The Immunohisto Chemical Examination of Changes that Take Place in PTEN Expression in Endometrioid Adenocarcinomas

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Abstract:

Objective: Endometrial carcinomas are the most commonly seen malignant tumors of woman reproductive system and while they are estrogen-dependent in 80-85% of the cases, they are estrogen-independent in 10-15 % of the cases. Endometrioid adenocarcinomas are aggressive tumors which are estrogen-independent. PTEN (phosphatase and tensin homolog) is a tumor suppressor gene which frequently mutates in endometrial carcinomas. It is known that PTEN plays a suppressor role in the growth of the tumor and an inhibitor role in the development and metastasis. **Material-Methods:** In the study, it was aimed to immunohisto chemically examine the changes of PTEN expression in young patients who were diagnosed with endometrioid adenocarcinoma. 2 groups were created in the study. **1st Group; Control group:** The group consisting of young people who were not diagnosed with endometrioid adenocarcinoma. **2nd Group; Patient Group:** The group consisting of young people who were diagnosed with endometrioid adenocarcinoma. **Result:** Patient age which is included in the hospital archive, CEA, p53, myometrial invasion, lymphovascular invasion, and cervical invasion values were statistically evaluated between the control group and patient group and within the patient group. As a result of the evaluation conducted with the immunohistochemical method, it was observed that PTEN expression statistically decreased in the patient group and furthermore, when the patient group is evaluated within itself, it was determined that H-score values of Grade 3 patient group were statistically and significantly lower than the Grade1 patient group. **Conclusion:** It was observed that there was a decrease in the PTEN expression in endometrioid adenocarcinomas and this decrease is in correlation with the grade.

Keywords:, Endometrium Carcinoma, H score, Immunohisto chemistry, PTEN

1. Introduction

Endometrial carcinomas are the most commonly seen malignancy in woman genitalia system and when the frequency rate is examined in developed countries, it is the fourth most common carcinoma type (1). Endometrial carcinoma is a carcinoma type which shows indications with hemorrhage in the early period and provides the opportunity of early diagnosis compared to carcinoma types such as ovarian carcinoma. A patient who was diagnosed with endometrium carcinoma should initially receive surgical staging. FIGO staging system is used in the surgical staging of endometrium carcinomas (Table1) (3, 4).

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Table1. FIGO staging of endometrium carcinoma

STAGE	EXPLANATION
Stage 0	If there is not a primary tumor. Existence of carcinoma in situ (preinvasive disease)
Stage I	The tumor is limited with corpus uteri
Stage IA	The tumor is limited with the endometrium
Stage IB	The tumor shows invasion in less than half of the myometrium.
Stage IC	The tumor shows invasion in more than half of the myometrium.
Stage II	There is cervix involvement, however, the tumor is still in the uterus.
Stage IIA	There is only endocervical glandular involvement.
Stage IIB	There is a cervical stromal invasion.
Stage III	There is local and/or regional spread.
Stage IIIA	Serosa and/or adnexal involvement (directly or metastatically) and/or carcinoma cells in abdominal irrigation fluid or acid were determined.
Stage IIIB	Vaginal involvement (directly or metastatically)
Stage IIIC	Existence of pelvic or paraaortic lymph node metastasis.
Stage IVA	Bladder and/or intestinal mucosa involvement.
Stage IVB	Existence of distant metastasis.

Endometrial carcinomas are classified into 2 sub-types as estrogen-dependent and estrogen-independent endometrial carcinoma. Estrogen-independent carcinomas are also defined as endometrioid adenocarcinomas and they are aggressive tumors which are observed with 10-15% frequency rate at later ages. These adenocarcinomas develop based on endometrial atrophy. They are usually estrogen and progesterone negative and p53 positive tumors. Their Ki-67 proliferation indexes are high (5, 6). PTEN which is a tumor suppressor gene is a gene which frequently mutates in endometrioid adenocarcinomas. PTEN is localized in the long arm of chromosome 10 (q) and codes a phosphatase named PIP3. PIP3 operates on the signal transduction pathway which regulates cell growth and apoptosis. PTEN also demonstrates a significant homology with two cytoskeleton proteins. This indicates that it is effective in cell adhesion and migration. It was demonstrated that PTEN plays a suppressor role in the growth of the tumor and an inhibitor role in the development and metastasis. In this study, it was aimed to evaluate the changes in PTEN protein expression which were stated in former studies in which it was determined that advanced age endometrioid adenocarcinomas develop in early stages, to compare with young patients who were diagnosed with endometrioid adenocarcinoma by using immunohistochemical methods.

2. Material-Methods

This study was conducted with the approval of Aydın Adnan Menderes University, Faculty of Medicine, Non-invasive Clinical Studies Ethics Committee dated 10.09.2014. Patients whose age range were 18-60 and who were operated in the Department of Obstetrics and Gynecology at Adnan Menderes University Training and Research Hospital and provide the study criteria were included in the study. PTEN antibody staining was performed in accordance with the kit protocol by taking 3-4 μm paraffin incisions from the biopsy samples of operated patients for immunohistochemical staining. The total sample size was determined with Power Analysis as 20 people in the patient group and 15 people in the control group. Two groups were determined in the study.

Control Group: Biopsy samples taken from patients who were not diagnosed with endometrial carcinoma between the age range of 18 and 60.
Patient Group: Biopsy samples taken from patients who were not diagnosed with type 2 endometrial carcinoma between the age range of 18 and 60.
Furthermore, CEA and p53 data which were in the hospital archive were recorded in order to be statistically evaluated.

2.1. Classification of Endometrial Carcinoma

The histological classification was performed as grade1, grade2, and grade3. The histological grading in endometrium carcinoma was conducted in accordance with the existence of solid areas. The surgical staging was conducted and recorded by using the surgical staging of FIGO (International Federation of Gynecology and Obstetrics).

2.2. Immunohisto chemical Evaluation

A total of 35 paraffin-embedded blocks were prepared for each group. Incisions of 0.5 microns were taken from blocks with a microtome and transferred into lysine lamina from the water bath. The tissues on the lysine lamina were drawn with Pab-pen after the dehydration process.

The samples were left for 10 minutes in room temperature by adding 3% of H_2O_2 (Hydrogen peroxide). After the waiting process, the samples which were washed with PBS (phosphate buffer saline) were blotted. Then, the samples on which the proper retrieval method was applied incubated for 20 minutes in 37 ^{0}C. Peroxidase sequencing solution was dripped on the samples which were rewashed with PBS for three times. After 6 minutes, the blocking solution was dripped on the samples which were washed with PBS and the samples were left in room temperature for 30 minutes. The block solution was carefully removed before the washing and was left in 4°C for one night by adding primary antibody which was diluted properly. The next day, the biotinylated secondary antibody which was compatible with the primary antibody was added on the preparations which were well-washed with PBS and were left in room temperature for 30 minutes in a closed humid container. Streptavidin peroxidase conjugate was added on the samples which were washed with PBS solution for 3 times and it was left in room temperature for 15 minutes in a closed humid container. DAB mixture was prepared in accordance with the number of samples as there would be 27 µl DAB substrate in 1000 microliter (µl) DAB dilution and at the end of the incubation process, DAB mixture was added on the samples which were washed with PBS and it was left for approximately 2 minutes. Preparations were washed with distilled water. Ground dyeing was performed with Harris hematoxylin. The samples were passed through ethanol and xylol series and closed with ental.

2.3. H Scoring

10 areas were chosen from each sample in the study group and cells in these areas were evaluated positively and negatively in accordance with the staining intensity and their percentage was taken for the immunohisto chemical evaluation. The staining intensity of cells that were stained positive was classified under 3 categories in accordance with the 'H-score method' as weak (+), median (++) and strong (+++) (8, 9).

The percentage of cells that were stained weak (+) positive was calculated for each sample and value was obtained by multiplying the result with 1. Then, the percentage of cells that were stained median (++) positive was calculated and the value was obtained by multiplying the result with 2. The percentage of cells that were stained strong (+++) positive was calculated and a third value was obtained by multiplying the result with 3. Then, these obtained results were added up and "H-score value" was obtained for each patient. The following formula was used in the calculation of H-score value for each patient:

H-score = 1 × (% of 1 + cells) + 2 × (% of 2 + cells) + 3 × (% of 3 + cells).

2.4. Statistical Analyses

All of the obtained data were statistically evaluated in SPSS 22.0 program. Normal Distribution analysis was conducted for each prognostic factor. The data which is non-normal distributed was evaluated with the Kruskal-Wallis test. Mann Whitney U test was conducted for paired comparisons. One-Way ANOVA variance analysis was performed for H-Score results which were analyzed with normal distribution and show normal distribution. The obtained values which were $p < 0.05$ were accepted as statistically significant.

3. Findings

While the age distribution was between 48 and 60 in the study, the average age was 54.2 in control group, 55.35 in the patient group and the total age average was 54.85 for all of the participants. FIGO staging system was used for histological grading. In the patient group, it was observed that 7 of the patients had grade 1 endometrioid adenocarcinoma (35%), 7 of them had grade 2 endometrioid adenocarcinoma (35%) and 6 of them had grade 3 endometrioid adenocarcinoma (30%). The staging was performed in accordance with FIGO for samples in the patient group. Stage I scoring in 1 patient (5%), stage IA scoring in 6 patients (30%), stage IB scoring in 4 patients (20%), stage IC scoring in 3 patients (15%), stage II scoring in 2 patients (10%), stage IIA scoring in 1 patient (5%) and stage IIB scoring in 2 patients (10%) were observed. Depth of myometrial invasion was examined in three stages and it was determined that the number of patients in the patient group who didn't have invasion was 6 (30%), patients who had less than half myometrial invasion were 4 (20%) and patients who had more than half myometrial invasion was 3 (15%). It was determined that patients who had cervical invasion among 20 patients in the patient group were 3 (15%).

The number of patients who had glandular invasion was 1 (5%) and the number of patients who had stromal invasion was 2 (10%). In Table 2, histological grading within the patient groups, FIGO staging, lymphovascular and cervical invasion values were given.

Table 2. Histological Grading within the Patient Groups, FIGO Staging, Lymphovascular, and Cervical Invasion Values

Patient Group	Histological Grade	Figo Stage	Lymphovascular Invasion	Cervical Invasion
Patient	Grade2	1B	Negative	Negative
Patient	Grade3	1B	Negative	Negative
Patient	Grade3	2	Negative	Negative
Patient	Grade2	2A	Negative	Positive
Patient	Grade1	1A	Negative	Negative
Patient	Grade3	1B	Negative	Negative
Patient	Grade2	1A	Negative	Negative
Patient	Grade1	1C	Negative	Negative
Patient	Grade2	1B	Negative	Negative
Patient	Grade3	2B	Negative	Positive
Patient	Grade2	2B	Negative	Positive
Patient	Grade1	1	Negative	Negative
Patient	Grade1	1B	Negative	Negative
Patient	Grade1	1A	Negative	Negative
Patient	Grade2	1C	Negative	Negative
Patient	Grade3	2	Negative	Negative
Patient	Grade1	1A	Negative	Negative
Patient	Grade3	1C	Negative	Negative
Patient	Grade1	1A	Negative	Negative
Patient	Grade2	1A	Negative	Negative

In Table 3, the distribution patient group within itself was demonstrated in accordance with the myometrial invasion values.

Table 3. The distribution of patient group within itself in accordance with the myometrial invasion values.

Patient	No Myometrial Invasion	Less than Half Myometrial Invasion	More than Half Myometrial Invasion
1		+	
2		+	
3	+		
4		+	
5	+		
6			+
7		+	
8	+		
9		+	
10	+		
11			+
12	+		
13			+
14	+		

When the immunohistochemical staining results performed with p53 and CEA antibodies were compared between the control group and patient group, it was observed that P53 expression in all of the Grade1, Grade2 and Grade3 patients increased significantly (p=0.000). When the patient group was evaluated within itself, the P53 expression was not statistically significant in the comparison between the grade1 and grade2 patient group (p=0.998).

It was also determined that when the grade1 and grade3 patient group was compared within itself, the difference was not statistically significant (p=0.706) and when the grade2 patient group was compared with grade3 patient group, the difference was not statistically significant (p=0.805). In Table 4, P53 expression values of control and the patient group were given and in Table 5, P53 expression values of the patient group within itself were given.

Table 4. P53 expression values of control and patient group

Group/P53	Median (Min.-Max.)
Control (n=15)*	0.000 (0.000-0.000)
Patient (n=20)*	31.20 (4-67)

*The statistical significance between the control and patient groups (p=0.000)

Table 5. P53 expression values of the patient group within itself

Group/P53	Median (Min.-Max.)
Grade1	28.71 (5-40)
Grade2	29.85 (4-67)
Grade3	35.66 (27-40)

When the staining results performed with CEA antibodies were compared between the control group and patient group, it was determined that CEA expression significantly increased in all of the Grade1, Grade2 and Grade3 patients (p=0.000). When the patient group was evaluated within itself, it was determined that the difference between the grade1 and grade2 patients were not statistically significant (p=0.870). When the grade1, grade2, and grade3 patient group was compared within itself, the difference was not statistically significant (p=0.270). In the comparison of grade2 patient group with grade3 patient group, the difference was not statistically significant (p=0.740). The CEA expression values of control and patient groups were given in Table 6 and the CEA expression values of the patient group within itself were given in Table 7.

Table 6. CEA expression values of control and patient groups

Group/CEA	Median (Min.-Max.)
Control (n=15)*	0.000 (0.000-0.000)
Patient (n=20)*	1.30 (1-2)

*The statistical significance between the control and patient groups (p=0.000)

Table 7. CEA expression values of the patient group within itself

Group/CEA	Median (Min.-Max.)
Grade1	1.14 (1-2)
Grade2	1.29 (1-2)
Grade3	1.50 (2-2)

3.1. Evaluation with the Immunohistochemical Method By Using PTEN Primary Antibody;

The H-score values obtained as a result of staining which was performed with PTEN primary antibody were compared between the control and patient groups. When the H-score values were evaluated in comparison with FIGO grade, it was observed that the H-score values of the control group were statistically and significantly higher than the Grade1 patient group (p=0.27), Grade2 patient group (p=0.000) and Grade3 patient group (p=0.000).

When the patient group was evaluated within itself, while there wasn't a statistically significant difference in the comparison of Grade1 and Grade2 patient group (p=0.341) and Grade2 and Grade3 (p=0.591), the H-score value of Grade1 patient group was statistically and significantly higher in the comparison of Grade1 and Grade3 patient group (p=0.033). Average H-score values and the lowest and highest H-score values of the groups were given in Table 8 and Graphic 1.

Table 8. Average H-score values of the groups

Group/PTEN IHC	Median (Min.-Max.)
Control	129.21(72.16-246.39)
Grade1	84.95(67.42-123.20)
Grade2	55.45 (24.23-85.57)
Grade3	32.59 (26.80-36.70)

The immunohistochemical PTEN gene expression levels of both groups are as follows in Graphic 1.

PTEN IHC
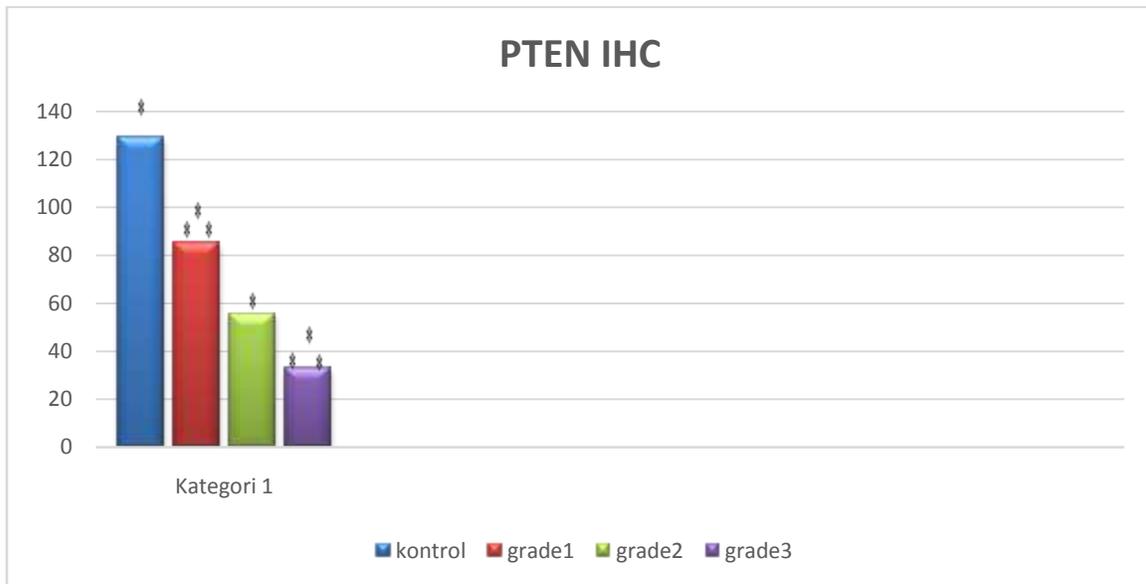
Graphic 1. The immunohisto chemical PTEN gene expression levels of control and patient groups
*the statistical significance between the control group and grade1 (P=0.270) grade2 (p=0.000), grade 3 (p=0.000) patient groups
**the statistical significance between the grade1 and grade3 patient group (p=0.033).
While it was observed that the values obtained after the IHC staining in the control group (++, +++) were higher, it was determined that (-) and (+) weak staining was more intense in the patient group from Grade1 to Grade3. The images captured in the x20 lens under the light microscope were given in Image 1.
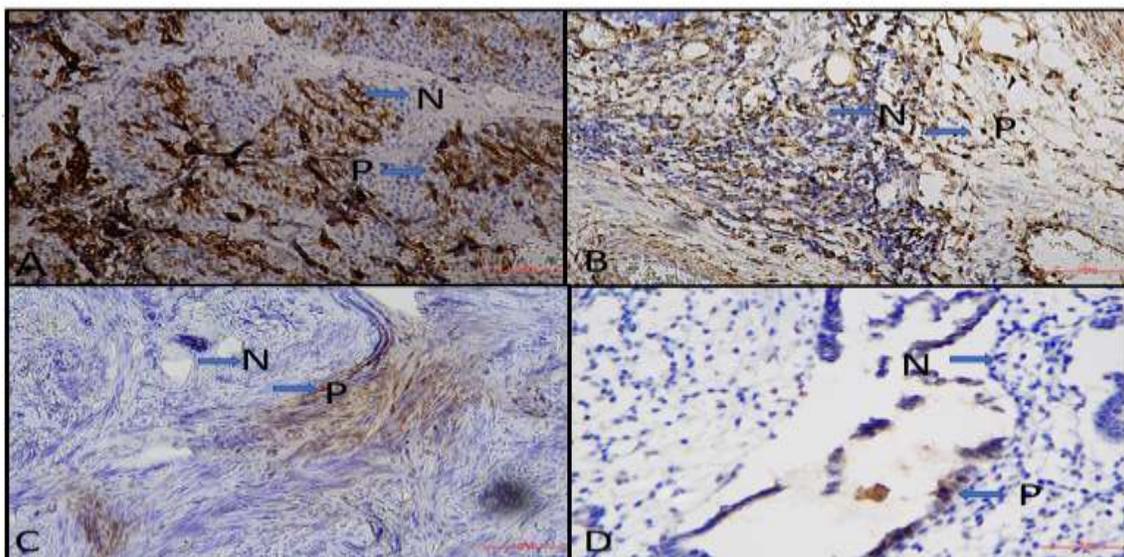

Image 1. PTEN, x20 zoom. (A; Control Group, B; grade1 endometrial carcinoma group, C; grade1 endometrial carcinoma group, D; grade1 endometrial carcinoma group, N; negative staining, P; positive staining)

4. Discussion:

PTEN expression in normal endometrium is a hormone-dependent condition which differs in accordance with the menstrual cycle. Especially, the high-level synthesis of PTEN in endometrium epithelium is estrogen-dependent. If there is an increase in estrogen level, PTEN expression increases in endometrium epithelium as well. For this reason, most of the studies were conducted on estrogen-dependent endometrium carcinomas. Another subject which draws attention in the conducted studies is the increase in PTEN levels without observing pathological changes in endometrium histology. Disruption in PTEN expression was also identified in cases where there weren't any histomorphological differences in the endometrium. While the increase in PTEN expression was observed as approximately 20% in hyperplasia after the pathological examination, this increase was 55% in endometrial intraepithelial neoplasms. The obtained results indicate that the mutations occurring in PTEN gene and the inactivity of PTEN gene are effective in the early stages of endometrioid adenocarcinoma pathogenesis, as they are effective in estrogen-dependent endometrial carcinomas (13, 14). Another important reason why the PTEN expression loss is more important in endometrial carcinogenesis compared to conditions observed in other malignancies is that it can be diagnosed in earlier stages of FIGO staging and thus, it enables early diagnosis and decreases in morbidity rate (14, 15, 16).

Immunohistochemical analyses are the most commonly used techniques in examining the stages of development and progression of endometrial carcinogenesis and the genetic changes in these stages. PTEN gene is the most frequently changing gene in endometrioid carcinomas and the mutation rate varies approximately between 30-50% (15, 17). The conducted studies emphasize that the decrease of PTEN activity is also observed in the early stages of endometrioid adenocarcinoma pathogenesis. In a study conducted with patients diagnosed with endometrial carcinoma, PTEN expression was evaluated immunohistochemically and it was observed that there was PTEN inactivity in most of the patients. In a study conducted by Mutter G. et al., loss of PTEN expression was observed in 61% of 33 patients (20 patients) diagnosed with endometrioid adenocarcinoma (14).

In another study conducted in 2012, the control group which consists of patients diagnosed with endometrial carcinoma and the patient group which consists of patients diagnosed with hyperplastic endometrium were evaluated in comparison and negative expression was observed in 12 patients, positive expression was observed under 50% in 13 patients and positive expression was observed over 50% in 4 patients among 29 patients who were diagnosed with endometrial carcinoma (18). In the study of Kapucuoğlu N. et al. which was conducted on the PTEN expression levels of 35 advanced aged patients who were diagnosed with endometrial endometrioid adenocarcinoma and PTEN inactivity was observed in 45.7% of the patients (16 patients) and it was concluded that PTEN gene expression has an important role in the early stages of endometrial carcinomas and the differences observed in PTEN expressions are important immunohistochemical indicators of endometrial carcinogenesis (19). According to the H-scoring results which were conducted on the obtained immunohistochemical images in the present study, it was determined that PTEN expression levels in the control group were significantly higher than patient groups. When the patient groups were compared within themselves in accordance with the histological grading, it was observed that the expression levels decreased as the grading increased.

In conclusion, it is considered that PTEN gene expression plays a role on early age endometrioid adenocarcinomas as it plays a role in estrogen-dependent endometrial carcinomas. However, it is also considered that as a result of conducting studies in large series with long-term monitoring in order to determine the prognostic importance of these indicators would enable these gene expressions to be important elements in treatment planning.

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