RESEARCH ARTICLE

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Role of Morphometry and Matrix Metalloproteinase-9 Expression in Differentiating between Atypical Endometrial Hyperplasia and Low Grade Endometrial Adenocarcinoma

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Abstract

Background: Endometrial carcinomas are common gynecologic malignancies worldwide. In Egypt they represent 2.6 %. We evaluated the role of morphometry and MMP-9 immunohistochemical expression to differentiate atypical endometrial hyperplasia from low grade endometrial adenocarcinoma. **Methods:** 60 cases of endometrial lesions that included 25 cases of complex endometrial hyperplasia with atypia, 25 cases of low grade endometrioid adenocarcinoma, in addition to 10 cases of proliferative endometrium as a control group. Morphometric measurements and D-score were evaluated. MMP9 was performed using streptavidin –biotin immunoperoxidase system. **Results:** D score was more than 1 in 100% of cases of proliferative endometrium. In atypical hyperplasia 28 % of cases had a D-score more than 1, 44% less than 0 and 28% of cases had a D score between 0 and 1 with uncertain prognosis. All carcinoma cases had D-score less than 0. MMP9 was positive in all cases of the study but differ in its degree of expression; proliferative endometrium with low expression. Atypical hyperplasia divided as 52% low expression and 48% high expression. Most of the Endometrial adenocarcinoma cases (92%) showed high expression. There was significant difference in expression of MMP9 in atypical endometrial hyperplasia and endometrial adenocarcinoma ($p > 0.001$). **Conclusion:** The relation between MMP9 expression and D-score value in cases of atypical endometrial hyperplasia was highly significant P>0.001Thus, incorporating both MMP9 immunoexpression and D-score value would increase the accuracy of diagnosis of atypical endometrial hyperplasia and low grade endometrial adenocarcinoma.

Keywords: Endometrial adenocarcinomas- atypical endometrial hyperplasia- D score- MMP9

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Introduction

Endometrial hyperplasia has a great clinical significance. It is thought to be a precursor lesion of endometrial carcinoma (Tavassoli and Devilee ,2003), where a continuum has been observed between atypical endometrial hyperplasia and endometrioid adenocarcinoma (Park et al., 2003).

Morphometric analysis, is a quantitative method with objective and reproducible results and could be a very useful supplement in the diagnosis, prediction of prognosis and treatment planning for certain types of cancer (Prvulović et al., 2010). MMP9, a member of the matrix metalloproteinases (MMPs), plays a critical role in breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as tumor metastasis. MMP9 is secreted from cells and once activated, is thought to degrade collagen in the extracellular matrix, which promotes spread of tumor cells (Yu et al., 2012). Our study aimed to evaluate the role of morphometry and MMP-9 immuno-histochemical expression in differentiating atypical endometrial hyperplasia from low grade endometrial carcinoma.

Materials and Methods

This is a retrospective study that was carried out at the pathology departments of National Research Centre and Faculty of Medicine, Zagazig University. Sixty formalin fixed, paraffin embedded tissue blocks of endometrial lesions comprised the material of this study. Studied cases were categorized into the following groups:

1. 10 cases of proliferative endometrium.
2. 25 cases of atypical endometrial hyperplasia.
3. 25 cases of low grade endometrial adenocarcinoma.

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Two sections of 4µm thickness were cut from each block. One section was stained with hematoxylin and eosin for histopathological evaluation, grading, and morphometric study. The other section was mounted on positively charged glass slides for immunohistochemical staining using anti-MMP9 antibody.

All specimens had confirmed pathological diagnoses. Computerized morphometric analysis was done at the pathology lab, MRCE unit, National research Centre using image analysis system Leica Qwin DW3000 (LEICA Imaging Systems Ltd, Cambridge, England) which consists of Leica DM-LB microscope with JVC color video camera attached to a computer system. Analysis covers gland architecture measurement and core measurement. In the selected specimens diagnosed as hyperplasia or carcinoma further selection of those endometrial areas exhibiting the most outspoken features of the deviation was done.

Immunohistochemical study

For immunostaining, the sections were deparaffinized and rehydrated through a graded series of alcohol. Endogenous peroxidase activity was blocked by freshly prepared 0.3% hydrogen peroxide in methanol for 20min. Microwave antigen retrieval was used, followed by incubation with MMP9 Rabbit Polyclonal Antibody (product code; Thermo Scientific/Lab Vision Corporation, Fermont , USA, clone no Cat. #RB-9234-R7, 1: 100 dilution) boiling tissue sections in 10mM citrate buffer, pH6.0 for 10-20 min followed by cooling at room temperature for 20 min. The Ultra vision LP polymer system (Lab vision, California, United States) and the chromogen diaminobenzidine were used to amplify and visualize the antigen–antibody complex.

For Morphometric Analysis

The morphometric analysis was carried out on hematoxylin and eosin-stained slides to measure the glandular area at 50x magnification and the nuclear area at magnification 400x. The areas to be measured are covered automatically by a green mask, which is called a binary image. The area of this binary image is then calculated which reflects the area of object to be measured. The reading of each measurement appears in micrometers and finally the mean area in all fields examined is determined.

Different variables measured in the D Score Equation

• *Glandular*

i)Volume percentage of stroma (VPS), which assesses the percentage of endometrial tissue composed of stroma (i.e., the inverse of glandular percentage, a measure of crowding). The volume percentage stroma equals 100 percent minus the volume percentage of glands.

ii) Gland outer surface density (OUTSD), which is a measurement of basement membrane length about the endometrial glands (measurement of gland complexity).

The formula for the calculation of the outer surface of endometrial glands is expressed as Surface per Volume (S/V), or surface density (mm^2/mm^3). Due to dimension reduction, such a surface is represented in the section as a line; and the surface density as surface line length per area (mm/mm^2). OUTSD equals Perimeter of endometrial glands in a field over the total area of that field.

• *Nuclear*

Nuclear Measurements were made at a total magnification 400x (an objective lens 40x and an eye lens with 10x). The selected nuclei had an intact wall that was outlined manually using a cursor and the shortest core axis of 100 cores were determined using a computer program.

The standard deviation of shortest nuclear axis (SDSNA) calculated automatically.

D-score calculation

Incorporating volume percentage stroma (VPS), standard deviation of shortest nuclear axis (SDSNA), and gland outer surface density (OUTSD) by this equation:

$D = 0.6229 + (0.0439 \times VPS) - (3.9934 \times Ln(SDSNA)) - (0.1592 \times OUTSD)$.

Where Ln stands for natural logarithm.

According to their results cases were divided into three categories (Khedr et al., 2008):

• D-score <0 (unfavorable or endometrial intraepithelial neoplasia) of which carcinoma may develops.

• D-score > 1 (favorable prognosis).

• 0< D-score ≤ 1 (uncertain prognosis) (Orbo et al., 2000).

Statistical Analysis

All data were collected, tabulated and statistically analyzed using SPSS 22.0 for windows (SPSS Inc., Chicago, IL, USA) and Med Calc 13 for windows (Med Calc Software bvba, Ostend, Belgium). Categorical qualitative variables were expressed as absolute frequencies (number) and relative frequencies (percentage). Categorical data were compared using Chi-square test or Fisher's exact test when appropriate. Spearman's rank correlation coefficient was calculated to assess relationship between various study variables. P-value < 0.05 was considered statistically significant (S), p-value < 0.001 was considered highly statistically significant (HS), and p-value ≥ 0.05 was considered statistically insignificant (NS).

Results

Demographic data: Patients diagnosed with proliferative endometrium ranged in age between 29 and 56 years with a mean of 46.5±8.6 years. In atypical endometrial hyperplasia, the age of the patients ranged between 31 and 60 years with a mean of 47.3±9.7 years; while in cases of endometrial adenocarcinoma, the age ranged between 45 and 70 years with a mean of 54.6±8.4 years.

Results of morphometric analysis: D-score was calculated for all cases. Its value was more than 1 (low risk) in all cases of proliferative endometrium (100%) and in 28% of cases of atypical endometrial hyperplasia. While it was less than 0 (high risk) in all cases of endometrial carcinoma (100%) and in 44% of cases of

Table 1. Relation between D-score and Different Histological Types

D-score	Total (N=60)	Group						Test	p-value
		Proliferative Endometrium (N=10)		Atypical Endometrial Hyperplasia (N=25)		Low Grade Endometrial Carcinoma (N=25)			
		No.	%	No.	%	No.	%		
<0	36	0	0%	11	44%	25	100%	48.745	<0.001
0-1	7	0	0%	7	28%	0	0%		(HS)
>1	17	10	100%	7	28%	0	0%		

Table 2. Relation between Intensity of MMP9 Expression and the Different Histological Types

MMP9 expression	Total (N=60)	Group						Test	p-value
		Proliferative Endometrium (N=10)		Atypical Endometrial Hyperplasia (N=25)		Low Grade Endometrial Carcinoma (N=25)			
		No.	%	No.	%	No.	%		
Low	25	10	100%	13	52%	2	8%	26.757	<0.001
High	35	0	0%	12	48%	23	92%		(HS)

atypical endometrial hyperplasia (Table 1). The D score could differentiate significantly between proliferative endometrium, atypical hyperplasia, and carcinoma (P<0.001).

MMP9 immunoexpression was positive in all the studied cases. In cases of proliferative endometrium, a weak cytoplasmic expression of MMP9 in the glandular compartment was noted (Figure1 and 3), with a single case showing both nuclear and cytoplasmic expression. All cases of proliferative endometrium showed positive stromal expression of MMP9 (Table 3).

In cases of atypical endometrial hyperplasia, the expression of MMP9 varied between low expression in 52% of cases and high expression in 48% of cases (Table 2). Most of the cases revealed cytoplasmic expression of MMP9 (72%) while only 28% of cases showed both nuclear and cytoplasmic expression. Positive stromal expression was noted in 80% of cases (Table 3). Most of endometrial adenocarcinoma cases showed high cytoplasmic expression of MMP9 (92%) with only 8% (2 cases) showing low expression (Table 2). In 44% of cases the expression was both nuclear and cytoplasmic (Figure 2 and 4). Stromal expression was noted in 36% of cases of endometrial adenocarcinoma (Table 3). A statistically high significant value was noted between the intensity of MMP9 immuno-expression in cases of proliferative endometrium, atypical endometrial hyperplasia and low grade endometrial carcinoma (p> 0.001) (Table 2).

Relation between MMP9 and D-score

The D-score categorizes cases of AEH into three groups,

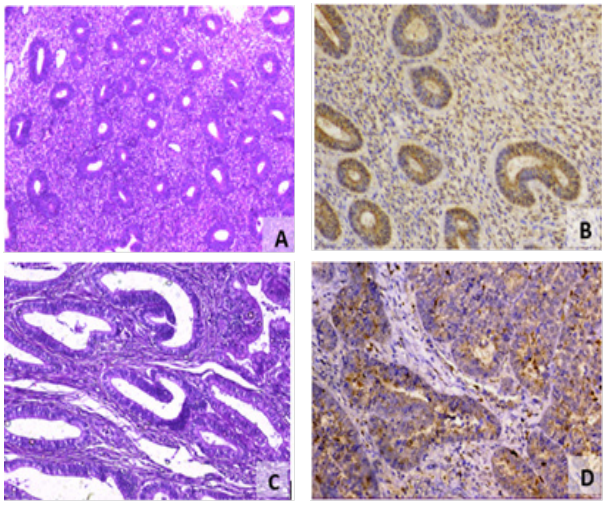

Figure 1. A Photomicrography of (A) Normal Proliferative Endometrium (HandE, x100), (B) Normal Proliferative Endometrium Showing Low Cytoplasmic Expression of MMP9 with Diffuse Stromal Expression (ABC, DABx200), (C) Complex Atypical Endometrial Hyperplasia (HandE x 200), (D) Complex Atypical Endometrial Hyperplasia: Showing Low Cytoplasmic Immunoexpression of MMP9 with Weak Stromal Expression (ABC, DABx400).

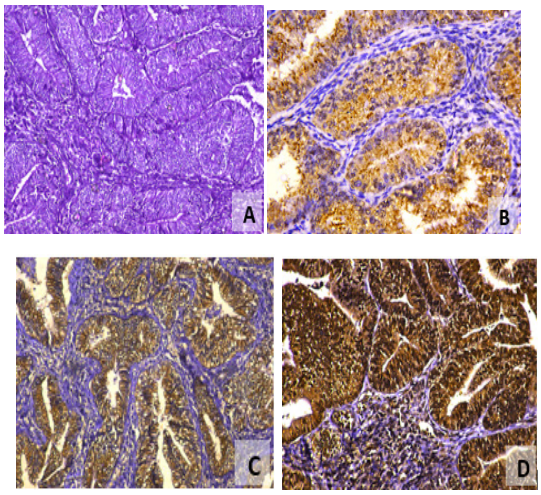

Figure 2. A Photomicrography of Low Grade Endometrioid Carcinoma. (A), (HandE x200); (B), Low Cytoplasmic Expression of MMP9 and Negative Stromal Expression (ABC, DAB x 400); (C), High Cytoplasmic Expression of MMP9 and Negative Stromal Expression (ABC, DAB x 200); (D), High Cytoplasmic and Nuclear Expression of MMP9 and Diffuse Positive Stromal Expression (ABC, DAB x 200).

Table 3. Distribution of MMP9 Expression in Proliferative, Atypical Hyperplasia and Low Grade Carcinomatous Endometrium Cases

MMP9 expression	Total	Group						Test	p-value
	(N=60)	Proliferative Endometrium (N=10)		Atypical Endometrial Hyperplasia (N=25)		Low Grade Endometrial Carcinoma (N=25)			
		No.	%	No.	%	No.	%		
Cytoplasmic	41	9	90%	18	72%	14	56%	4.082	0.130
Cytoplasmic and nuclear	19	1	10%	7	28%	11	44%		(NS)
Negative stromal	21	0	0%	5	20%	16	64%	17.099	<0.001
Positive stromal	39	10	100%	20	80%	9	36%		(HS)

Table 4. Relation between Expression of MMP9 and Results of D-score in Atypical Endometrial Hyperplasia Group

MMP9 expression	Total	D-score						Test	p-value
	(N=25)	<0 (N=11)		0-1 (N=7)		>1 (N=7)			
		No.	%	No.	%	No.	%		
Low	13	1	9.1%	5	71.4%	7	100%	15.634	<0.001
High	12	10	90.9%	2	28.6%	0	0%		(HS)

Table 5. Expression of MMP9 in Uncertain Group of Atypical Endometrial Hyperplasia (AEH)

	D-score	
	R	p-value
Scores of MMP9	-0.81	>0.001 (HS)

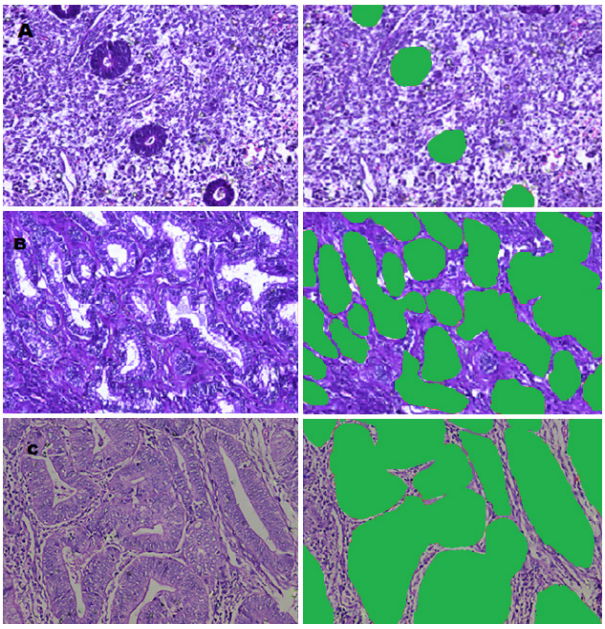

Figure 3. Morphometric Glandular Measurment of Area % of Glands Using Image Analysis System in a Cases of (A) Proliferative Endometrium, (B) Atypical Hyperplasia and (C) Endometrial Adenocarcinoma

namely low risk(<0), high risk(>0) and cases of uncertain prognosis(0-1); while MMP9 expression categorizes them into Cases of low expression reflecting low risk and others of high expression reflecting high risk (Table 4).

The relation between MMP9 expression and D-score value in cases of atypical endometrial hyperplasia was highly significant (P>0.001). Also correlating MMP9 expression to the uncertain group of cases of atypical endometrial hyperplasia was highly significant (P>0.001) (Table 5).

MMP9 proved a sensitivity of (92%), specificity of

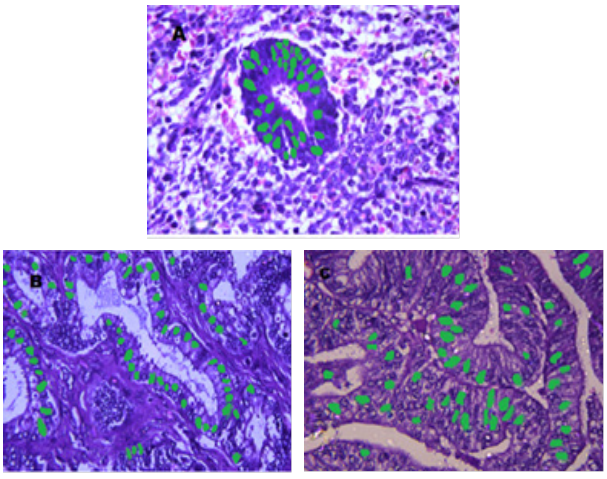

Figure 4. Morphometric Nuclear Area Measurement for (A) Proliferative Endometrium, (B) Atypical Hyperplasia and (C) Endometrial Adenocarcinoma, Revealing a Large Nuclear Area in Carcinoma Compared with Hyperplasia

Table 6. Validity of MMP9 and D Score in Diagnosis of Low Grade Endometrial Carcinoma; ROC Cuarve Analysis

Cut-off values	SN % (95% CI)	SP % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	Accuracy (95% CI)	AUROC (95% CI)
MMP9 score > 5	92% (74-99)	65.7% (47.8-80.9)	65.7% (47.5-81.1)	92% (73.5-99.1)	76.7% (58.7-88.4)	0.828 (0.709-0.913)
D-score ≤ -1.8	80% (59.3-93.2)	91.4% (76.9-98.2)	87% (66.4-97.2)	86.5% (71-95.6)	86.6% (69.6-96.1)	0.947 (0.857-0.988)

(65.7%) and overall accuracy of (76.7%). While D-Score proved a sensitivity of (80%), specificity of (91.4%) and overall accuracy of (86.6). Thus incorporating both MMP9 immunoexpression and D-score value would increase the accuracy of diagnosis of atypical endometrial hyperplasia and low grade endometrial carcinoma (Table 6).

Discussion

Endometrial carcinoma is the most common malignancy of the female genital tract (Siegel et al., 2016), and its most frequent type is Endometrioid "type 1" carcinoma (Bokhman, 1983) which represents 80% of newly diagnosed cases of endometrial cancer (Talhouk and McAlpine, 2016). It is often preceded by atypical endometrial hyperplasia which is thought to be a precursor lesion. Accurate and sensitive recognition of these precursors has great clinical value as an early warning of cancer risk and a potential target for preventative treatment. Mutter (2012), According to the 2014 WHO classification of endometrial hyperplasia, EIN is synonyms of AEH. It is a clinically relevant diagnosis that is intended to direct treatment (Kurman, 2014). It is, therefore, mandatory to explore diagnostic tools that would be helpful in distinguishing atypical hyperplasia from low grade carcinoma especially among endometrial biopsies.

The D-score has been developed in the early 1980s not to mimic subjective WHO 94 diagnostic classes, but as a prognostic test in predicting future or concurrent carcinoma (Mutter and Group, 2000).

In this study, the D score value in proliferative endometrium was >1 (low risk), while it was <0 (high risk) in carcinoma. The D-score values in cases of AEH were more than 1 (low risk) in 28 % of cases, less than 0 (high risk) in 44% of cases and between 0 and 1 (uncertain group) in 28 % of cases. The D- score value can, thus, help as a prognostic tool to give an idea about the fate of AEH and the probability of its transformation into endometrial carcinoma. It could differentiate significantly between proliferative endometrium, atypical hyperplasia, and carcinoma ($P<0.001$).

This is in agreement with a previous study of Baak et al., (1988), They showed that in patients with simple or complex hyperplasia, an even lower D-score of >0 or >1 seems to be an appropriate and safe way to indicate a very low probability of cancer development when compared with the WHO classification. Mutter et al., (2000) found that the D score in endometrial hyperplasia is a more sensitive and specific marker for cancer prediction than the WHO classification.

The architectural feature of volume percentage stroma (VPS) has the greatest predictive value among the parameters used to calculate the D-score. As glands become more crowded, the VPS drops below a threshold of 55%. This establishes formal architectural features as useful cancer predictive criteria in addition to cytology. In this study the differences in volume percentage stroma were statistically significant ($P= 0.001$) comparing atypical endometrial hyperplasia and well differentiated adenocarcinoma of the endometrium.

A multi-step endometrial carcinogenesis involving coordinated intervention of hormonal regulation, gene mutation, adhesion molecules, apoptosis, imbalance between metalloproteinases (MMPs), and tissue inhibitors of MMPs (TIMPs) is currently accepted (Graesslin et al., 2006). Matrix metalloproteinase 9 (MMP 9) is a zinc containing protease with the capability to degrade most components of the extracellular matrix (ECM) and basement membranes (Farina et al., 2012). MMP-9 has roles in almost all aspects of tumor biology (Farina and Mackay, 2014).

In this study the levels of MMP9, as defined by its immunohistochemical expression, were higher in cases of endometrial carcinoma than in atypical hyperplasia, with a 92% (23/25) in endometrial cancer cases to 48% and 0% in atypical endometrium and proliferative endometrium cases respectively, suggesting the involvement of MMP9 in the pathogenesis of endometrial carcinoma. This finding is in agreement with (Amalinei et al., 2011; Yu et al., 2012) who stated that the level of MMP9 is increased in endometrial carcinoma when compared to benign lesions.

The pattern of MMP9 expression varies between different types of cancer. Rao et al., (1996) found that MMP9 is expressed by malignant epithelium in tumors of the brain; while Sugiura et al., (1998) reported on stromal expression of MMP9 in their study on neuroblastomas. Tissue specific differences in MMP9 expression may reflect local microenvironments, possibly in response to matrix components or the result of signals derived from an inflammatory reaction which often accompanies the establishment of a malignant tumor (Di Nezza et al., 2002).

In this study, MMP-9 is expressed in both epithelial and stromal cells of endometrial tissue in all cases of proliferative endometrium, most cases of atypical hyperplasia (80%) and some of the cases of endometrial carcinoma (36%). This comes in agreement with the study of (Di Nezza et al., 2002; Graesslin et al., 2006) who found that MMP9 is expressed by stromal cells particularly in areas of close proximity to the endometrial cancer cells.

The loss of MMP9 stromal expression in many of the endometrial carcinoma cases studied can have different possible explanations, including the fact that all cases studied were of low grade malignancy Kamat et al., (2006) related MMP9 stromal expression to increased aggressiveness this calls for a comparison in the expression of MMP9 between low and high grade endometrial adenocarcinomas. Another possibility is the fact that a desmoplastic stroma in cases of malignancy is a proliferation of fibroblasts with subsequent secretion of collagen (Liu et al., 2012). The stroma is sometimes replaced by homogenized collagen where fibroblasts become less prominent or less active with expected decrease in MMP9 expression. This can provide an explanation to the lack of stromal reaction is some of our cases. These cases are theoretically expected to show an indolent course; a hypothesis that has to be proven by follow up of females diagnosed with low-grade endometrial adenocarcinoma, with a hyalinized stroma negative for MMP9.

In this study the D-score categorizes cases of AEH

into three groups, namely low risk, high risk and cases of uncertain prognosis; in accordance with previous studies by (Baak et al., 1988; Mutter et al., 2000; Izadi Mood et al., 2009) while MMP9 expression categorizes them into cases of low expression denoting low risk and others of high expression denoting high risk; in accordance with (Yu et al., 2012; laas et al., 2014).

D-Score proved a sensitivity of 80%, specificity of 91.4% and overall accuracy of 86.6%. These results are close to those of Khedr et al., (2008) who proved a sensitivity of 84.6%, specificity of 66.6% and overall accuracy of 76.5%. Results, however, differ from the findings of Baak et al., (2005); Izadi Mood et al., (2009) who proved a sensitivity of 100%, specificity of 82%. MMP9 proved a sensitivity of 92%, specificity of 65.7% and overall accuracy of 76.7%, in agreement with Lopata et al., (2003) who proved a sensitivity of 98%, yet with a higher specificity of 91%.

The variation in sensitivity of D-score between this study and that of Baak et al., (2005) could be explained by the fact that this study focused only on atypical endometrial hyperplasia and low grade adenocarcinoma, whereas their study addressed all types of hyperplasia and all grades of adenocarcinoma. Low grade carcinoma sometimes reveals features simulating the normal epithelial cells making the D score not as sensitive as in differentiating higher grades of carcinoma.

Thus, in conclusion incorporating both MMP9 immunoexpression and D-score value would increase the accuracy of diagnosis of atypical endometrial hyperplasia and low grade endometrial carcinoma.

In differentiating cases of AEH from EC, we recommend calculating the D- score as a first step. Cases of uncertain prognosis should be further evaluated by MMP9 immunostaining thereby categorizing them into either low or high risk groups.

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