4
Disordered proliferative phase	10	0	3	4	0	3	9
Simple hyperplasia	9	3	1	1	2	2	7.5
Atypical hyperplasia	3	1	2	0	0	0	2.6
EIN	3	2	1	0	0	0	1.3
Carcinoma in situ	1	0	0	0	0	1	16
Carcinoma	11	0	0	0	2	9	13.09
Total	57	12	14	8	8	15	59.16

Ki-67 showed maximum expression in carcinoma in situ and carcinoma followed by DPP. The expression was reduced in hyperplasia and markedly reduced in secretory phase.

Table 7: Comparison of Bcl-2 and Ki-67 in various endometrial lesions

Endometrial lesions	Mean Bcl-2 score	Mean Ki-67 score
Proliferative phase	7.6	7.27
Secretory phase	1.3	2.4
Disordered proliferative phase	11.2	9
Simple hyperplasia	8.8	7.5

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Atypical hyperplasia	4	2.6
EIN	2.6	1.3
Carcinoma in situ	12	16
Carcinoma	12.36	13.09

Both Bcl-2 and Ki-67 showed higher expression in carcinoma carcinoma in situ, DPP and lowest in secretory phase .

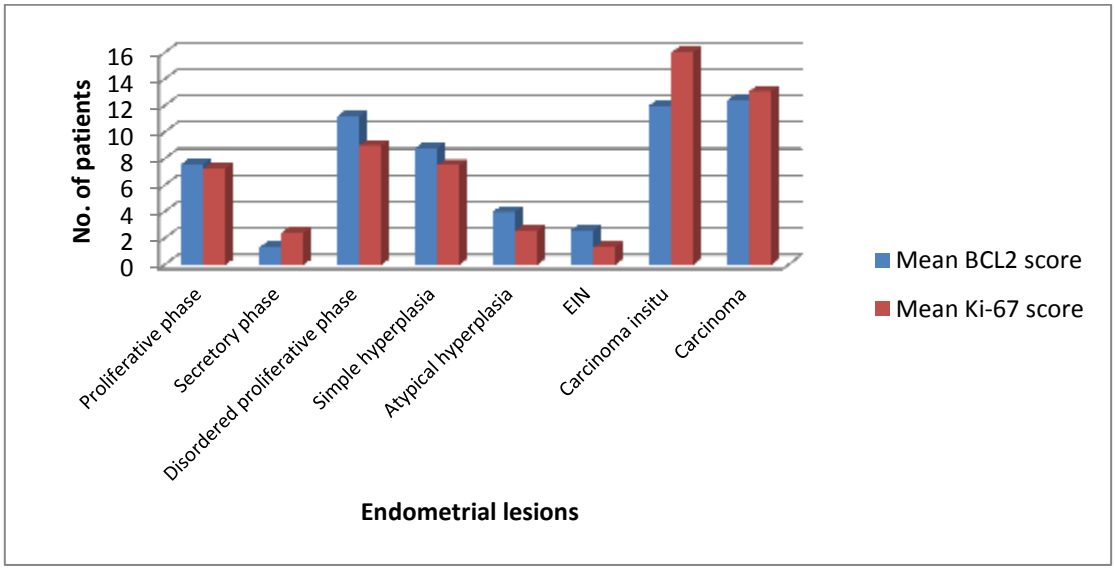

Figure no 1: Comparison of expression of Bcl-2 and Ki-67 in various endometrial lesions

Table 8: Expression of Bcl-2 and Ki-67 in cyclical endometrium

Endometrial lesions	No. of cases	Mean BCL2 score	Mean Ki-67 score
Proliferative phase	11	7.6	7.27
Secretory phase	9	1.3	2.4

Both Bcl-2 and Ki-67 expression were high in the proliferative phase and showed decreased expression in secretory phase.

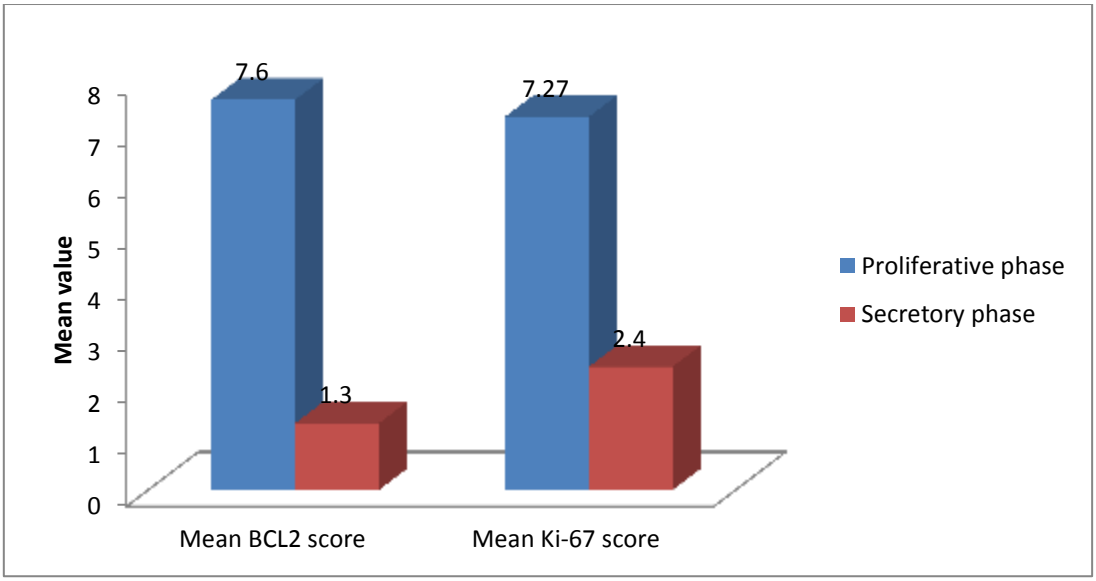

Figure no 2 : Expression of Bcl-2 and Ki-67 in cyclical endometrium

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Table 9: Expression of Bcl-2 and Ki-67 in DPP and endometrial hyperplasia

Endometrial lesions	No. of cases	Mean BCL2 score	Mean Ki-67 score
Disordered proliferative phase	10	11.2	9
Simple hyperplasia	9	8.8	7.5
Atypical hyperplasia	3	4	2.6

Highest expression of both Bcl-2 and Ki-67 were seen in disordered proliferative phase than hyperplasia.

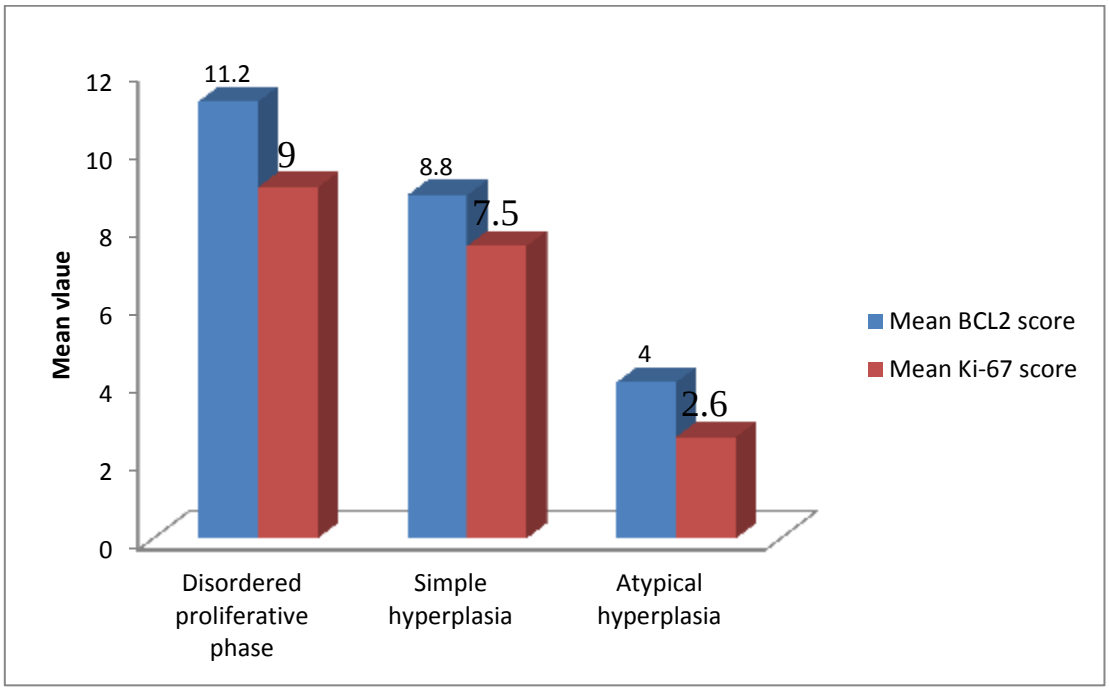

Figure 3: Expression of Bcl-2 and Ki-67 in DPP and hyperplasia

Discussion

A total of 57 endometrial samples were studied for Bcl-2 and Ki-67 expression which included 19.30 % of proliferative endometrium, 15.79% of secretory endometrium, 17.54% of disordered proliferative phase, 15.79% of simple hyperplasia,5.26 % of atypical hyperplasia,5.26 % of EIN, 1.75% of carcinoma in situ and 19.30% carcinoma endometrium. In this study age of the patients ranged from 20-75 years. Nearly half of them belonged to perimenopausal age group (50.5%), followed by the reproductive age group(35%), which is similar to the studies done by Soleymani et al[53] and Sweta et al[54] that can be explained due to the increased incidence of intrauterine lesions in perimenopausal age group. This disagrees with the study done by Deka et al[55] in which reproductive age group was most commonly affected that can be explained by the increased incidence of pregnancy related complications in their study group. In this study , mean Bcl-2 score was 7.6 in proliferative phase and 1.3 in secretory phase whereas mean Ki-67 score was 7.27 in proliferative phase and 2.4 in secretory phase. Thus both Bcl-2 and Ki-67 expression were high in the proliferative phase and did show decreased expression in secretory phase that can be explained due to onset of progesterone production during the secretory phase and increased oestrogen stimulation during the proliferative phase. According to study done by Mertens H J MM[56] et al in 30

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endometrial samples of ovulatory cyclical endometrium, Bcl-2 expression was high in proliferative phase and decreased significantly in the secretory phase, especially in the glandular epithelial cells. Ki-67 also showed the same cyclical pattern with a later onset. According to a study done by T. E. Vaskivuo[57] et al using 39 endometrial samples the results of Bcl-2 expression were increased in proliferative phase and decreased in secretory phase. Ki-67 was detected predominantly in the proliferative phase. A study done by X J Tao[58] et al stated that Bcl-2 immunoreactivity was maximal during the proliferative phase and decreased in thesecretory phase. A study done by A. Gompel[59] et al in 49 endometrial samples of which 26 were proliferative endometrium and 23 were secretory endometrium and stated that Bcl-2 staining peaked at proliferative phase and disappeared with the onset of secretory phase. So results of this study were consistent with that of these previous studies. In this study 10 cases of Disordered proliferative phase showed mean score 11.2 of Bcl-2 that was slightly higher than mean Bcl-2 score of hyperplasia which was 6.2, indicating that anti-apoptotic activity is increased in DPP. This observation was not in concordance with previous studies done by Apostolou et al[60] and Morsi et al[61], that can be explained due to prolonged oestrogen exposure. DPP is characterized by dilated glands that are interspersed among normal proliferative glands. In this study the scoring was done on areas stained with maximum intensity. Due to this, variations in intensity of scoring of Bcl-2 in DPP is unavoidable. Hence furthermore larger studies with higher number of DPP cases subjected to Bcl-2 analysis are recommended to standardize the scoring system for disordered proliferative endometrium. However in this study the risk of progression towards hyperplasia still persists. In this study the expression of Ki-67 was increased in DPP. This observation was in concordance with previous studies done by Morsi et al[60] and Apostolou et al[61]. This indicates that cell proliferation contributes in the pathogenesis of DPP Of the total 12 cases of hyperplasia 9 cases were of simple hyperplasia, 3 were of atypical hyperplasia. In simple hyperplasia mean score was 6.2 for Bcl-2 and 4.8 for Ki-67. In atypical hyperplasia mean score was 4 for Bcl 2 and 2.6 for Ki- 67. In hyperplasia and endometrial carcinoma, the Bcl-2 score showed increased expression in ascending order of frequency from atypical hyperplasia to simple hyperplasia and malignancy. This observation was in concordance with previous studies done by Arjunan et al[62], Morsi et al[60], and Ambros RA et al[63]. This indicates that hyperplastic states which are under the influence of unopposed oestrogenic stimulation, have decreased apoptotic activity. In a recent study done by Travaglino et al, has stated that Bcl-2 protein loss appeared as a highly specific marker of endometrial precursor lesion, with high diagnostic accuracy. Thus, the finding of Bcl-2 protein loss in endometrial hyperplasia might be a novel indication for treatment and follow-up, especially when precancerous features are ambiguous at histological examination [64]. Studies by Morsiet al[60], Kokawa et al[65] and Nunobiki et al[66] noted that Bcl-2 expression to be higher in simple hyperplasias compared with atypical hyperplasias. The finding of decreased Bcl-2 expression in atypical hyperplasia suggests a possible role for Bcl-2 in promoting the malignant transformation of hyperplastic cells. As soon as nuclear atypia was observed, Bcl-2 expression was difficult to detect. In addition, it has been shown in various studies that Bcl-2 overexpression plays an important role in epithelial tumor development 3 cases of EIN and 1 case of carcinoma in situ were studied and expression of both Bcl-2 and Ki 67 increased from EIN to carcinoma in situ. However the sample size is low and more cases need to be studied.

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On comparing Bcl-2 staining and Ki-67 in DPP, hyperplasia and malignancy, the mean score of Bcl-2 expression was higher when compared to Ki-67 mean score. This observation was similar to previous study done by Arjunan et al[62]. But in the study done by Apoustolou et al[61] Ki67 expression was higher than Bcl-2.Hence further studies are needed to understand the role of Bcl-2 and Ki-67 in tumor pathogenesis.

Limitation of the study

Immunohistochemistry is a highly meticulous procedure. The antigen retrieval which is an important step is influenced by various factors such as use of old blocks, tissue fixed in formalin for long period, inadequate time of heating, pH of the buffer etc. Further if sample size is increased better results can be obtained.This study accounted a low incidence of endometrial carcinoma hence various histologic grades and various subtypes could not be studied in detail. Hence this study could be further expanded by including various grades and subtypes of endometrial malignancy and evaluate them histopathologically and immunohistochemically.

CONCLUSION

In cyclical endometrium, Bcl-2 expression was maximum in the proliferative phase and decreased in the secretory phase. Ki-67 expression in cyclical endometrium was maximum in the proliferative phase and decreased in secretory phase.Comparison of Bcl-2 and Ki-67 values in proliferative phase shows a positive correlation statistically. Thus the proliferative endometrium is characterized by decreased apoptotic and increased mitotic activity in response to estrogen. This proves the association of hyper estrogenic states in causing increased proliferation leading to neoplasm. In secretory phase as the endometrium prepares itself for shedding there is increased apoptosis which results in loss of Bcl-2 expression. Mitotic activity is also significantly reduced resulting in decreased Ki-67expression.

Bcl-2 and Ki-67 have been found to be reliable markers to indicate the progression of the disease and might be a novel indication for treatment and follow-up, especially when precancerous features are ambiguous at histological examination.

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