Research Article

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PIK3CA and TP53 MUTATIONS and SALL4, PTEN and PIK3R1 GENE EXPRESSION LEVELS in BREAST CANCER
MEME KANSERİNDE PIK3CA ve TP53 MUTASYONLARI ve SALL4, PTEN ve PIK3R1 GEN EKSPRESYON SEVİYELERİ

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Abstract

Objective: A high frequency of PI3K signalling pathway abnormalities and TP53 mutations are critical in the development and progression of breast cancer (BCa). We aimed to detect PIK3CA and TP53 mutations via an expression analysis of PIK3R1, PTEN and SALL4 and correlate the expression of these genes with clinical parameters of BCa.

Materials and methods: PIK3CA and TP53 mutations in BCa samples were analysed by High-Resolution Melting (HRM) analysis, followed by Sanger sequencing, and the expression levels of PIK3R1, PTEN and SALL4 were evaluated by RT-PCR methods.

Results: The frequency of PIK3CA and TP53 mutations was 42% and 38% according to the HRM and Sanger sequencing. There was a significantly high frequency of these mutations in ER(+), N0 and HER2(−) tumour samples. PIK3R1 and PTEN expression levels were high in tumour samples, whereas SALL4 expression was low. In patients with TP53 mutations, PIK3R1 expression was low, and this finding was statistically significant. PIK3R1 and PTEN expression levels showed statistically significant, respectively in G3 grades, ER(+)(+), (PR)(+), HER2(+) and ER(+).

Conclusions: We suggest that these candidate genes could be potential prognostic biomarkers of BCa and that they should be considered in the evaluation of clinical parameters of BCa.

Keywords: Breast cancer; PI3K; PIK3CA; HRM; Candidate genes.

Öz

Amaç: Yüksek frekanstaki PI3K sinyal yolağı anormal-likleri ve TP53 mutasyonları, meme kanseri gelişimi ve ilerlemesinde kritik öneme sahiptir. Bu çalışmanın amacı PIK3CA ve TP53 mutasyonları ile PIK3R1, PTEN ve SALL4 ekspresyon analizi ve bunların meme kanser klinik parametreleri ile ilişkisini belirlemektir.

Gereç ve Yöntem: Meme kanser örneklerinde Yüksek Çözün-ürülükli Erime (HRM) analizi ile PIK3CA ve TP53 mutasyonları analiz, Sanger dizi takibi ve PIK3R1, PTEN ve SALL4 ekspresyon seviyeleri RT-PZR yöntemile değerlendirildi.

Sonuçlar: HRM analizi ve dizilemeyle, PIK3CA ve TP53 mutasyon frekansı %42 ve %38 olarak bulundu. Bu mutasyonlar sırasıyla ER(+), N0 ve HER2(−) tümör örneklerinde yüksek ve istatiksel olarak anlaşımlı görüldü.
SALL4 ekspresyonu tümör örneklerinde düşük olmasına karşın, PIK3R1 ve PTEN ekspresyon seviyeleri yüksekti. TP53 mutasyonu taşıyan hastalarda PIK3R1 ekspresyonu düşük ve istatiksel olarak anlamlı bulundu. PIK3R1 ve PTEN ekspresyon seviyeleri sırasıyla G3 dereceli, OR(+) ve PR(+) ve HER2(+) ve OR(+)’lerde istatiksel olarak anlamlı olduğu gösterildi.

Sonuç: Biz bu aday genlerin meme kanserinde progreso-
tik bir özellik olma potansiyelini ve böylece bunların meme kanserinde klinik parametrelerin değerlendirilmesinde dikkat edilmesi gerektiğini ileri sürürüz.

Anahtar Kelimeler: Meme kanser; PI3K; PIK3CA; HRM; aday genler.

Introduction

Breast cancer (BCa) is one of the most common diseases in women, affecting 1.3 million women worldwide each year [1, 2]. Several studies revealed mutations and abnormal expression of a number of genes (e.g. BRCA1/2, PIK3CA, TP53, PTEN and CDH1) that are known to play a key role in genomic stability and cancer signalling pathways, including BCa. Research demonstrated that abnormal expression of some of these genes contributed to proliferation, invasion, recurrence and tumour formation (metastasis) in BCa [3]. Detection of genetic aberrations involved in BCa development could provide a theoretical basis for BCa prevention, diagnosis, prognosis and therapy.

Aberrations in the PI3K pathway are among the most common genomic abnormalities in BCa [4]. PI3Ks are essential molecules in this cascade and ultimately cause to tumour cell growth [5]. They are heterodimers comprised of regulatory (p85) and catalytic (p110) subunits that consist of α, β, δ, and γ isoforms [6]. The most common alterations in BCa are PIK3CA (p110α) mutations in the PI3K pathway and the TP53 gene, with alterations in these genes affecting about 27% of BCa patients [7]. The PI3K pathway, which includes the PI3K regulatory PIK3R1 and PTEN, is the most frequently altered pathway in cancer. Spalt-like transcription factor 4 (SALL4) have also crucial role in the proliferation of cancerous cells [8].

In the present study, we investigated how activating mutations in PIK3CA (p110α) and TP53 and the gene expression profile of regulatory subunits PIK3R1 (p85α), PTEN and SALL4 mediated PI3K activation in BCa. The aim of the current study was to detect PIK3CA and TP53 gene mutations, together with SALL4, PTEN and PIK3R1 gene expression levels, and then find the association of the obtained results with BCa clinical parameters. The findings may shed light not only on the association of these mutations and gene expression profiles with BCa progression and development but also on potential prognostic biomarkers of BCa.

Materials and methods

Patients, samples and histopathological evaluation

BCa tissues were obtained from 145 Turkish patients who underwent surgery in the Marmara University School of Medicine during 2010 and 2012 (n = 66) and 2016 and 2017 (n = 79). The clinical characteristics of the patients are summarized in Supplement Tables 2 and 3. The histological diagnosis was confirmed by a pathologist. The histopathological evaluation criteria have been described in our previous study [9]. Also, for describing BCa subtypes we used the St. Gallen classification system [10].

Our current research was admitted by the research and ethics commission of Marmara University of Medical Sciences.

DNA extraction

After tissue homogenization, genomic DNA was isolated from frozen tissue (−80°C) using a HP-PCR Template Preparation Kit (Roche, Germany), with regard to the kit’s instructions. The concentrations were detected by spectrophotometry and stored at −20°C until used.

PIK3R1, PTEN and SALL4 gene expression analysis

After tissue homogenization, total RNA was isolated from frozen tissue (−80°C) using a HP RNA Tissue Kit (Roche), with regard to the kit’s instructions. The total RNA concentration was detected by spectrophotometry. After evaluating the concentration and purity of total RNA using a spectrophotometer, the total RNA was reverse transcribed.

PIK3R1, PTEN and SALL4 transcription were quantified by RT-PCR using LightCycler® 480 Probes Master (Roche) according to the manufacturer’s protocol. RT-PCR and 5 μL of cDNA were used in a reaction volume of 20 μL (4 μL of dH2O, 10 μL).
LC® 480 Probes master (2X), 1 μL of Real-Time ready Assay (20X) with each sample analysed in duplicate. The beta-actin reference gene was used as an internal control to normalize variability in expression levels. The primer probe mix for each gene was purchased as a Real-Time Ready Custom Single Assay (Roche) and used with regard to the kit’s instructions.

HRM analysis

The HRM assay and analysis protocol were described in our previous study (Dirican et al. 2014). HRM was accomplished using the LightCycler® 480 (Roche) platform and an LC480 HRM Master Mix reagent kit (Roche), with 5 μL of gDNA and 0.4 μL of each primer in a final volume of 20 μL, in accordance with the manufacturer’s instructions. Primers for PIK3CA (exons 9 and 20) and TP53 (exons 5–8) genes (Table 1) were used as described previously [11, 12], and samples with a shift in the HRM analysis and melting temperature curves were then sequenced. The cycling conditions were 95°C (10 min), 45 cycles of 60°C (20 sec), 72°C (25 sec), 95°C (5 sec) and a final extension at 40°C for 10 min.

The data were analysed using LightCycler® 480 (Roche) software. The melting curves were normalised and temperature shifted to allow the samples to be directly compared.

Table 1: Primer pairs used for HRM assay and Sanger Sequencing.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
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<td><strong>PIK3CA sequencing</strong></td>
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<tr>
<td>exon 9-F</td>
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<td>exon 9-R</td>
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<td>exon 9-RP</td>
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<td>exon 20-RP</td>
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<tr>
<td><strong>TP53 HRM and sequencing</strong></td>
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<tr>
<td>exon 8-RP</td>
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Sanger sequencing

Following HRM screening, for the purification of PCR products used an ExoSAP Purification kit, and DNA sequencing was performed with an ABI3500 (Applied Biosciences) sequencer. Then, 2 μL of PCR product was treated with 1 μL of ExoSAP reagent. The treatment was carried out at 37°C for 30 min, followed by an incubation period at 80°C for 1 min to irreversibly inactivate both enzymes. The BigDye v3.1 Sequencing Kit provides a streamlined workflow by eliminating the PCR clean-up step and improving resolution of sequencing data at the 5’ end. PCR sequencing was then performed using 10 μL of PCR products. PCR sequencing was performed using the BigDye v3.1 kit under the following conditions: 96°C for 1 min, followed by 25 cycles each at 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min, followed by cooling at 4°C. Subsequently, 10 μL of PCR sequencing products were precipitated using 3 μL of ethanol/EDTA, together with sodium acetate, followed by centrifugation to remove excess ethanol. After air drying, the DNA products were solved in 10 μL of dH2O at 95°C for 3 min in a thermal cycler.

Statistical analyses

All tests were performed using Graphpad Instat and SPSS.16.0 (SPSS Inc., Chicago, IL, USA). The correlations of PIK3CA and TP53 gene mutations with clinical parameters and demographic data were evaluated using Fisher’s exact test. The x2 test was used to detect trends. Gene expression was analysed by comparing relative expression using the ∆∆Ct method. A nonparametric test (Mann–Whitney U test) was used to compare groups for categorical variables. Median (interquartile range) values are presented for variables. A p value of 0.05 or less was approved as statistically significant.

Results

PIK3CA gene mutation frequency in BCa tissues

Table 2 shows the results of the HRM analysis of PIK3CA mutations in the BCa samples (n = 79) obtained between 2016 and 2017. The HRM assay was used to distinguish wild-type/mutation BCa samples. Supplement Figure 1A shows the results of the HRM assay for PIK3CA exon 9, and Supplement Figure 1B presents the findings for
PIK3CA exon 20. The difference in the melting behaviour of heterozygous mutations was due to heteroduplex formation. Heteroduplexes melted earlier and therefore peaked above the wild-type baseline. HRM mutation-positive samples (n = 37) were identified by Sanger sequencing. In total, 33 mutations were detected in 79 BCa samples. According to the results, 21 of 33 mutations were in exon 20, and 12 mutations were in exon 9. The exon 20 and exon 9 mutations were identified as H1047L, H1047R, Q546R, E545K and E542K by Sanger sequencing (Figure 1 and Table 2).

TP53 gene mutation frequency in BCa tissues

Direct Sanger sequencing was used to confirm possible mutations in the samples (n = 108). The results of the HRM assay for tumour DNA are shown in Supplement Figure 1C–F and for TP53 exons 5–8. Supplement Table 1 presents the results of the analysis of exons 5–8 in 145 BCa tumours. In total, 67 (38%) mutations were identified in the BCa samples. Seven of these mutations were in exon 5, eight mutations were in exon 6, 21 mutations were in exon 7, and 31 mutations were in exon 8. The mutations in exons 5–8 were identified as R275H, S166, Y163C, R213R, V217A, H214R, R209T, M243T, T256T, G245S, R248Q, G245G, E298K, R290H, H296R, R273H, C275R, E270C, P301P and E294D by Sanger sequencing (Figure 2).

Correlation between TP53 mutation status and clinicopathological characteristics

Nod, ER and PR status exhibited a significant correlation with TP53 mutations (p = 0.029, p = 0.03 and p = 0.02, respectively). In terms of clinical profiles, BCa subtypes (luminal A, luminal B, luminal-HER2 and basal type) and age (p > 0.05) were not significantly correlated with an increased frequency of TP53 mutations (Supplement Table 3).

Expression of PIK3R1, PTEN and SALL4 mRNA in BCa tumour and non-tumour tissues

PIK3R1, PTEN and SALL4 mRNA were analysed in 112 BCa samples. PIK3R1 and PTEN gene expression levels were higher in tumour tissue than in non-tumour tissue (p < 0.0001 and p = 0.0059, respectively). On the contrary, the SALL4 gene level was lower in tumours than in non-tumour tissue (p = 0.0378) (Supplement Figure 2A–C).

Correlation between PIK3CA mutations and the genes expression status

In patients with PIK3CA mutations, the PIK3R1 gene expression level was significantly higher than those in patients without PIK3CA mutations, with a statistically significant difference (p = 0.1561, p = 0.7119 and p = 0.1684, respectively) (Supplement Figure 3A–C).

Correlation between TP53 mutations and the genes expression status

In patients with TP53 mutations, the PIK3R1 gene expression level was significantly lower than that in patients who did not carry TP53 mutations (p = 0.0169). However, PTEN and SALL4 gene expression levels in patients with

<table>
<thead>
<tr>
<th>Exon</th>
<th>Nucleotide</th>
<th>Codon</th>
<th>Domain</th>
<th>n (%)</th>
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<td>Q546R</td>
<td>Helical</td>
<td>1 (1.3)</td>
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<tr>
<td>20</td>
<td>A3140G</td>
<td>H1047R</td>
<td>Kinase</td>
<td>17 (21)</td>
</tr>
<tr>
<td>20</td>
<td>A3140T</td>
<td>H1047L</td>
<td>Kinase</td>
<td>4 (5.06)</td>
</tr>
</tbody>
</table>

The total number of mutations identified 33
The total number of the patients carrying the mutation 33
The number of samples examined 79
Percentage of samples with the mutation 42%
Figure 1: Sequence variants detected in PIK3CA mutations in BCa.
(A) An E542K in exon 9 (B) An E545K in exon 9 (C) A Q546R in exon 9 (D) A H1047R in exon 20 (E) A H1047L in exon 20.

Figure 2: Sequence variants detected in TP53 mutations in BCa.
(A) A R175H in exon 5 (B) A S166 in exon 5 (C) A Y163C in exon 5 (D) A R213R in exon 6 (E) A V217A in exon 6 (F) A H214R in exon 6.
TP53 mutations were lower than those in patients without TP53 mutations, although this finding was not statistically significant (Supplement Figure 4A–C).

Correlation between the gene expression status and clinicopathological characteristics

The median of gene expression was determined. Gene expression higher than the median value was considered high expression, whereas gene expression below the median value was considered low expression. High expression of PIK3R1, PTEN and SALL4 was detected in 47.7%, 52.4% and 51.7% of BCa samples, respectively. PIK3R1 expression levels exhibited a significant correlation with the histological grade (G3), ER(+) and HER2(−) tumours (p < 0.05). The PTEN expression level was significantly correlated with ER status (p < 0.05). However, SALL4 expression was not significantly correlated with clinical parameters (p > 0.05) (Supplement Table 4 and 6).

Discussion

In this study, PIK3CA and TP53 gene mutations were analysed using an HRM assay, and PIK3R1, PTEN and SALL4 gene expression analyses were performed using the RT-PCR. This study aimed to investigate whether these genes could be used as prognostic biomarkers in the diagnosis of BCa. To date, several mutations and gene expression analysis studies have proposed various candidate genes to use as biomarkers in BCa prognosis. However, as most of these gene variation studies were based on non-cancerous tissues, their results have limited clinical value. Therefore, in the present study, PIK3CA and TP53 mutations and PIK3R1, PTEN and SALL4 mRNA expression levels were analysed in cancerous and healthy fresh tissues.

Many studies have reported a high frequency of PIK3CA and TP53 mutations in BCa and demonstrated the important role of these mutations in tumorigenesis. According to the COSMIC [7] report, the mutation frequency of both PIK3CA and TP53 was about 27%. In this study, the results of the HRM assay of PIK3CA and TP53 mutations were confirmed by Sanger sequencing. The frequency of PIK3CA and TP53 mutations was 42% (33/79) and 38% (55/145), respectively. In a previous study, we analysed 101 BCa samples and detected 31 PIK3CA mutations using a HRM assay [9]. As most PIK3CA mutations have been found in exon 9 and exon 20, in current study we analysed PIK3CA exon 9 and exon 20 gene regions and found that mutations were localized in 36% (12/33) of cases in exon 9 and in 64% (21/33) of cases in exon 20. On the other hand, 67 TP53 gene mutations were localized in exons 5–8 sequence regions. The distribution of these 67 mutations in these regions was as follows: 4.8% (7/61) in exon 5, 5.5% (8/61) in exon 6, 14% (20/145) in exon 7 and 18% (26/145) in exon 8. In a study that included 309 BCa patients, Shimoi et al. [13] reported a frequency of PIK3CA mutations of 33% in all subtypes and a frequency of approximately 30–40% in all subtypes. However, they found no statistically significant difference in the mutation frequency in the different subtypes among the 309 BCa samples. Takeshita et al. [14] detected PIK3CA exon 9 and exon 20 mutations in plasma ctDNA in three and seven of 33 patients, respectively. Chen et al. [15] found a somatic mutation in PIK3CA in 44% of Chinese BCa patients. In their study, PIK3CA (32%) and TP53 (29%) genes were the most common mutations in HR(+) BCa patients. In another study, TP53 mutations were related with shorter recurrence-free survival, progression-free survival and overall survival [16]. Although the incidence of PIK3CA mutations in the present study was significantly high in ER(+) patients, no valuable statistical results in terms of the mutation frequency was found in luminal A, invasive ductal carcinoma, T2 tumour stage, G2 histological grade, nod 0, aged >50 y, PR(+), HER2(−) and >14 Ki67 level in patients.

Hotspot mutations of PIK3CA, create one of the major routes of activation of the PI3K signal and have been disclosed to be correlated with ER(+) condition [17]. According to previous research, high frequency of PIK3CA and FAT1 were detected in HR(+) patients, whereas high frequency of PIK3CA and ERBB2 mutations were detected in HER2(+) patients [18]. In a COX model, a ctDNA TP53 + PIK3CA gene mutation pattern successfully predicted disease progression within 6 months (17). Bartels et al. [19] concluded that CDKN2A loss and the PIK3CA mutation characterized a subgroup of metaplastic BCa patients with myoepithelial and spindle cell differentiation. PIK3CA mutations were found in 47% of menopausal women. Moreover, postmenopausal women with PIK3CA wild-type tumours had significantly worse disease-free survival [20]. On the other hand, in the current study, the incidence of TP53 mutations was significantly high in nod 0, ER(+) and HER2(+) BCa patients; however, no valuable statistical results in terms of the mutation frequency was found in luminal B, invasive ductal carcinoma, T2 tumour stage, G3 histological grade, >50 age, PR(+) and >14% Ki67 level in patients. In triple negative BCa, Shi et al. [21] in
multiple negative BCa detected very few recurrent mutations, including TP53 and PIK3CA, together with a long tail of individually rare. In another study, TP53 mutations were correlated with HER(+) BCa and younger age at diagnosis [22]. In a univariate analysis, Luo et al. [23] showed that MKI67, vascular invasion, CD117, CD117+/TP53 missense mutations and TP53 missense mutations were associated with overall survival of patients with TNBC.

In the present study, to analyse and detect all possible PIK3CA and TP53 gene mutations in BCa patients, we used a HRM assay, which offers a quick and cost-effective advanced methodology for detection of hotspot mutations. In the HRM assay, the mutations were identified based on different coloured peaks, and HRM variants were subjected to DNA sequencing using a DNA analyser to identify nucleotide variations. In the study, Sanger sequencing confirmed 89.2% of PIK3CA mutations and 62.04% of TP53 mutations detected in the HRM analysis, demonstrating that the method is a very forceful for detection of PIK3CA mutations. In regard to the outcome, cases with a mutation allele burden were better differentiated in reannealing than the melting step, as well as better separation of the wild-type from the mutant cases. As the length of the PCR products was closely, the Tm shift in curves was directly concerned to sequence variations. The HRM assay methodology was much more effective for PIK3CA gene mutation detection than TP53 gene mutation detection. This finding may be related to the lengths of the exons of the genes analysed.

According to the literature, PIK3R1 suppressed growth, invasiveness and metastatic properties of BCa cells. In addition, PIK3R1 was negatively regulated by miR-21, a direct target of miR-21 [24]. Andrade et al. [25] showed that among differentially expressed genes in normal epithelium, LCIS and ILC, using 23 patient-matched triplets of N, LCIS and ILC are demonstrated to be PIK3R1, GOLM1 and GPR137B genes. Cizkova et al. [26] reported under-expression of the PIK3R1 gene in 283 (61.8%) samples. Their study found that PIK3R1 underexpression was correlated with poorer metastasis-free survival. Also they reported that PIK3CA mutations were correlated to better metastasis-free survival. In the same study, by merging the PIK3CA mutation with PIK3R1 expression levels, four prognostic groups were showed with markedly different metastasis-free survival [26]. On the other hand, PIK3R1 gene expression levels in tumour tissue were higher than in non-tumour tissue in the present study. Moreover, PIK3R1 expression levels showed a statistically significant correlation with the G3 histological grade and ER(+), PR(+), HER2(+) and ER(+) status. PIK3R1 gene expression levels were higher in patients with PIK3CA mutations than in those without PIK3CA mutations, although the results were not statistically significant. The PIK3R1 gene expression level in patients with TP53 mutations was lower than that in patients without these mutations, with a statistically significant difference.

In previous research, loss of PTEN expression was related to ER(−), PR(−) and TNDC and marginally linked with distant metastasis. There was no correlation between PTEN loss and recurrence-free survival or overall survival [27]. Xu et al. [28] showed that mRNA expression levels of PTEN were significantly decreased in adjacent healthy tissues as compared with those in BCa tissues. In their research, miR142-5p and PTEN expression levels were positively and negatively correlated, respectively, with tumour size and metastasis. In the other study, 4% of patients with a PIK3CA mutation and/or low PTEN expression levels had a pCR as confront with 39% of patients with wild-type PIK3CA and high PTEN expression levels [29]. Furthermore, low PTEN levels and PIK3CA mutations predicted resistance to neoadjuvant lapatinib and trastuzumab treatment in the absence of chemotherapy in patients with HER2 overexpressing BCa [29]. According to the present our study, PTEN gene expression levels in tumour tissue were higher than those in healthy tissue. PTEN gene expression levels were higher in patients with PIK3CA mutations than in those without PIK3CA mutations, although the results were not statistically significant. PTEN expression levels showed a statistically significant correlation with the G3 histological grade and ER(+), PR(+), HER2(+) and ER(+) status.

Chen et al. [30] reported that SALL4 was upregulated in a drug-resistant BCa cell line, MCF-7/ADR. High cytoplasmic expression of SALL4 was analysed in BCa tissues as compared with that in healthy tissues. In addition, high SALL4 expression was associated with advanced tumour invasion, lymph node stage, ER, PR, HER2 and TNBC [31]. Itou et al. [32] showed that SALL4 overexpression suppressed the expression of adhesion gene CDH1 and positively regulated the CDH1 suppressor ZEB1. In the same study, SALL4 suppressed intercellular adhesion and maintained cell motility after cell-cell interactions and cell division, which resulted in a dispersed phenotype. In another study, the SALL4 mRNA expression level was high in 86.1% (31/36) of breast tumours, with no obvious correlation with clinicopathological factors. Moreover, SALL4 was expressed at a high level even in early clinical stages of cancer [33]. However, in our study the SALL4 gene expression level in tumour tissue was lower than that in healthy tissue. Although SALL4 was amplified in 62% of ER(+) patients, the finding was not statistically significant. SALL4 gene expression levels were higher in patients with
PIK3CA mutations than in those without PIK3CA mutations, although the results were not statistically significant.

In the present study, we identified a high frequency of PIK3CA and TP53 mutations using a HRM assay in a Turkish BCa population. Previous studies reported that the expression levels of three genes (PIK3R1, PTEN and SALL4) played a significant role in BCa. The present study is the first to investigate the effects of five genes in the PI3K pathway. The mutation and gene expression analysis results shed light on the association of these genes with various histological and molecular parameters in BCa.

**Conclusion**

**PIK3R1, PTEN and SALL4** gene expression analysis in patients with PIK3CA and TP53 mutations may be beneficial for the diagnosis of early-stage BCa in patients who have a high risk of a poor outcome. This study suggests that PIK3CA and TP53 gene mutations may be potential prognostic biomarkers of BCa and that PIK3R1, PTEN and SALL4 genes may be used in order to choose the best BCa treatment planning. We propose that these results should be considered in the evaluation of clinical parameters of BCa in the future.

**Conflicts of interest:** The authors declare no conflict of interest.

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**Ethical approval:** The ethical approval certificate was taken for this preclinical study from Marmara University Faculty of Medicine (09.2015.264).

**References**


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