Expression of Ki67 and PTEN in benign endometrial hyperplasia and Endometrial Intraepithelial Neoplasia

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Summary

Uterine carcinoma is the most common neoplastic disease in the female genital tract and progress from a common precursor lesion, atypical hyperplasia. The current study used immunohistochemistry to detect the Ki67 and PTEN expression in 49 endometrial cases distributed as normal, disordered proliferative endometrium, benign endometrial hyperplasia and endometrial Intraepithelial Neoplasia EIN samples. IHC statistical analysis recorded significant differences P≤0.01 all samples in EIN sample especially between normal and atypical hyperplastic lesions. Complete loss of PTEN expression was 66.6% in atypical hyperplastic lesion samples then 13.3% in non- atypical hyperplastic cases. PTEN intensity in non- atypical hyperplastic cases was significant p= 0.0392 compared with normal and atypical hyperplastic tissue which was highly significant intensity P 0.0059, P 0.0023 respectively. In Ki67, all endometrial cases were highly significant P≤0.01. All atypical EH (EIN) showed positive Ki67 expression with high significant p value (0.0074) and 66.6% of cases was in score 3 compared with 3.3% of benign EH. The high percentage of both Ki67 scoring and intensity with null PTEN expression compared with the other hyperplastic lesion confirm the probability of these cases as a precancer lesion, precursor for uterine carcimoma.

Key words: Benign hyperplasia; EIN; PTEN; Ki67

Introduction

Endometrial hyperplasias (EH) consider one of the gynecological lesions that common in most women around the world [1]. It is described by unequal proliferation of an increasing in endometrial glands (gland-to-stroma ratio) when compared with proliferative endometrium [2], there are two groups categorized into EH; non-atypical (benign) and atypical hyperplasia (neoplastic) which mentioned as endometrial intraepithelial neoplasia EIN that is suggested as the precursor of endometrial carcinoma [3]. The important association between EIN and endometroid progression is the important medical for EH diagnosis [4].

The negatively influences action of PTEN in PI3K-AKT pathway reflect the role of PTEN in introducing the carcinogenesis route [5]. As unrestricted estrogens promote proliferation of endometrial glands, glands with PTEN loss may undergo clonal increase and atypical hyperplasia or even carcinoma [6]. The lower expression of PTEN protein in endometrial

Cuest.fisioter.2025.54(2):1-8

precancer and cancer tissues may confirm the loss PTEN expression theory that suggest it as a marker for initial diagnosis through the endometrial cancer progress [7]. Several studies have demonstrated that PTEN is lost as an early event that indicated progression to endometrial carcinoma [8]. In the other side Ki67 which is one of the most well-known biomarker proteins used to identify proliferating [9]. Various studies to date require demonstrated the significance of Ki-67 as a prominent and indispensable diagnostic instrument in pathology for uterine hyperplasia in particular and proliferative tumors in general [10]. This marker is specifically expressed by dividing cells and has a strong relationship to cell growth, this protein's IHC detectable expression is utilized to assess tumor proliferation activity and biological behavior [11]. Its expression in the cell cycle reflects the status of cell proliferation and associated with tumor growth, metastasis, and diagnosis [12]. Thus, we aimed in this paper to observe Ki67 and PTEN expression in hyperplastic tissue (hyperplasia without a typia and endometrial hyperplasia with atypia (EIN).

Materials and Methods

Tissue specimens: 49 endometrial tissue from uterine hysterectomy were collected during March 2023 from the Department of Pathology in Al Karama Teaching Hospital in Wasit Province, Iraq, distributed as; 10 normal endometrium, 12 disordered proliferative endometrium, 15 benign endometrial hyperplasia and 12 endometrial hyperplasia with atypia \ (EIN). The whole hyperplasia’s samples were diagnosed due to the EIN diagnostic and classification criteria [13-14].

Immunohistochemistry: IHC method was achieved by the streptavidin-peroxidase due to the company’s guidelines. The primary polycolonal antibody (PTEN) and Ki67 was purchased from E-LAB-Sciences, China. The sample of endometrial stroma cells and endometrial carcinoma were used as positive controls for PTEN and Ki67 expression respectively. The sections subjected to the standard protocol: deparaffinized sections then boiled in sodium citrate solution, pH 6 in order to de-masking antigen. The endogenous peroxidase blocking was done via incubating of sections in 3% oxygenated water for 30 minutes at room temperature, and sections washed in distilled water for 10 minutes and then in 1% phosphate-buffered saline (PBS) for 5 minutes. Blocking of nonspecific sites. Then sections incubated with primary antibodies, for overnight at 40c in a refrigerator. Then the secondary antibody was applied at room temperature for 30 minutes and washed in 1% PBS (5 minutes for each 3 path), Streptavidin–HRP

(Horseradish peroxidase) at room temperature for 30 minutes, then washed in 1% PBS. The sign was noticed by using 3.3'-Diaminobenzidine (DAB). Then tissues contrasted with Mayer's Hematoxylin. The intensity percentage of positive cells was measured under high power microscope (40x). Ki67 and PTEN intensity presented cytoplasmic or nuclear brown staining or both of them, loss of PTEN immune expression in both nuclear and cytoplasmic level refers as (PTEN null gland) [15-18]. The expression on PTEN staining at the nuclear and cytoplasm epithelium level on different states was recorded as; phase 0: (-) negative expression, phase 1: (+1) poor expression, phase 2: (+2) moderate expression, phase3 (+3) strong expression. While ki67 immunostaining calculate through counting the positive nuclei in 100 epithelial cells in 10 high powered fields and the total number of positive cells was expressed as a percentage of 1000. Then they are graded as follows based on their positivity and the intensity as 1 - 25% - week, 26 - 50% moderate, 51 - 75% strong and >76 very strong [19,20].

Statistical analysis: The Statistical Analysis System- SAS (2018) program was used to detect the effect of difference groups in study Parameters. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability). T-test was used to significant compare between means in this study. Frequencies and percentages were employed to describe categorical variables, whereas mean SD was utilized to characterize continuous variables (Al-Nuaimi et al., 2020).

Results

Of the 49 cases, 27 endometrial cases diagnosed as hyperplasia distributed as 15 in benign endometrial hyperplasia, 12 in hyperplasia with atypia\EIN. Diagnoses of hyperplasia's sections were done due to (EIN WHO classification) and pathological criteria, endometrial hyperplasia without atypia or benign hyperplasia was described by a more glandular crowded than normal endometrium, and by slight alterations of the glands construction. Abundant of glands were capricious in size and shape, some with a glandular distention up to a cystic, with a glandular cystic feature hyperplasia (figure 1, A). While in hyperplasia with atypia, glands appear in an irregular outline, papillary intra-glandular proliferations, with stratified epithelium, loss of polarity, evident of nuclear atypia, and atypical mitoses. In most cases, the glands were particularly irregular shape and size, organized "back to back" (figure 1, B).

All endometrial cases were statistically significant p-value 0.01 to PTEN expression. On comparing the reaction of PTEN in normal, disordered proliferative, EH with and without atypia; entirely cases of studied normal endometrial and EH that lacked any sign of cytological atypia presented strong cytoplasmic expression of PTEN, while most of atypical EH samples presented decreased intensity of PTEN expression . Complete loss of PTEN immunoreactivity was found

in 66.6% of atypical hyperplastic tissue and 13.3% of non atypical hyperplastic lesions while normal and disordered proliferative endometrial cases not recorded complete loss (Table 2). This study showed that Pten intensity was high significant within the normal, disordered and a typical endometrial cases with p- value 0.01 while less in non atypical endometrial cases with p – value 0.05 (Table 3). A highly significant association was observed when the expression of PTEN in normal endometrial tissues was compared to that in EIN specimens. Almost all the included normal endometrial specimens showed strong PTEN immunostaining, while none of the studied EIN specimens presented strong expression. PTEN staining was evaluated in cases of EH without atypia versus with atypical, (13\15) cases of EH without atypia recorded positive PTEN immunostaining and the higher expression was showed in +1 intensity (10\15), +2 intensity (3\15) while (3\15) cases showed negative staining, in the other side, the great majority of atypical EH specimens (8/12 cases) showed negative staining and (4\12) as +1 intensity while no specimens (0\12) showed strong positive staining (tables 3). So PTEN intensity in non -atypical endometrium recorded was statistically significant p-value (0.0392) while highly significant the other endometrial cases ($P \leq 0.01$). The statistical analyzed within intensity levels showed that the (mild intensity) in all cases was significant (p- value = 0.489) compared with moderate and strong intensities which were high significant $P \leq 0.01$ as $P=0.0047$ and 0.0002 respectively.

Regarding the extent of the response of the studied samples to the ki67 proliferative marker, the results showed that the entire (10\10) 100% of normal endometrium showed negative ki67 expression, only (2\12) 16.6% of disordered proliferative endometrial sections showed positive reaction and (10\12) 83.3% with negative reaction. In endometrium with non atypical hyperplasia (5\15) 33.3% of cases showed positive reaction and the other 10 (66.6%) samples recorded negative expression. Wheres all atypical hyperplastic samples showed positive expression (12\12) 100% (Table 4). The percentages of Ki67 expression among different endometrial lesions recorded negative expression (10\10) 100% in normal endometrial sample. The highest percentage recorded in endometrium samples with atypical hyperplasia, all atypical EH samples (10\10) showed positive expression distributed as (2\10 in scores 2) (2\10 in score 4) and the highest number in score3 (8\10). in endometrium samples with non atypical hyperplasia only (5\15) sample showed positive ki67 expression distributed as (2\15) in score1, (2\15) in score 2 and (1\150 in score 3 while (10\15) appeared negative reaction to ki67. the lower ki67 reaction recorded in disordered proliferative endometrium samples as only (2\12) showed

positive cytoplasmic reaction without any nuclear reaction. It's noted that Ki67 scoring in atypical endometrial cases was increased significantly with p- value (0.0074) compared with the other endometrial cases (Table 4). In the other side, the intensity of Ki67 immunstaining results showed that (8\12) sample of endometrium with atypical hyperplasia recorded (intensity3) and the remaining samples were distributed equally, two for each 1 and 2 intensities. The intensity of the positive ki67 immunostaining samples of endometrium with non atypical hyperplasia were recorded in (intensity 3) in all positive samples as (5\15) intensity 2. In disordered proliferative endometrium samples, there was only (2\12) recorded (intensity 1) and the others showed no intensity while all normal endometrial sample (10\10) showed (intensity 0). The statistical analyzed of Ki67 intensity between endometrial cases showed significantly increasing in atypical hyperplastic tissue especially in (intensity 3) in (0.0067) p- value (Table 5).

Microscopic section analysis of IHC Ki67 immunostaining area in normal endometrium showed negative expression at both nuclear and cytoplasmic level (score0, intensity 0) (figure 5, A) and as low to moderate cytoplasmic expression in in Benign EH (score 1, intensity 2), which is noticed that ki67 cytoplasmic expression was more obvious than nuclear expression in Benign EH at scoring and intensity levels (figure5, B). While in EIN sections which is recorded the highest ki67 expression, the IHC Ki67 immunostaining area in endometrium with EIN hyperplasia showed strong nuclear and cytoplasmic expression (score 4, intensity 3) (figure 6, A) and strong cytoplasmic expression and low nuclear expression (score 2, intensity 2). In general both glandular and stromal expression to ki67 was higher in EIN sample than Benign EH (figure 7). In (figure 8) comparison between Ki76 and PTEN immunostaining in both EIN endometrium and benign endometrial hyperplasia specimens, the IHC staining confirm that strong Ki67 intensity and low or negative PTEN expression related with atypical endometrium hyperplasia according to the proliferative and apoptosis activity.

Table 1: Comparison between Endometrial cases in results of PTEN immunostaining

Cases	No.	PTEN Negative	PTEN positive	
			1+	2+
Normal endometrium	10	0 (0%)	1(10%)	9 (90%)
Disordered proliferative	12	0 (0%)	4 (33.3%)	8 (66.6%)

Benign EH	15	2 (13.3%)	10 (66.6%)	3 (20%)
EIN		8 (66.6%)	4 (33.3%)	0 (0%)
Chi-Square (P-value)	---	7.508 ** (0.0087)	9.361 ** (0.0017)	8.955 ** (0.0024)
**** (P≤0.01).**				

Table 2: Immunohistochemical of PTEN null expression between study cases

cases	**N.**	**Null PTEN Numbers\ percentage**	
Normal endometrium	10	0 (0 %)	**** (P≤0.001)**
Disordered proliferative	12	0 (0 %)	
Benign EH	15	2 (13.3 %)	
EIN	12	10 (83.3%)	

Table 3: PTEN intensity in endometrial study samples

Endometrial cases	**No.**	**PTEN intensity**				**Chi-Square (P-value)**
		Negative	Mild	Moderate	Strong	
Cyclical endometrium	10	0 (0%)	0 (0%)	0 (0%)	10 (100%)	9.175 ** (0.0023)
Disordered proliferative	12	0 (0%)	1 (8.3%)	2 (16.6%)	9 (75.0%)	8.694 ** (0.0041)
Benign EH	15	2 (13.3%)	3 (20.0%)	8 (53.3%)	2 (13.3%)	5.317 * (0.0392)
EIN	12	8 (66.6%)	3 (25.0%)	1 (8.3%)	0 (0%)	7.402 ** (0.0059)
Chi-Square (P-value)	---	7.261 ** (0.0054)	3.365 * (0.489)	7.516 ** (0.0047)	9.374 ** (0.0002)	---
**** (P≤0.05), ** (P≤0.01).**						

Table 4: The percentage of Ki67 expression among different lesions of endometrium cases

Endometrial cases	**No.**	**Proportion score**					**Chi-Square (Pvalue)**
		Score1	Score2	Score3	Score4	Negative	
Normal endometrium	10	-	-	-	-	**10 (100%)**	9.97 ** (0.0003)
Disordered proliferative	12	**2 (16.6%)**	-	-	-	**10 (83.3%)**	9.61** (0.0005)
Hyperplasia without atypia	15	**2 (13.3%)**	**2 (13.3%)**	**1 (6.6%)**	-	**10 (66.6%)**	9.07** (0.0012)
Hyperplasia with atypia	12	-	**2 (16.6%)**	**8 (66.6%)**	**2 (16.6%)**	-	7.65 ** (0.0074)
**** (P≤0.01).**							

Table 5: Endometrial cases in connection with the color intensity of Ki67 immunostaining

Endometrial cases	**No.**	**Ki67 Intensity**				**Chi-Square (P-value)**
		I=0	I=1	I=2	I=3	
Normal Endometrium	10	10 (100%)	0 (0%)	0 (0%)	0 (0%)	9.71 ** (0.0004)
Disordered Proliferative	12	10 (83.3%)	2 (16.6%)	0 (0%)	0 (0%)	9.58 ** (0.004)
Hyperplasia without atypia	15	10 (66.6%)	0 (0%)	5 (33.3%)	0 (0%)	9.53 ** (0.0006)
Hyperplasia with atypia	12	0 (0%)	2 (16.6%)	2 (16.6%)	8 (66.6%)	7.89 ** (0.0067)
**** (P≤0.01).**						

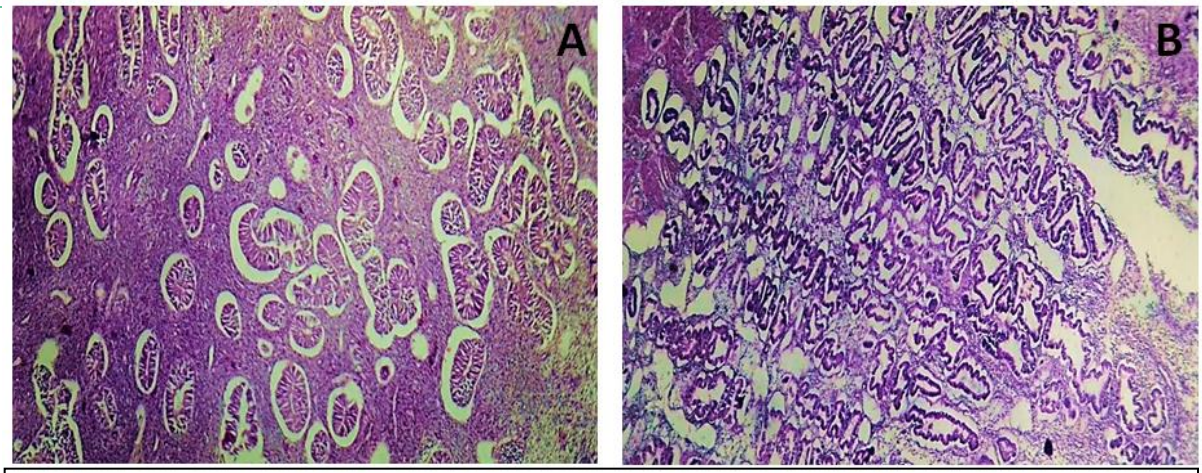

Figure 1: Microscopic section of endometrium showed (A) non-atypical hyperplasia (B) endometrium with atypical hyperplasia. H&E stain, 40X.

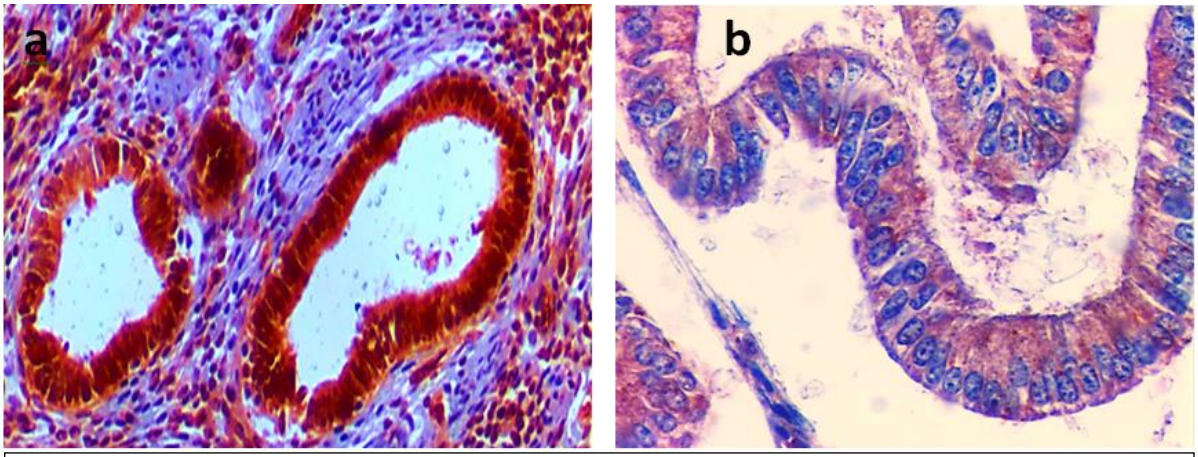

Figure 2: Microscopic section of IHC PTEN immunostaining area in (A) normal endometrium showed strong nuclear and cytoplasmic expression (B) moderate cytoplasmic expression in in Benign EH. 200x.

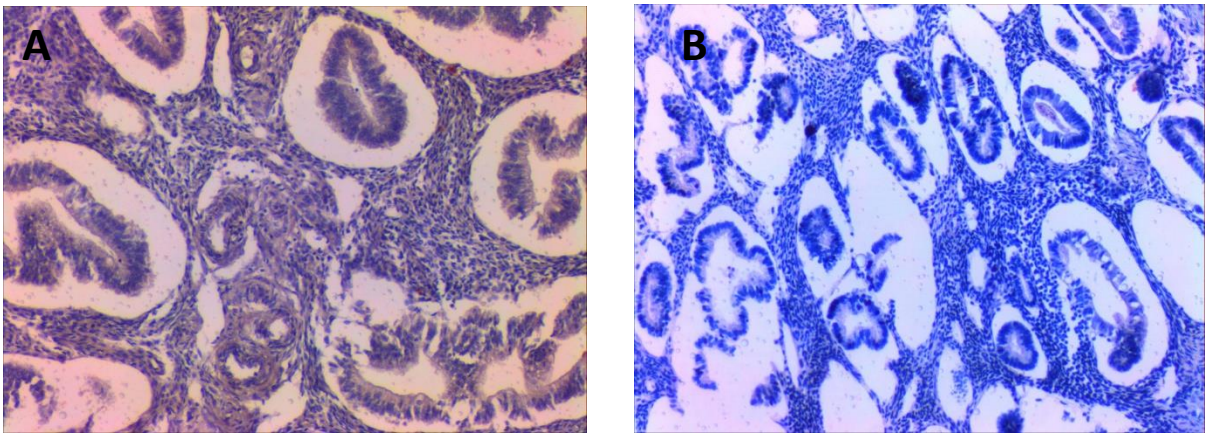

Figure 3: Microscopic section of IHC PTEN immunostaining area in endometrium with EIN hyperplasia (A) very low cytoplasmic expression (B) no cytoplasmic expression. 100x.

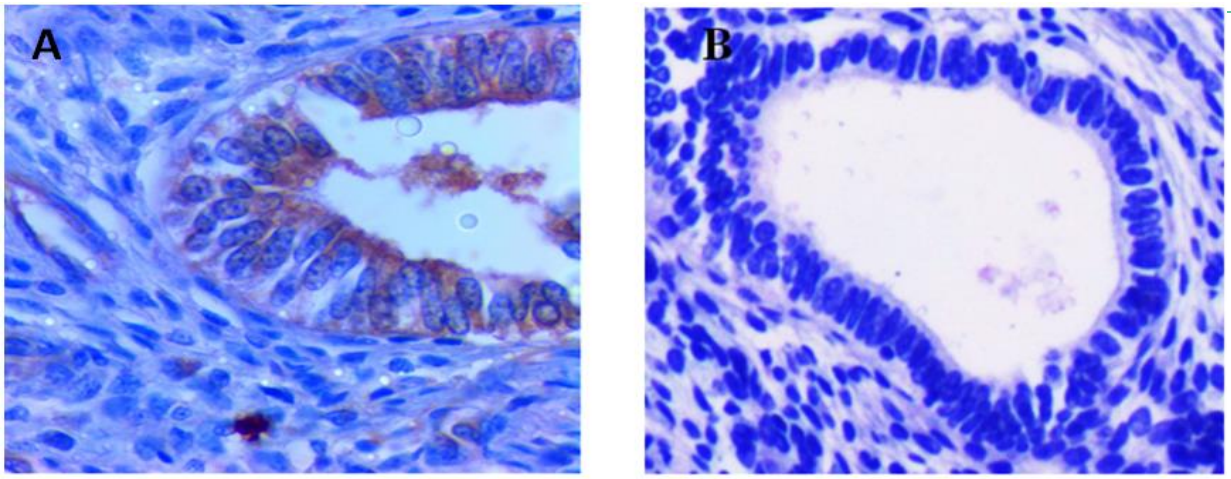

Figure 4: Microscopic section of IHC PTEN expression showed moderate intensity of glandular epithelia in Benign EH sample (A). No PTEN expression (null intensity) in endometrial gland and stromal of EIN sample (B). 400x.

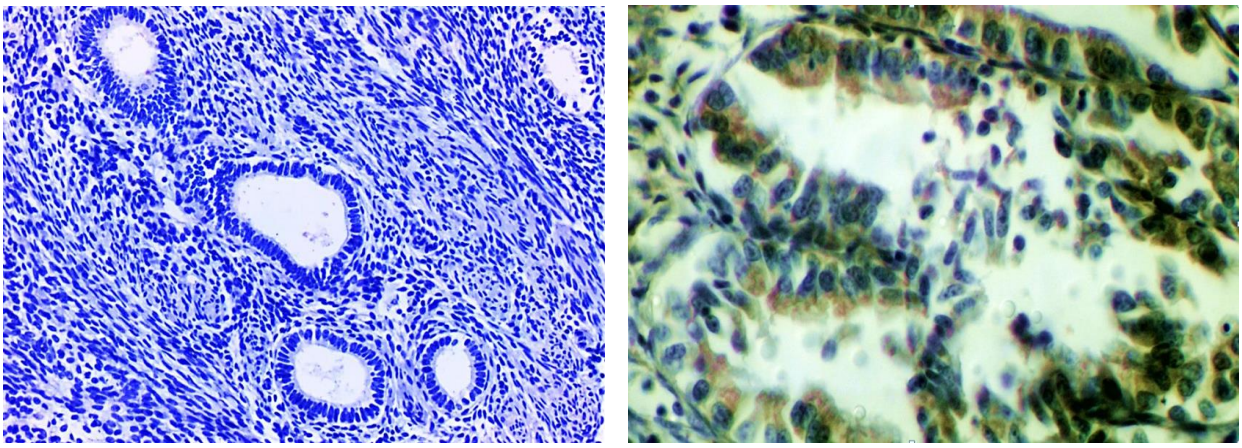

Figure 5: Microscopic section of IHC Ki67 immunostaining area in (A) normal endometrium showed negative nuclear and cytoplasmic expression (score0, intensity 0) (B) moderate cytoplasmic expression in in Benign EH (score 1, intensity 2). 100x.

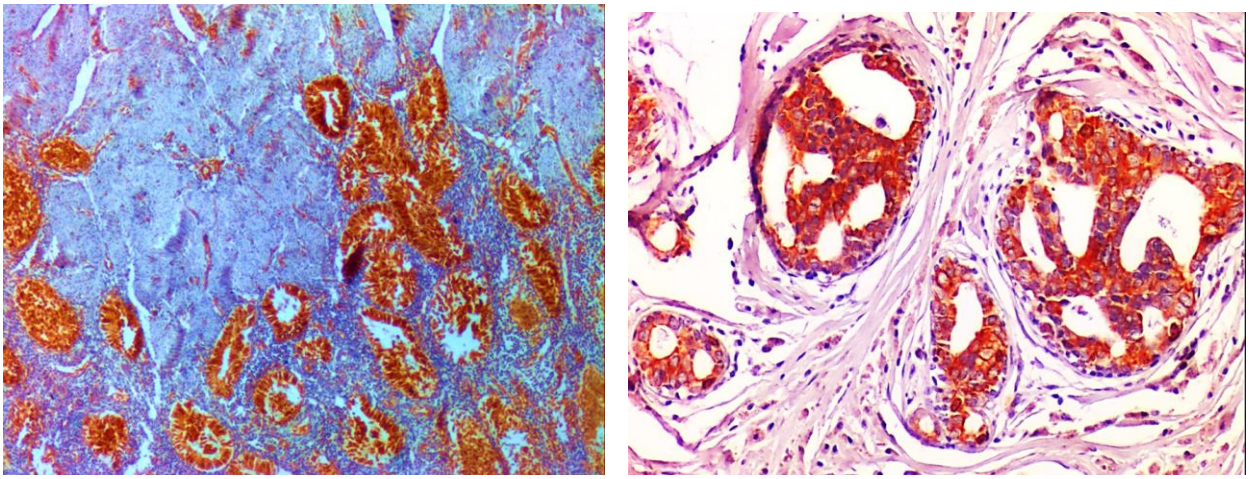

Figure 6: Microscopic section of IHC Ki67 immunostaining area in endometrium with EIN hyperplasia (A) strong nuclear and cytoplasmic expression (score 4, intensity 3) 40x, (B) strong cytoplasmic expression and low nuclear expression (score 2, intensity 2). 200x.

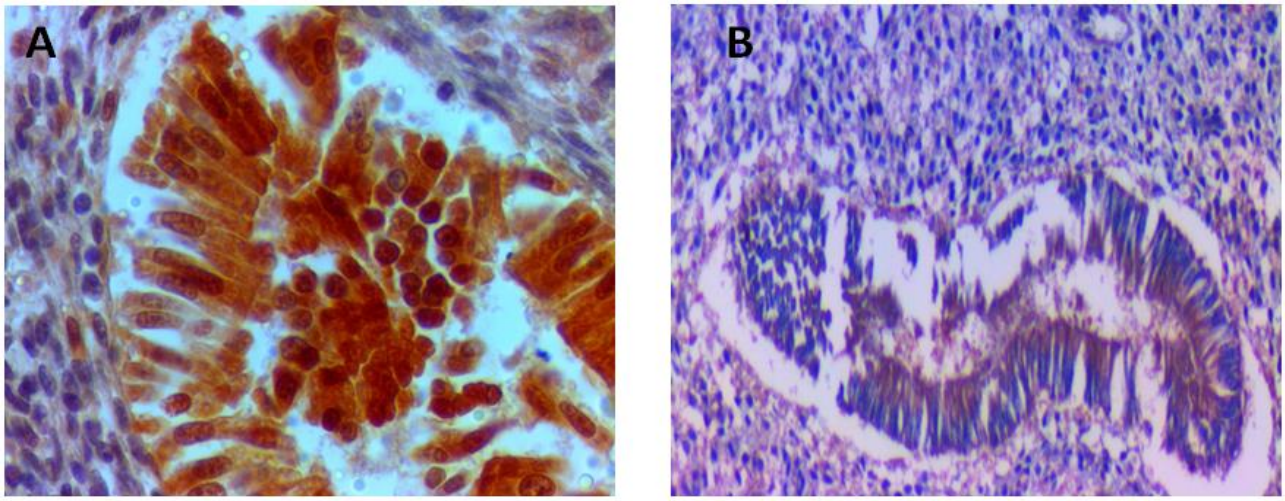

Figure 7: Microscopic section of IHC Ki67 immunostaining compared between benign EH and EIN. (A) strong intensity in glandular and stromal endometrium with EIN (A.) low\ moderate cytoplasmic intensity in gland and stromal endometrium with Benign EH (B) 40x.

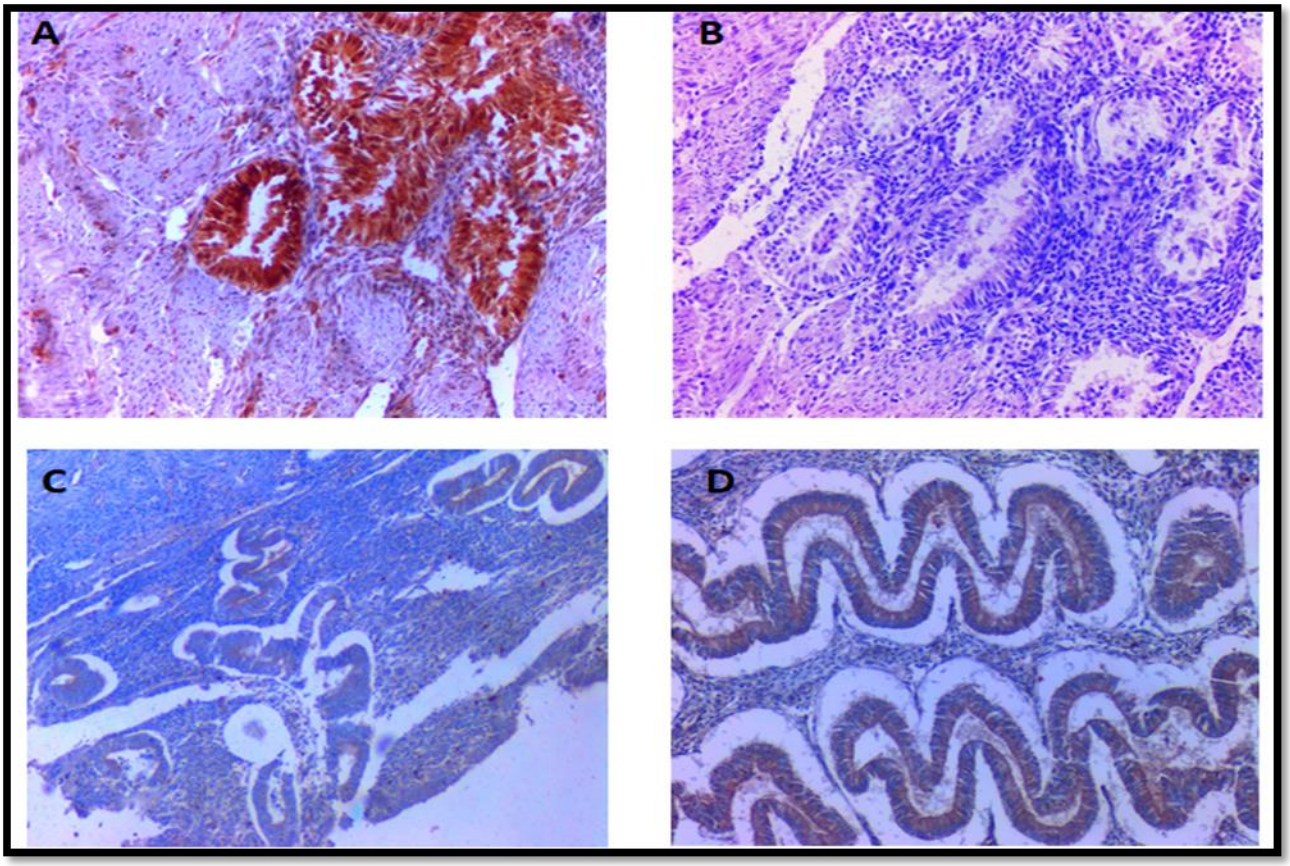

Figure 8: Microscopic sections of endometrium showed differences in ki67 and PTEN expression between benign and EIN hyperplasia. **(A)** EIN showed strong Ki67 expression**,(B)** loss18PTEN expression while **(C)** glandular endometrium with benign EH showed low PTEN intensity, **(D)** moderate PTEN expression.

Discussion

Endometrial hyperplasia is morphological views representing a stage of proliferation endometrial glands with or without cytonuclear atypia that most frequently development to a malicious lesion. The problems of differential diagnosis were considered through the atypical hyperplasia, particularly when showing signs of endometrioid carcinoma. Atypical hyperplasia and endometrial carcinomas are most often identified on endometrial biopsy sections. In medical practice, there is some times an inconsistency between the diagnoses of atypical hyperplasia on the endometrial biopsy and the slice picked after surgery, with a various expression of confident immunohistological markers [21].

In this study, the PTEN immunoexpression analysis showed highest stage intensity (3) at nuclear and cytoplasmic level in the glandular epithelium of normal endometrium, but no expression at nuclear level hyperplasias. At cytoplasmic levels, normal endometrial record the highest expression and decreasing in hyperplasia without atypia until reach (0 stage) in EIN. Some author's such as Stoenescu and his team [22] records strong PTEN nuclear expression in hyperplasias with and without atypia, at cytoplasmic reaction, PTEN expression was (o negative) in EH without atypia, which are different our results. But the same author recorded similar results which were accepted with our findings related to normal, cytoplasmic and null PTEN expression in EIN samples. Some authors refers that the strong immunoexpression found only in non-typical hyperplasia [23]. Some researchers presented in their revisions that PTEN loss expression can be used as a key in the diagnosis of atypical hyerplasia, as the atypical glands are strong negative in contrast to the normal which are strong PTEN positive [24], which are accepted with our findings. Other authors showed that the loss of PTEN was noticeable in benign and cancerous endometrium, but with different intensity [25]. Completely loss of PTEN expression in non atypical hyperplasia not recoded in our results, Lakhani *et al*. [26] indicate that PTEN loss expression is rarely occur in non atypical hyperplasia, and if happened, require closer continuation to estimate for atypical or cancer of endometrium.

The (PI3K-AKTpathway) is affected with null PTEN reaction and cause un controlling in proliferation and apoptosis of cells which lead to neoplasm progress [27]. Matias-guiu et al. [28] record some mutation and decrease of PTEN expression through the hyperplastic phase. Prat *et al*. [29] refers to the same records related to inactivation of PTEN through the hyperplasias types, As well as, Doll *et al*. [30]. Mutter and his team, [31] establish diminished expression in 75% of cases at the precancer stage. Samulak et al. [32] reported that loss PTEN expression was increase in cancer and precancer of endometium compared with normal and nontypical hyperplasia. The first deta analysis to examine the effectiveness of PTEN as predictive marker was recorded by

Raffone et al.[33], he recommended that PTEN reaction should be reevaluated and highlighting the requirement to recover its diagnosis[33].

On the other hand, Ki67 protein expression is supposed to be a valued marker for cell growth and proliferation [34]. As the growth of tumor tissue depends on its proliferative activity [35], the expression of the Ki67 proliferative marker was calculated in this study to examine the potential diagnostic roles of it in hyperplastic tissues. The gradual and progressive increase in the expression of Ki67 levels among the examined endometrial tissues may be due to the physiological effect of estrogenic stimuli. Consequently, when these two cellular proteins are decontrolled, they probably have a role in endometrial carcinogenesis [36]. While the high mean score of Ki67 in young women is related to an elevated rate of cellular proliferation and clarifies a highly aggressive type of carcinoma [37-39]. Ki67 data was accepted with Farhood et al. [40] when they record that ki67 mean score was higher in atypical hyperplastic tissue than non atypical one. Also Geethanjali [41] and Al-Nuaimy et al. [20] in his thesis confirm the results in this study when he refer that all hyperplastic tissues recorded Ki67 positive expression and the ki67 mean score was higher in atypical hyperplastic tissue than non atypical. But Al-Nuaimy et al. [20] findings about Ki67 intensity in atypical hyperplastic tissue were unaccepted with our finding when he recorded moderate intensity of Ki67 expression instead of strong intensity that appears in our immunostaining analysis. As exposed in this study, Stoenescu, showed that cases of hyperplasia and carcinoma showed more iKi67 intensity staining in comparison with those of benign situations [22].

Conclusion

Nuclear and cytoplasmic analyses of PTEN marker express diverse reactivity between normal endometrium and hyperplasias, also between hyperplasia with and without atypia. Completely loss PTEN expression was recoded in hyperplasia with atypia which may indicate for precancer lesion. A significant connotation between Ki67 expression and poor tumor features concluded. Significant differences was shown between the expression of the Ki67 in sections of hyperplasia with atypical feature and without aypical , suggestion the using of Ki67 as diagnostic and prognostic tool in endometrial hyperplasia with atypia and carcinoma. Further estimation of the features related with the IHC manifestation, collected with an elongated term monitoring of women, and could be beneficial in thoughtful the progressions that happened in the development of endometrial lesions to neoplastic.

Acknowledgment

Thanks to all women who contribute with their data in this work. Special thanks to the staff of pathology and gynecology departments in Al Karama Teaching Hospital in Wasit Province, Iraq for their kind help.

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