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Investigation of *PTEN* Gene Expression Levels in Patients with Different Stages and Grades of Breast Cancer

Farklı Evre ve Derecelerdeki Meme Kanserli Hastalarda *PTEN* Geninin İfadelenme Düzeylerinin Araştırılması

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ABSTRACT

Background: Breast cancer is the most common malignancy in women that cause death genetic and environmental factors affect the development of breast cancer. In many studies, it has been determined that *PTEN* is an important tumor suppressor gene for breast cancers and its loss induces carcinogenesis in the breast.

Materials and Methods: In our study, mRNA expression levels of *PTEN* gene were determined in tumor tissues and peripheral blood samples of 31 cases diagnosed with breast cancer, and breast tissues and peripheral blood samples of 5 healthy individuals. Real-time polymerase chain reaction method was used for the analysis.

Results: In this study, *PTEN* gene expression in tumor tissues and peripheral blood samples of breast cancer patients were determined compared to controls. The mRNA level of the *PTEN* gene in peripheral blood samples significantly downregulated (0.501-fold) ($p<0.05$), while it upregulated (1.109-fold) in tumor tissues ($p>0.05$). There was a contrast between *PTEN* gene expression behavior between blood and tissue. In addition, *PTEN* gene expression was downregulated in almost all peripheral blood samples of patients with breast cancer stage IIA. Additionally, it appears that *PTEN* gene expression was upregulated in tumor tissue in every histological grade while downregulated in peripheral blood.

Conclusion: In general, when our results are evaluated, we think that the decrease in the mRNA levels of the *PTEN* gene (down regulation) in the peripheral blood of breast cancer cases may be a potential finding in early stage diagnosis. These data will shed light on new comprehensive studies.

Keywords: Breast cancer, gene expression, different stage, biomarker, *PTEN*

ÖZ

Amaç: Meme kanseri, kadınlarda en sık görülen ve ölümle sonuçlanmasına neden olan malignitedir. Meme kanserinin gelişimine genetik ve çevresel faktörler etki etmektedir. Yapılan birçok çalışmada *PTEN*'nin meme kanserleri içinde önemli bir tümör baskılayıcı gen olduğunu ve kaybının memede karsinogenezi indüklediği belirlenmiştir.

Gereç ve Yöntemler: Çalışmamızda *PTEN* geninin mRNA ekspresyon seviyeleri, meme kanseri tanısı alan 31 olgunun tümör dokuları ve periferik kan örnekleri ile 5 sağlıklı bireye ait meme dokuları ile periferik kan örneklerinde belirlendi. Analizler için eş zamanlı-polimeraz zincir reaksiyonu yöntemi kullanıldı.

Bulgular: Bu çalışmada, meme kanserli olguların tümör dokularında ve periferik kan örneklerinde ifade edilen *PTEN* geninin, kontrol grubuna göre mRNA düzeylerindeki değişimler belirlenmiştir. Periferik kan örneklerinde *PTEN* geninin mRNA düzeyi, kontrole göre önemli derecede azalmışken (0,501 kat) ($p<0,05$), tümör dokularında artmıştır (1,109 kat) ($p>0,05$). Kan ve doku arasında *PTEN* geni ekspresyon davranışları bakımından zıtlık söz konusudur. Bunun yanı sıra, meme kanseri Evre IIA'da bulunan olguların periferik kan

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ÖZ

örneklerinin neredeyse tamamında *PTEN* gen ekspresyonu azalmıştır. İlaveten, her histolojik gradda tümör dokusunda *PTEN* gen ekspresyonu artmışken, periferik kanda azalmıştır.

Sonuç: Genel olarak sonuçlarımız değerlendirildiğinde, meme kanserli olguların periferik kanında *PTEN* geninin mRNA düzeylerindeki azalmanın (down regülasyon), erken evre teşhisinde potansiyel bir bulgu olabileceğini düşünmekteyiz. Bu veriler yeni geniş kapsamlı çalışmalara ışık tutacaktır.

Anahtar Kelimeler: Meme kanseri, gen ekspresyonu, farklı evre, biyobelirteç, *PTEN*

Introduction

Today, with the contribution of the developments in the molecular field, breast cancer is considered as a heterogeneous disease that shows differences in morphological structure, biological behavior and response to treatment. Molecular studies have gained momentum in the last 10 years, and it has become possible to evaluate thousands of genes simultaneously with the cDNA (complementary deoxyribo nucleic acid) microarray and "next generation sequencing" methods. A new molecular classification was made in breast cancers by extracting gene expression profiles from tumor tissue samples.

Tumor suppressor *PTEN* (phosphatase and tensin homolog) is a dual-specific phosphatase that acts as a negative regulator of the PI3K-AKT-mTOR pathway, thus controlling a variety of processes related to cell survival, proliferation, and growth (1).

Expression of HER family oncoproteins is increased in solid tumors compared to normal tissue. Cerb-B2 (HER2); it was found to be increased in stomach, breast, endometrium, ovarian carcinomas and vulvar Paget's disease. To show HER receptor expressions, IHC methods and fluorescent *in situ* hybridization (FISH) methods showing chromosomal amplification is used (2,3).

A gene localized to the long arm of chromosome 10 (10q 25) encodes the Ki-67 protein. This proteins expressed in all active phases (G1, S, G2 and mitosis) in the cell cycle, except the G0 phase, where cells do not proliferate (4). Positive nuclear staining for Ki-67 by immunohistochemistry is an important determinant for measuring cell proliferation. This ratio has been reported to be particularly high in aggressive tumors. High Ki-67 rate has been shown as a poor prognostic factor in many tumors (breast, lung, esophagus, kidney, prostate and endometrial cancer, malignant melanoma, non-Hodgkin lymphoma, glial tumors) (5).

Estrogen receptor (ER) and progesterone receptor (PR) evaluation is of fundamental importance for the pathological evaluation of breast carcinoma. ER is a nuclear transcription factor activated by estrogen. About 75% of breast cancers are positive for hormone receptors. Some low-grade invasive carcinomas and secretory carcinoma are typically negative. The ER does not predict the metastatic potential of the tumor but indicates that it will have a calm clinical course and a longer time to recurrence. ER positive tumors mostly metastasize to the bone, soft tissue and genitourinary system, while ER negative tumors metastasize to the visceral organs and brain. ER and PR are poor prognostic but strong predictive factors for endocrine therapy (6).

In this study, relative gene expression analysis was applied to show the *PTEN* gene behavior in the presence of breast cancer. While we were analysing the samples, we considered also both stages and grades of breast cancer.

Material and Methods

Sampling

Thirty-one breast cancer patients and 5 healthy control samples were included in our study. Tumor tissues were obtained during the breast operation performed in Department of General Surgery, Afyonkarahisar Health Sciences University, between June 2019-June 2021. Also, peripheral blood sample was taken into a 5 cc EDTA tube for RNA isolation. Patients who had previously received chemotherapy or immunotherapy for breast cancer were not included the study.

Determination of Histological Grade and Immunohistochemical Examination

Immunohistochemical examination was performed on sections prepared from paraffin blocks of tissues fixed in 10% formalin solution. Immunohistochemical application for each antibody was applied to all cases in a single session and was studied together with positive and negative controls. It was carried out by the method of detection of streptavidin-biotin-peroxidase (performed using the Leica Bond-max automated immunohistochemistry staining device). ER and PR status were determined by quick score. The quick score was calculated using a semi-quantitative method based on the percentage of nuclei that react and the intensity of immunostaining. The percentage of staining was evaluated as follows: 0= no nuclear staining, 1=<1% nuclear staining, 2=1-10% nuclear staining, 3=11-33% nuclear staining, 4=34-66% nuclear staining, 5=67-

100% nuclear staining. Staining intensity was evaluated as follows: 0= no staining, 1= weak staining, 2= moderate staining, 3= strong staining. The quick score was obtained by summing the percentage of staining and the degrees obtained from the staining intensity (7). When evaluating HER2 expression, it was scored in 4 groups (8):

Score 0: No staining in tumor cells or less than 10% of cells with membranous staining.

Score 1: More than 10% of tumor cells have pale staining on part of the cell membrane.

Score 2: More than 10% of tumor cells have weak-to-moderate staining of the entire cell membrane.

Score 3: More than 10% of tumor cells have strong staining of the entire cell membrane.

In cases with nuclear staining for p53 and Ki-67 expression, the percentage of tumor cells with nuclear staining is evaluated.

Medical pathology department routinely examines histological grade, axillary lymph node involvement and defined immunohistochemical parameters. Nottingham modification of the Bloom-Richardson system was used (9).

The staging of the patients was routinely done by the general surgery department according to the TNM staging (10).

Genetic Analysis

EZ-RNA Total RNA extraction kit (BI, Israel, Cat. No: 20-400-100) was used for RNA isolation. iScript Reverse Trancription Supermix (Biorad, USA, Cat. No: 170884) was used for reverse transcription. Genetic analysis was performed by real-time polymerase chain reaction (PCR) method using the Rotor Gene-Q (Qiagen, Hilden, Germany) and iTaq Universal SYBR Green Supermix (Biorad, USA, Kat. No: 1725122). *GAPDH* gene was used as a housekeeping gene for normalization. Oligomere Biotechnology (Ankara, Türkiye) designed oligonucleotide primers (Table 1).

Statistical Analysis

All data analyses were performed using REST 2009 V2.0.13 and SPSS v.19 software which use pairwise fixed reallocation randomization test (11) where $p<0.05$ is deemed to represent a statistically significant result. REST 2009 Software is a standalone tool for analysis of gene expression data from quantitative, real-time PCR experiments. The analysis or quantitation of relative gene expression uses expression of reference genes to normalize expression levels of genes of interest in different samples.

SPSS v19 software Pearson's correlation was used to determine linear relationship between ER/PR values and gene expression behaviour.

Results

Cases

Thirty-one breast cancer patients and 5 healthy control samples were included in our study. Table 2 shows the clinical and demographic characteristics of the breast cancer cases. Information on prognostic parameters such as, stages, histological subtype and immunohistochemical ER, PR, HER2, Ki-67 and p53 staining results obtained from patients pathology reports. Table 3 shows the surgical stage, histological grade, immunohistochemical status of all breast cancer patients.

RT-qPCR Analysis

The cDNA molecules synthesized from the RNA molecules isolated from the patients and the control group

Table 1. Primer sequences

Gene	Base sequences 5'→3'	Base lenght	Tm
***PTEN*-F**	TGGATTCGACTTAGACTTGACCT	23	59 °C
***PTEN*-R**	GGTGGGTTATGGTCTTCAAAAGG	23	61 °C
***GAPDH*-F**	CATTGCCCTCAACGACCACTTT	22	64 °C
***GAPDH*-R**	GGTGGTCCAGGGGTCTTACTCC	22	64 °C

-F: Forward, -R: Reverse, T: Tymin, G: Guanine, A: Adenine, C: Cytosine, Tm: Melting temperature

Table 2. Clinical and demographic characteristics of breast cancer patients

n		**31**			
Age, mean ± SD		53.2±12.0			
27-65, n (%) 66-90, n (%)		26 (83.9%) 5 (16.1%)			
Surgical staging		**Histological grade**		**Tumor location**	
Stage I Stage IIA Stage IIB Stage IIIA Stage IIIB	10 (32.3%) 14 (45.2%) 4 (12.9%) 2 (6.5%) 1 (3.2%)	Grade I Grade II Grade III	11 (35.5%) 13 (41.9%) 6 (22.6%)	Left breast Right breast	14 (45.2%) 17 (54.8%)

SD: Standard deviation

tissues and bloods were amplified in accordance with the relevant protocols. At the end of each reaction, melting curve analyzes were performed and it was confirmed that there was no dimerization in the primers and that the fluorescence values obtained belonged to the relevant gene region (Figure 1). Each analysis was performed in 3 replicates, both as an intraassay and an interassay. The fold change values obtained were calculated logarithmically, and their increase or decrease was prepared in graphic form.

Table 3. Surgical stage, histological grade, immunohistochemical status of all breast cancer patients

Patient no	Surgical stage	Histological grade	ER	PR	HER2	Ki-67	P53
P1	IIB	I	6	-	-	5%	10%
P2	IIA	I	-	-	-	60%	10%
P3	IIA	I	6	8	-	2-3%	10%
P4	I	II	6	8	-	3+%	10%
P5	2B	II	7	8	-	7-8+%	5%
P6	I	I	8	8	-	40%+	50%
P7	IIA	III	7	8	-	20+%	1-2%
P8	I	II	7	7	-	20%	80%
P9	IIA	III	-	-	-	80+%	100%
P10	IIA	I	6	6	-	2-3%	5%
P11	IIA	II	8	8	-	4-5%	10%
P12	IIA	II	8	5	Positive	20%	70%
P13	IIA	I	8	8	-	10%	20%
P14	I	I	8	8	-	1-2%	2-3%
P15	IIA	I	6	6	-	30%	10%
P16	IIIA	II	8	8	-	15%	1-2%
P17	IIA	III	8	-	-	30%	40%
P18	I	II	8	8	-	5%	1-2%
P19	IIA	I	8	8	-	1-2%	1-2%
P20	I	II	-	-	-	80%	60%
P21	IIA	I	7	6	-	30%	-
P22	IIIA	I	8	8	-	60%	10%
P23	2B	II	-	-	-	80%	80%
P24	I	II	7	7	-	2-3%	5%
P25	I	II	8	8	-	20%	20%
P26	IIB	III	4	4	-	60%	-
P27	IIA	II	6	8	-	5%	-
P28	IIA	III	7	7	-	15%	-
P29	I	II	-	-	-	20%	-
P30	IIIB	II	7	8	-	7-8%	-
P31	I	III	6	8	-	10%	-

mRNA Analysis of *PTEN* Gene of All Breast Cancer Patients

PTEN gene expression in tumor tissues and peripheral blood samples of breast cancer patients was determined compared to controls. The mRNA level of the *PTEN* gene in peripheral blood samples was significantly downregulated (0.501-fold) ($p<0.05$), while it was upregulated (1.109-fold) in tumor tissues ($p>0.05$) (Figure 2) (fold regulation changes are shown at Log10 level).

mRNA Analysis of *PTEN* Gene in Breast Cancer Patients at Different Stages

PTEN gene expression in tumor tissues of patients with stage I breast cancer was upregulated (1.693-fold), while it was downregulated (0.813-fold) in peripheral blood samples ($p>0.05$). *PTEN* gene expression in peripheral blood samples of patients with stage IIA breast cancer was significantly downregulated (0.813-fold) ($p<0.001$). It was downregulated in almost all patients with stage IIA (Table 4). In addition, it was downregulated (0.927-fold) in tumor tissues ($p>0.05$). *PTEN* gene expression in tumor tissues of

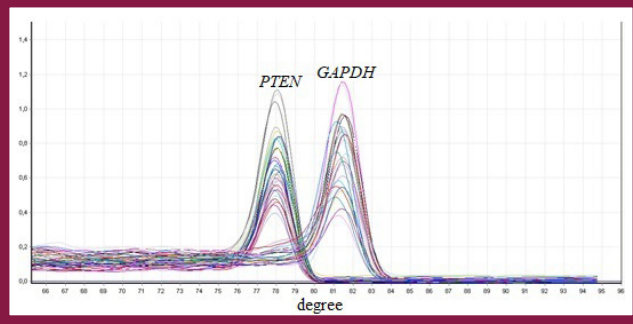

Figure 1. Example of *CDH1* and *GAPDH* melting curve

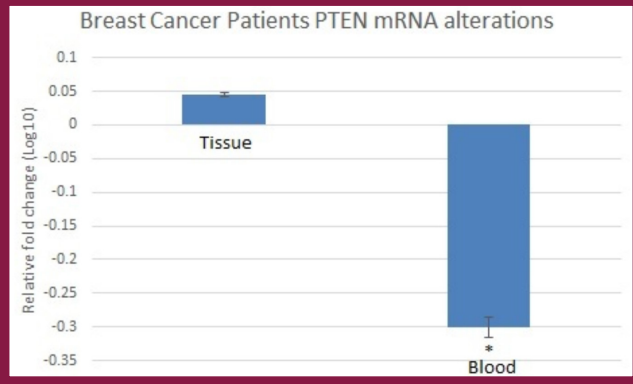

Figure 2. Alterations of *PTEN* gene expression in tumor tissues and peripheral blood samples of breast cancer patients compared to control. *GAPDH* was used as the reference gene for normalization (*$p<0.05$)

stage IIB breast cancer cases was upregulated (1.084-fold), while it was downregulated (0.937-fold) in peripheral blood samples (p>0.05). *PTEN* gene expression in tumor tissues and peripheral blood samples of patients with stage IIIA breast cancer was upregulated (1.112-fold and 1.332-fold, respectively) (p>0.05). *PTEN* gene expression in tumor tissues of stage IIIB breast cancer cases was significantly upregulated (2.231-fold) (p<0.001), while it was significantly downregulated (0.371-fold) in peripheral blood samples (p<0.05) (Figure 3, 4).

mRNA Analysis of *PTEN* Gene Expression of Breast Cancer Cases in Grade I-II-III

PTEN gene expression in tumor tissues of grade I breast cancer cases was upregulated (1.272-fold) (p>0.05), while it was significantly downregulated (0.554-fold) in peripheral blood samples (p<0.05). *PTEN* gene expression in tissue samples of grade II breast cancer cases was upregulated (1.114-fold), while it was downregulated (0.897-fold) in peripheral blood samples (p<0.05). *PTEN* gene expression in the tissue samples of grade III breast cancer cases was upregulated (1.116 fold), while it was downregulated significantly (0.302-fold) in the peripheral blood samples (p<0.05) (Figure 5, 6).

Evaluation of *PTEN* Gene Expression Changes with ER and PR Statuses

The ER and PR status of patients with breast cancer who had downregulation in tumor tissues were evaluated.

Table 4. *PTEN* gene expression levels of stage IIA breast cancer patients

Patient no	Surgical stage	*PTEN* expression blood
P2	IIA	Down
P3	IIA	Up
P7	IIA	Down*
P9	IIA	Down*
P10	IIA	Down
P11	IIA	Down
P12	IIA	Down*
P13	IIA	Down*
P15	IIA	Down*
P17	IIA	Down*
P19	IIA	Down*
P21	IIA	Down*
P27	IIA	Down*
P28	IIA	Down*

*It shows significant change in *PTEN* gene expression levels compared to control. Down means: Decreased mRNA level, Up means: Increased mRNA level

There was a significant and strong correlation between the downregulation of the *PTEN* gene in tumor tissues and the positivity of ER and PR values (Pearson correlation, r=0.770, p<0.01). Likewise, there was a significant and strong correlation between the downregulation of the *PTEN* gene in peripheral blood samples and the positivity of ER and PR values (Pearson correlation, r=0.772, p<0.01). In addition, there was a significant and strong correlation between the positivity of the ER and PR values of the cases in which the *PTEN* gene was significantly downregulated in peripheral blood samples (pearson correlation, r=0.672, p<0.01). There was one case with HER2 positive. This case was in stage IIA. *PTEN* gene expression level was downregulated in breast tumor tissue and peripheral blood samples of this patient. This downregulation was significant in peripheral blood (p<0.001). In addition, when triple negative patients ER (-), PR (-), HER2(-) were evaluated, downregulation of *PTEN* gene

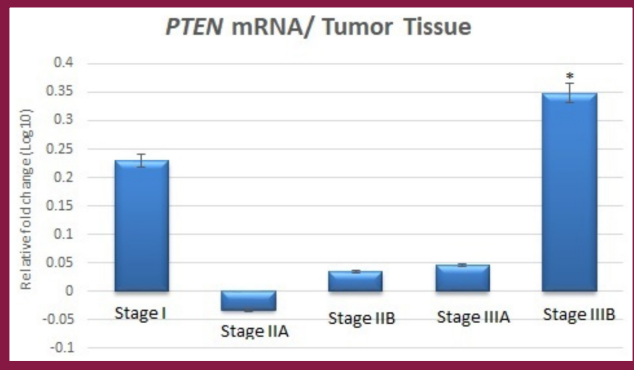

Figure 3. Alterations of *PTEN* gene expression in tumor tissues samples of breast cancer patients at different stages compared to control. *GAPDH* was used as the reference gene for normalization (*p<0.001)

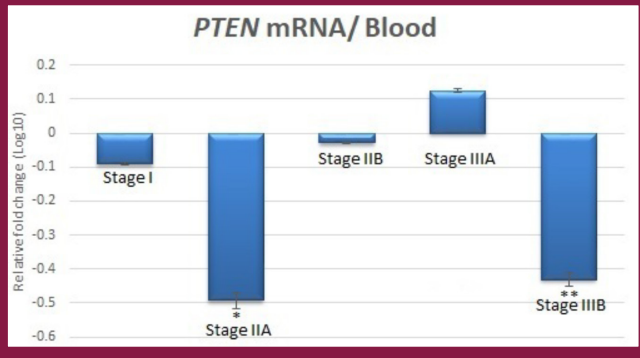

Figure 4. Alterations of *PTEN* gene expression in peripheral blood samples of breast cancer patients at different stages compared to control. *GAPDH* was used as the reference gene for normalization (*p<0.001, **p<0.05)

expression was observed in all peripheral blood samples of these patients (Table 5).

Discussion

Molecular biology and genetics represent one of the most important and interesting topics in medical oncology, thus providing a global and detailed knowledge of the molecular changes involved in tumor progression, leading to a better understanding of the carcinogenesis process, the discovery of new prognostic markers and therapeutic targets (12).

Gene expression analysis has determined global gene dosage sensitivity in cancer. Thousands of genetic variants so far. It has been associated with cancer, other diseases, and complex traits. It has been found that disease-related single nucleotide polymorphisms and somatic copy number changes frequently affect gene expression levels (13,14,15,16).

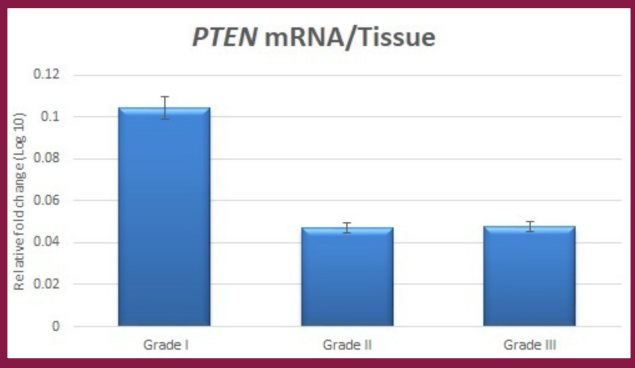

Figure 5. Alterations of *PTEN* gene expression in tumor tissues of breast cancer patients at different grades compared to control. *GAPDH* was used as the reference gene for normalization

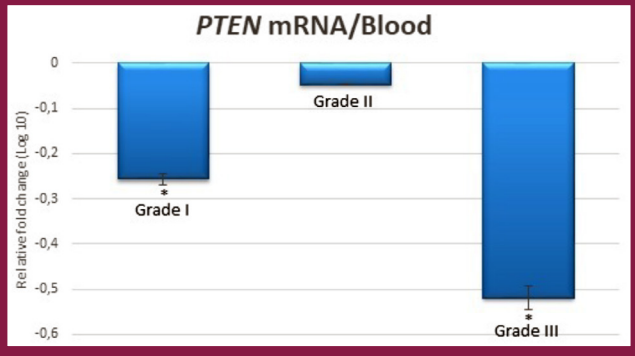

Figure 6. Alterations of *PTEN* gene expression in peripheral blood samples of breast cancer patients at different grades compared to control. *GAPDH* was used as the reference gene for normalization (*p<0.05)

Deletion and decreased *PTEN* function as a result of mutation have been detected, in sporadic cancer and hereditary cancer syndromes. An also, germline mutations causing loss of *PTEN* function have been detected in patients with Cowden's disease (17,18,19). In many studies, it has been determined that *PTEN* is an important tumor suppressor gene for breast cancers and its loss induces carcinogenesis in the breast. Similarly, in our study, *PTEN* gene expression were significantly decreased in blood samples of patients with breast cancer. Specially, this downregulation draws attention in high percentage with stage IIA patiens. Contrary to this, upregulation was detected in more samples of our tumor series, but not significant. There was a contrast between blood and tissue in terms of *PTEN* gene expression behaviors. Additionally, we found that *PTEN* gene expression was upregulated in tumor tissue at every histological grade, while it was downregulated in peripheral blood. The major differences in gene expression levels in tumor tissue samples and peripheral blood samples may reflect tissue specific regulatory mechanisms for this tumor supressor gene. Although we couldn't detect this decrease in tumor tissues, the decrease in blood was compatible with the literature. Changes of the *PTEN* gene in somatic cells are common in certain cell lines and primary tumors, including thyroid tumors, brain tumors, prostate carcinomas, endometrial carcinomas, and melanoma. However, it has been reported that such changes are less common in breast cancer, kidney carcinomas, and head and neck squamous cell carcinomas (20).

Depowski et al. (21) reported loss of PTEN protein expression in 48% of 151 breast tumors. In their analysis, they reported that loss of PTEN protein expression, node positivity, stage, tumor grade were associated with disease-related deaths. Loss of PTEN protein expression has been reported to correlate with predicted lymph node metastasis and loss of ER staining. Loss of PTEN protein expression has not been reported to correlate with stage, tumor grade,

Table 5. Surgical stage, histological grade, immunohistochemical status of breast cancer patients with triple negative

Patient no	Surgical stage	Histological grade	ER	PR	HER2	*PTEN* expression blood
P2	IIA	I	-	-	-	Down*
P9	IIA	III	-	-	-	Down*
P20	I	II	-	-	-	Down
P23	IIB	II	-	-	-	Down
P29	I	II	-	-	-	Down*

*It shows significant change in *PTEN* gene expression compared to control. Down means: Decreased mRNA level, Triple negative means: ER (-), PR (-) HER2(-)

disease relapse, or loss of PRs. These results show that the proposed *PTEN* gene is a candidate tumor suppressor in breast cancer and future studies are needed for this marker (21). Additionally, Depowski et al. (21) confirmed a significant loss of *PTEN* expression in 17 of 25 ER-negative tumors and an almost significant loss of *PTEN* in PR-negative tumors. The underlying reason for this correlation is unclear, but loss of *PTEN* expression appears to be due to an aggressive ER/PR negative phenotype (21). In addition, a significant relationship was found with PR loss (22). Contrary to this findings, in our study, there was a significant and strong correlation between the downregulation of the *PTEN* gene expression and the positivity of ER and PR values in both blood and tumor tissues. Dicrepancies may be resulted from the relatively few sample number. However, Shoman et al. (23) findings support our results. They reported strong association between downregulation of *PTEN* expression in ER-α-positive tumors (23). *PTEN* loss is shown in many cancers and in the triplet negative group of breast cancers (24). Similarly to this report, when triple negative patients [ER (-), PR (-) and HER2 (-)] were evaluated, downregulation of *PTEN* gene expression was observed in all blood samples of these patients in our study. Due to the lack of hormonal treatment and anti-HER-2 treatment alternatives in triplet negatives, drug studies have been carried out on these patients by targeting these pathway activations due to *PTEN* dysfunction. Loss of *PTEN* was observed at a rate of 66% in the triplet-negative subgroup of patients with recurrent breast cancer, while it was 28% and 22% in the hormone-positive and HER2 groups, respectively (25).

Study Limitations

The main limitation of this study was the sample size. The predicted downregulation was not observed in the tumor tissues. We think that this was due to the low number of patients with advanced stages of breast cancer.

Conclusion

When the results regarding the mRNA changes of *PTEN* in breast cancer patients were examined, the downregulation of the *PTEN* gene expression in peripheral blood samples draws attention. We think that the decrease in *PTEN* gene mRNA level in blood samples of breast cancer patients may be a potential finding of breast cancer. These data will contribute to future studies.

Ethics

Ethics Committee Approval: This study was approved by the Ethics Committee of Afyonkarahisar Health Sciences University (04.05.2018/144).

Informed Consent: All patients provided informed consent.

Peer-review: Internally and externally peer-reviewed.

Authorship Contributions

Concept: H.A.Ö., E.S.A.S., Y.A., Design: H.A.Ö., E.S.A.S., Y.A., Data Collection or Processing: H.A.Ö., M.Ç., M.A., Ç.T., Analysis or Interpretation: H.A.Ö., E.S.A.S., Ç.T., Literature Search: H.A.Ö., E.S.A.S., Writing: H.A.Ö., E.S.A.S.

Conflict of Interest: The authors declare that there are no conflicts of interest.

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