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Original Article

Pathology Section

Evaluation of Loss of Paired Box 2 Gene Expression in Endometrial Hyperplasia for Detecting Endometrial Intraepithelial Neoplasia

DAVSHEEN BEDI, AMARJIT SINGH KATARIA, SUJATA SHARMA, VAISHALI VERMA, SURINDER PAUL

ABSTRACT

Introduction: Endometrial Hyperplasia (EH) is a non-neoplastic proliferation of endometrial glands, resulting from the action of unopposed estrogen, which if unchecked for long, can cause certain genetic alterations that eventually lead to the development of endometrial adenocarcinoma. *Paired Box 2 (PAX2)* is a gene coding for a transcription factor required during embryonic development, which has been found to mutate early during endometrial carcinogenesis.

Aim: Our study aimed at evaluating loss of *PAX2* gene expression in different types of EHs and analyzing its utility in detecting Endometrial Intra-epithelial Neoplasia (EIN).

Materials and Methods: It was a cross-sectional study conducted in the duration of 1.5 years, from January 2014 to June 2015. Total 50 cases diagnosed as EH were categorised as one of the four WHO sub-types. Further, these were catergorised as per EIN classification. Thereafter, *PAX2* immunohistochemical staining was applied and percentage loss of *PAX2* staining was evaluated and the results obtained were analysed statistically. Mainly Chi-square test and ANOVA test were applied.

Results: Simple hyperplasia without atypia was found to be the most common sub type where as simple hyperplasia with atypia the least common. 29/33 (87.89%) cases of simple hyperplasia without atypia showed <50% loss of *PAX2* expression and 5/6 (83.33%) complex hyperplasia with atypia cases showed *PAX2* loss of >50%. 13/14 (92.86%) of the EIN cases showed *PAX2* loss >50%, thus showing a more consistent loss of *PAX2*.

Conclusion: Hence, it is concluded that >50% loss of *PAX2* staining indicates early endometrial carcinogenesis, and is suggested as an aid in the diagnosis of EIN.

Keywords: Biomarker, Endometrial carcinogenesis, Endometrial pre-cancer lesion

INTRODUCTION

The spectrum encompassing disordered proliferative endometrium, EH, atypical EH/EIN) and Type 1 endometrial adenocarcinoma, results from the action of unopposed estrogen [1-3]. In this continuum of endometrial changes, genetic and molecular level alterations occur much before the changes that can be appreciated through the light microscope [4]. *PAX2* is a gene required during embryonic development [5], which has recently been found to mutate early during endometrial carcinogenesis [6-9]. Though the role of Phosphatase and Tensin Homolog (PTEN) gene as a tumour suppressor has been well established in endometrial carcinoma [10-12], *PAX2* is relatively a newer gene. Our study aimed at evaluating and comparing the loss of *PAX2* gene expression in different types of EH, and exploring its possibility as a novel biomarker for detecting EIN.

MATERIALS AND METHODS

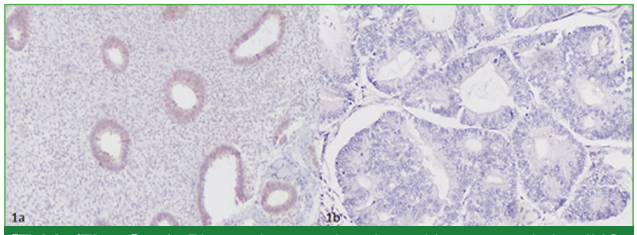
This is a cross-sectional study conducted in the Department of Pathology at Government Medical College, Amritsar, Punjab, India, over a period of 1.5 years from January 2014 to June 2015. Fifty cases diagnosed as EH on routine haematoxyline and eosin staining, were included in the study. The approval of the members of the thesis and the ethical committee of the institute was taken before the start of the study. Written, informed consent from the patients was also taken. Endometrial curettings/hysterectomy specimens diagnosed as endometrial carcinoma, simple proliferative or secretory endometrium were excluded from the study. Each of the fifty cases was reassessed and classified as one of the four 1994 WHO (World Health Organisation) sub-types of EH, namely simple hyperplasia without atypical, simple hyperplasia with atypia, complex hyperplasia without atypia and complex hyperplasia with atypia [1]. Further, each of the fifty cases were classified as per EIN classification criteria (namely architecture, cytology, size >1 mm, excluding mimics and cancer) into EH and EIN [3].

One paraffin block with best representative tissue was selected for each of the 50 cases and thereafter immunostaining with anti-*PAX2* antibody (Aviva Systems Biology, USA) was done.

The slides were stained in 5 batches, 10 slides per batch, with one positive control (slide of proliferative endometrium) and one negative control (slide of endometrioid endometrial adenocarcinoma) in each batch. Sections with 3-5 µm thickness were cut, mounted on freshly prepared 0.01% poly-L-lysine coated slides. Slides were dried overnight at 37°C. Slides were dewaxed by keeping at 70-75ºC on hot plate for 10 minutes, and then put in xylene (2 dips for 5 minutes each) to remove the melted wax and then hydrated in ethyl alcohol. The slides were then dipped in distilled water for 2 minutes to remove the alcohol. The excess distilled water was wiped out using a blotting paper. Endogenous peroxidase activity was blocked by adding freshly prepared 0.3% hydrogen peroxide in methanol (peroxidizer) for 10 minutes followed by three washings in Tris Buffer Saline (TBS) of 2 minutes each. Antigen retrieval was done as per the specifications of the kit. Slides were immersed in citrate buffer (pH=6) and put in a microwave oven for 4 cycles of 5 minutes each. The slides were then allowed to come to room temperature and immersed in TBS for 2 minutes. The excess fluid was wiped out from around the tissue sections using a blotting paper, and circles were marked around the tissue sections using an IHC PAP (Immunohistochemistry Peroxidase-anti-Peroxidase) pen. This provides a hydrophobic barrier around the sections, such that reagents remain within the circle marked, thus we have better results, lesser overflowing of reagents over the entire slide and less wastage of reagents as they remain within the circle marked. Then the slides were put in a moist chamber and incubated with protein block for 15 minutes. Anti-PAX2 primary antibody provided by Aviva Systems Biology, USA was prepared into a ready to use form, by removing it from the refrigerator and diluting it in the ratio of 1:50 with the diluent provided by the company. After 15 minutes of protein block, the excess fluid over the slides was drained, and the ready to use diluted primary antibody was added over each tissue section, approximately 100 µL per slide (varying with the size of the sections), and then the sections were incubated in the moist chamber for 2 hours. After 2 hours, the sections were washed with TBS twice for 2 minutes each. Sections were then incubated with enzyme HRP (Horse Radish Peroxidase) linked universal secondary antibody provided by Biogenex for 30 minutes in the moist chamber following which again 2 washings with TBS were given. Thereafter, Diaminobenzidine (DAB) solution was added on to the sections and incubated in the moist chamber for 3 minutes. Slides were then washed in distilled water for 3-4 minutes. Haematoxylin counter staining was done for 2-3 minutes and sections were washed under tap water and then dried and dehydrated in ascending concentrations of alcohol. Clearing was done in xylene, sections mounted with DPX (Dibutyl Phthalate Xylene), and cover slips were put. Sections were then viewed under the light microscope (10x, 40x).

Brown coloured nuclear staining of the endometrial glands was assessed. While the positive control tissue had positive nuclear staining in all the endometrial glands of the section [Table/Fig-1a] the negative control had no nuclear staining in any of endometrial glands of the section [Table/Fig-1b]. A gland was scored as *PAX2* negative when the stain was absent in the nuclear compartment of all cells or atleast in ≥ 90% of the cells in that gland [12]. For percentage assessment, the total number of glands in each slide was counted and then amongst them, the unstained glands or *PAX2* null glands were counted. The ratio of null glands to total number of glands was taken and expressed as percentage to derive the percentage loss of *PAX2* in each case.

The scoring system of loss of *PAX2* has not been well defined in literature so far. However, guidelines laid down by some studies on scoring of immunohistochemical staining [13,14] and by a study that was done as part of poster presentation at John Hopkins Institute, USA [8] and another study on *PTEN* expression in EH [12], were used as basis for IHC scoring in the present study [Table/Fig-2].



[Table/Fig-1]: a) Photomicrograph of positive control for IHC: Proliferative endometrium (*PAX2*, 100X); b) Photomicrograph of negative control for IHC: Endometrial adenocarcinoma (*PAX2*, 400X)

IHC Score	Percentage Loss of PAX2 Staining
0	>95%
+1	75-95%
+2	50-75%
+3	25-50%
+4	<25%

[Table/Fig-2]: IHC Scoring For *PAX2*.

STATISTICAL ANALYSIS

Data were analysed statistically using Chi square and ANOVA test.

RESULTS

The age range for the 50 cases of EH (43 endometrial curettings, 7 hysterectomy specimens), was 22-80 years with majority in the age group 41-60 years 30/50 cases, with the most common presenting complaints being abnormal uterine bleeding (menorrhagia, polymenorrhea) and post menopausal bleeding (38/50).

The 50 cases were sub-classified as per the 1994 WHO classification and the distribution of the cases [Table/Fig-3]. Thereafter, according to the EIN criteria 14/50 cases were found to be EIN cases, the remaining being EH.

Subsequently, anti-*PAX2* immunostaining was performed and its distribution in the different WHO types was studied [Table/Fig-4]. While 29/33 cases of simple hyperplasia without atypia showed <50% loss of *PAX2* expression, 5/6 complex hyperplasia with atypia cases showed *PAX2* loss of >50%. Similarly, loss of *PAX2* staining was analysed in the EIN classification. Majority of the EH cases (33/36) showed a percentage loss of *PAX2* <50%, whereas among the 14 EIN cases 13 showed a >50% *PAX2* loss [Table/Fig-5].The results were found to be highly significant.

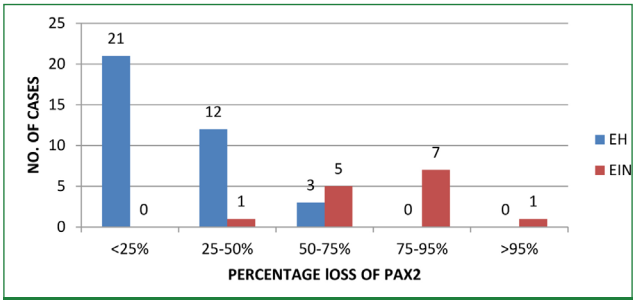
Average percentage loss of *PAX2* staining was also calculated for both WHO and EIN sub-types, and data analysed with ANOVA tests, and results were found to be significant [Table/Fig-6,7].

WHO Sub-type	Number of cases	Percentage
Simple Hyperplasia without Atypia	33	66%
Complex Hyperplaia without Atypia	07	14%
Simple Hyperplasia with Atypia	04	8%
Complex Hyperplsia with Atypia	06	12%
Total	50	100 %

[Table/Fig-3]: Showing distribution of WHO sub-types.

Percentage loss of PAX2 Staining	SH without Atypia	CH without Atypia	SH with Atypia	CH with Atypia	Total
<25%	20	0	1	0	21
25-50%	9	2	1	1	13
50-75%	4	3	1	0	8
75-95%	0	2	1	4	7
>95%	0	0	0	1	1
Total	33	7	4	6	50

[Table/Fig-4]: Percentage loss of *PAX2* Staining in WHO sub-types.
*χ^2 = 38.573; df = 12; p <0.001: Highly Significant; (SH= Simple Hyperplasia, CH= Complex Hyperplasia)



[Table/Fig-5]: Percentage loss of *PAX2* staining in the EIN classification sub-types.
*χ^2=36.121; df=4; p <0.001; (EIN=Endometrial Intraepithelial Neoplasia, EH=Endometrial Hyperplasia)

Sub-types	Total no of Cases	Average Percentage Loss of PAX2 (%)	ANOVA
SH without atypia	33	23.64 ± 15.51	F value= 21.824; p <0.001; Highly significant
CH without atypia	7	56.29 ± 17.60	
SH with atypia	4	47.50 ± 29.73	
CH with atypia	6	79.00 ± 17.38	

[Table/Fig-6]: Average percentage loss of *PAX2* In WHO sub types
*(SH= Simple Hyperplasia, CH= Complex Hyperplasia)

Sub-type	Total No of cases	Average Percentage loss of PAX2 (%)	p value	Significance
EIN	14	69.71 ± 17.50	<0.001	Highly significant
EH	36	23.94 ± 15.24		

[Table/Fig-7]: Average percentage loss of *PAX2* in EIN and EH cases.
*(EIN= Endometrial Intraepithelial Neoplasia, EH= Endometrial Hyperplasia)

The WHO-EIN concordance [Table/Fig-8] was found to be highly significant with a p value < 0.001. The WHO subtype that showed the highest percentage of EIN diagnosis was Complex hyperplasia with atypia i.e. 5 out of 6 making 83.33%, and the subtype showing the least EIN diagnosis was simple hyperplasia without atypia i.e., 1 out of 33, constituting 3.03%. These findings in our study corresponded with other studies on WHO-EIN concordance [15,16] .

WHO Sub-type	No. of Cases	No of Cases Diagnosed as EIN	Individual Percentage of EIN Diagnosis in each WHO sub-type
SH without atypia	33	1	3.03%
CH without atypia	7	5	71.43%
SH with atypia	4	3	75%
CH with atypia	6	5	83.33%
Total	50	14	28%

[Table/Fig-8]: Concordance between WHO sub types and the EIN classification.
*χ^2 = 30.250; df = 3; p <0.001: Highly Significant; (SH= Simple Hyperplasia, CH= Complex Hyperplasia, EIN= Endometrial Intraepithelial Neoplasia, EH= Endometrial Hyperplasia)

DISCUSSION

EH is a common non-neoplastic condition characterised by excessive proliferation of glands over stroma, commonly affecting women in perimenopausal age groups. There are different types of hyperplasias, each having a different risk of progression to cancer [17-20]. While simple hyperplasia is treated medically, the complex one and those with atypia, having a higher risk of developing into carcinoma, are advised to undergo a prophylactic hysterectomy [21,22]. Hence, it's important to correctly classify the hyperplasia in order to decide the further management of the patient, but the problem arises in cases where a clear cut subtyping based on morphology alone is not possible. Several drawbacks of

the 1994 WHO classification scheme were encountered, with poor reproducibility among pathologists being one of them, with some degree of interobserver and intraobserver variation in the diagnosis of the four sub-types. Maximum diagnostic disagreement was seen in the cytologic atypia ($p<0.0001$) as per a study by Allison KH et al., [23].

The EIN system is a new system that divides endometrial hyperplastic changes into two groups: EH and EIN. This system was proposed by Mutter and the Endometrial Collaborative Group in 2000, which is an International Group of Gynaecologic Pathologists [24]. EIN has five diagnostic criteria and all 5 must be met in order to make a diagnosis of EIN. These are architecture (area of glands greater than stroma), cytology (the crowded focus of glands shows cytology different from the backgound normal glands), maximum linear dimension of this crowded focus >1 mm, exclusion of benign mimics (like secretory endometrium, polyp, repair, etc.,) and exclusion of adenocarcinoma [24,25]. Mutter GL, suggested that the EIN classification system is more in agreement with current concepts of premalignant endometrial disease and it would certainly aid in more uniform patient management [26]. Studies conducted on the WHO and EIN concordances, i.e., what percentages of the 4 WHO sub types of EH come out to be EIN positive [15,16,27], have concluded that most of atypical hyperplasia, and more of complex atypical is diagnosed as EIN, and the simple hyperplasia without atypia has the least chances of being diagnosed as EIN. Our study also showed similar WHO-EIN concordance.

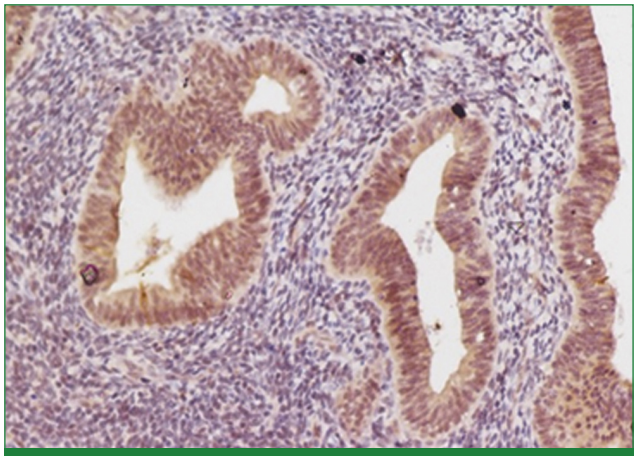
In 2014, WHO came up with the new classification for EH, dividing it into 2 categories namely, hyperplasia without atypia and atypical hyperplasia/EIN [28]. This two tier classification is more consistent and more relevant for patient management. Whether in the older 4 tier WHO classification or EIN or the newer two-tier WHO classification, there are always the borderline cases where putting them into any one type purely on morphology becomes difficult for a pathologist.

Many studies have shown that changes occur in the endometrium at the molecular and genetic level at a very early stage, even before any morphological changes can be detected with light microscopy [29,30]. These changes are in the form of mutations in genes like *PTEN, PAX2, HOXB13 (homeobox B13*) etc., which can be detected with the aid of immunohistochemistry [6,29,30]. Whereas, role of *PTEN* has been well-documented as a tumour suppressor gene in many studies on endometrial carcinoma, *PAX2* has emerged as a newer gene in this regard [6,9-12] .

PAX (Paired homeobox) genes is a family of 9 genes, encoding a group of transcription factors which play an important role in determination of lineage during embryonic development and *PAX2* is a member of this family [31]. *PAX2* gene encodes a protein that is involved in the development of the eye, ear, central nervous system, genito-urinary tract. *PAX2* has recently been studied in regard to its role in endometrial carcinogenesis. While most studies conclude it to be a tumour suppressor [6-9], one study mentions it as a proto-oncogene, whose expression increases in endometrial carcinogenesis [32].

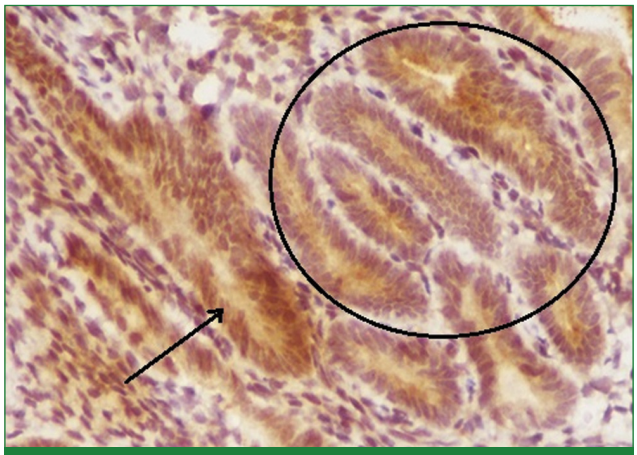
In our study, we found that expression of *PAX2* gene decreased significantly for complex hyperplasias, more so for atypical hyperplasias. While the simple hyperplasia without atypia showed a *PAX2* loss of <50% for about 87.8% of its cases, complex hyperplasia with atypia showed *PAX2* loss of >50% in 83.3% cases. The other two sub-types namely, complex hyperplasia without atypia and simple hyperplasia with atypia showed a variable *PAX2* loss. The EIN cases, in comparison showed a more consistent *PAX2* loss. Thus, a >50% *PAX2* loss can be inferred to be a significant cut-off criteria for endometrial carcinogenesis, and can be suggested as a novel biomarker for the same.

PTEN has been well documented as a tumour suppressor gene in endometrial carcinoma in literature [10-12]. However, *PTEN* immunostaining has a pan cellular distribution and it stains both glands and stroma of the endometrial tissue, thus making interpretation difficult and confusing at times [9]. In contrast, *PAX2* has a strong nuclear staining pattern of only the endometrial glands and not the stroma, thus making the glands stand out in comparison to the stroma, the latter also acting as an internal negative control and all of this makes *PAX2* easy to score [Table/Fig-9].



[Table/Fig-9]: Photomicrograph showing brilliant nuclear positivity in endometrial glands, compared to the negative stromal nuclei, which act as the internal negative control (*PAX2*, 400X).

A pathologist commonly encounters cases where all microscopic features don't fall into a single category of EH, and it becomes difficult to classify clearly into one WHO sub-type. Even in the EIN classification, there are certain borderline cases where distinguishing EIN from EH might be a tricky affair. From our study, we suggest that *PAX2* immunostaining can be used as an aid in the diagnosis of such cases, where in a *PAX2* loss of >50% would suggest a case with atypia and/or EIN. Thus, while *PAX2* immunostaining can't replace morphological diagnosis completely, but it can definitely be an aid in the morphological diagnosis, especially in doubtful and difficult cases [Table/Fig-10], where *PAX2* loss



[Table/Fig-10]: Photomicrograph showing EIN focus with PAX2 loss (encircled), with a positive gland on the left side (arrow) for comparison (PAX2, 400X).

is delineating an EIN focus. With our statistically significant results, we can conclude that a loss of *PAX2* staining more than 50% can be suggested as a marker for EIN diagnosis, especially in doubtful cases.

LIMITATION

Though, we understand the limitations of this study as the sample size is small (50 cases) and it is a cross-sectional study, we suggest more research and prospective study in this direction with larger case numbers to have more certain results, so that *PAX2* can be used clinically on a routine basis as a diagnostic aid for EIN.

CONCLUSION

We suggest a possibility of using *PAX2* as a biomarker for EIN diagnosis, with loss of *PAX2* more than 50% as a cut off. This may surely prove to be of immense help to pathologists in clear cut decision making in doubtful cases and to clinicians for better management of the patients with EH. It will also aid in picking up those few crucial cases that might look benign on light microscopy and are harbouring the pre-cancerous mutations, thus catching a pre-cancer at its very early stage. Clinically, this may prove to be of great aid deciding the next step in management in patients with EH.

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AUTHOR(S):

1. Dr. Davsheen Bedi
2. Dr. Amarjit Singh Kataria
3. Dr. Sujata Sharma
4. Dr. Vaishali Verma
5. Dr. Surinder Paul

PARTICULARS OF CONTRIBUTORS:

1. Senior Resident, Department of Pathology, PGIMER, Chandigarh, India.
2. Professor, Department of Pathology, Government Medical College, Amritsar, Punjab, India.
3. Professor and Head, Department of Obstetrics and Gynaecology, Government Medical College, Amritsar, Punjab, India.
4. Medical Officer, Department of Pathology, Civil Hospital, Hoshiarpur, Punjab, India.
5. Professor and Head, Department of Pathology, Government Medical College, Amritsar, Punjab, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:

Dr. Davsheen Bedi,
S-50, Sanjeevani Hostel,
PGIMER, Chandigarh-160012, India.
E-mail: davsheen28@gmail.com

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