RESEARCH ARTICLE

Detection of PIK3CA Gene Mutations with HRM Analysis and Association with IGFBP-5 Expression Levels in Breast Cancer

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Abstract

Breast cancer is the second most common cancer and second leading cause of cancer deaths in women. Phosphatidylinositol-3-kinase (PI3K)/AKT pathway mutations are associated with cancer and phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) gene mutations have been observed in 25-45% of breast cancer samples. Insulin growth factor binding protein-5 (IGFBP-5) can show different effects on apoptosis, cell motility and survival in breast cancer. We here aimed to determine the association between PIK3CA gene mutations and IGFBP-5 expressions for the first time in breast cancer patients. Frozen tumor samples from 101 Turkish breast cancer patients were analyzed with high resolution melting (HRM) for PIK3CA mutations (exon 9 and exon 20) and 37 HRM positive tumor samples were analyzed by DNA sequencing, mutations being found in 31. PIK3CA exon 9 mutations (Q546R, E542Q, E545K, E542K and E545D) were found in 10 tumor samples, exon 20 mutations (H1047L, H1047R, T1025T and G1049R) in 21, where only 1 tumor sample had two exon 20 mutations (T1025T and H1047R). Moreover, we detected one sample with both exon 9 (E542Q) and exon 20 (H1047R) mutations. 35% of the tumor samples with high IGFBP-5 mRNA expression and 29.4% of the tumor samples with low IGFBP-5 mRNA expression had PIK3CA mutations (p=0.9924). This is the first study of PIK3CA mutation screening results in Turkish breast cancer population using HRM analysis. This approach appears to be a very effective and reliable screening method for the PIK3CA exon 9 and 20 mutation detection. Further analysis with a greater number of samples is needed to clarify association between PIK3CA gene mutations and IGFBP-5 mRNA expression, and also clinical outcome in breast cancer patients.

Keywords: PIK3CA - PI3K - IGFBP5 - HRM assay - Turkish breast cancer - mutation

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Introduction

Breast cancer remains as the most second common cancer among women in Turkey, and the cancer incidence in Turkey is 35.8/100,000 according to the data of Ministry of Health (http://www.ketem.org/istatistik.php). The phosphoinositide 3-kinase (PI3K) family generates 3'-phosphoinositides that activates various cellular targets that are important for cell differentiation, cell proliferation, survival and migration (Cantley, 2002; Osaki et al., 2004; Sui et al., 2014). PI3Ks have oncogenic potential and several components of this signaling pathway are altered in human cancers (Li et al., 2006).

Activation of PI3K pathway has been directly linked to cancer through somatic mutations or amplifications of PIK3CA (Ligresti et al., 2009; Vogt et al., 2010; Tong et al., 2012; Liu et al., 2014), and loss of function of tumor suppressor PTEN (Georgescu, 2010). It was not until 2004 that cancer-specific activating mutations were reported in PIK3CA, which encodes the p110α isoform of PI3K (Campbell et al., 2004; Samuels et al., 2004). However, no mutations in non p110α isoforms have been detected thus far (Samuels and Velculescu 2004; Wood et al., 2007; Thomas et al., 2007; Parsons et al., 2008; TCGA 2008).

Somatic mutations in the PI3K subunit p110α occur in a variety of cancer types (He et al., 2013). PIK3CA mutations seem to be clustered mainly in three regions of the gene, exon 1 coding for p85, exon 9 coding for helical and exon 20 coding for kinase domains. Almost all tumor activating mutations (>90%) in human tumors occur in exon 9 (helical domain E542K and E545K) and in exon 20 (kinase domain H1047R) (Liedtke et al., 2008; Kandula et al., 2013). These mutations occur at frequencies extending from 5 to 25% in several common cancers, PIK3CA was mutated in 32% of colon, 27% of brain, 25% of gastric, 4 of 5 glioblastomas (27%), 3 of 12 breast cancer (8%) and 4% of lung cancers (Samuels et al., 2004).

In recent years, studies have demonstrated that the IGF (insulin-like growth factor) pathway can play a crucial role in the cell growth, differentiation, developmental
processes and malignant cell transformation (Valentinis and Baserga, 2001; Karamouzis and Papavassiliou, 2012). Moreover, IGF-I and IGF-II are potential mitogenic and survival factors, especially they play important roles for both normal and cancer cells. Their effects on cell proliferation are mediated by the type I IGF tyrosine kinase receptor (IGF-IR). It has been shown that high serum levels of insulin-like growth factor I (IGF-I) are associated with an increased risk of sporadic breast cancer (Pan and Hong, 2014). A study has demonstrated that, by binding to receptors, the IGF family can initiate the cell signal transduction pathway. This may play an important role in tumor occurrence and development (Grimberg and Cohen, 2000). The biological effects of IGFs, after binding to their receptors, are regulated by a series of specific insulin-like growth factor binding proteins (IGFBP-1,-2,-3,-4,-5,-6 and -7) (Hermani et al., 2013; Huang et al., 2014). Functional role of IGFBP5 in breast cancer is controversial and complicated. There are numerous studies on the role of IGFBP5 in survival and apoptosis of both normal and cancer cells. Recent studies have linked insulin-like growth factor (IGF)-binding protein 5 (IGFBP-5) to metastasis by revealing increased IGFBP-5 expression in metastatic patients (Pekonen et al., 1992; McGuire et al., 1994; Huynh, 1998; Mita et al., 2007). One study showed IGFBP5 expression is inversely related with tumorigenesis of breast cancer (Polanco et al., 2010), where in another study, IGFBP-5 is shown to be associated with increased survival of breast cancer patients (Ahn et al., 2010).

In light of the foregoing, this study was designed to describe the association between PIK3CA mutations and IGFBP-5 mRNA expressions in breast cancer for the first time. Therefore, we aimed to detect the frequency of PIK3CA mutations using a new method-high-resolution melting analysis (HRM), which has recently been used to detect unexpected mutations (Studer et al., 2009).

Moreover, we want to evaluate the associations between PIK3CA mutations with clinical, pathological, survival status and molecular features of breast cancer patients. We hypothesized that activation of this pathway through somatic mutations may be associated with increased IGFBP-5 mRNA levels and metastasis.

Materials and Methods

Patient Samples and Histopathologic Evaluation

A total of 101 patients with breast cancer, who diagnosed at the Department of General Surgery Marmara University School of Medicine from July 2010 to January 2012, were included in this study. Patients were at the age of 26 to 83. All samples were obtained under approved ethical protocols and informed consent from all subjects were take. As shown in Table 1 patients clinical informs.

Tumor histology and tumor grade were evaluated at primary diagnosis and were extracted from pathology reports. Tumors were graded according to the Bloom-Richardson grading modified system (Frierson et al., 1995). Human epidermal growth factor receptor 2 (Her2) status was determined by immunohistochemical analysis using the Dako HercepTest kit (Dako, Carpinteria, CA).

Moreover, if the Her2 score was 2, fluorescence in situ hybridization was performed further (Wadodo et al., 2014). For progesterone (PR) receptor status, the PR receptor monoclonal antibody PgR 636 (Dako, Wiesentheid, Germany) was used; for estrogen (ER) receptor status, the ER receptor monoclonal antibody clone SP1 (NeoMarkers, Fremont, CA) was used.

DNA extraction

The DNA was extracted from frozen tissue (-80 °C) using (Roche) Genomic DNA Kit (Roche), according to the manufacturer’s protocol. The DNA was qualitatively assessed by agarose gel electrophoresis and was quantified spectrophotometrically, to confirm that A260/A280 value between 1.8 and 2.0.

High-resolution melting analyses (HRM)

Real-time PCR protocols were conducted in Roche Light-Cycler® 480 (LC480) Multiwell plates (96-well white) on the LC480 instrument (Hoffman-La Roche, Basel, Switzerland) using the LC480 HRM Master Mix reagent kit (Roche). Real-time PCR programmes and reaction mixes were optimized to generate the required quantities of amplicon that is necessary for full melting analysis. DNA quantification of stock sample aliquots were detected using a Nanodrop 1000 spectrophotometer (Thermo Scientific). DNA template samples were diluted to 20-30 ng/μL. For each assay, two different primer sets (high performance liquid chromatography purified) were selected to flank the hot-spot mutation regions (exon 9 and exon 20) of the PIK3CA gene. Amplicon lengths ranged from 96 bp to 100 bp, depending on the HRM assay. Primers (Table 1) were ordered as described by Ney et al. (2012). Each individual sample (total 100 samples) was run in HRM analysis, depending on the total sample size in the run. Each real-time PCR reaction was conducted in a 15μL final volume containing 7.5μL of the Taq enzyme mix (No. 1 reagent from the Roche HRM Master kit containing Taq polymerase, dNTPs, buffer system and saturating dsDNA binding dye), 2.4μL of 25mM MgCl₂ solution (No. 2 reagent from the Roche HRM Master kit, final Mg concentration 2.5 mM), 0.4μL of each primer and 1.8μL of de-ionized water (No. 3 reagent from the Roche HRM Master kit) and 5μL of DNA template (20-30 ng). Fluorescence values for each sample replicate in each PCR cycle were recorded through the SYBR Green (483-533 nm) channel using the default LC480 data acquisition settings (Dang et al., 2012). The melting curves were analyzed by Gene Scanning Software (Roche Diagnostics) with normalized, temperature-shifted curves and finally

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–&gt;3’)</th>
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<tbody>
<tr>
<td>PIK3CA-9-F sequencint</td>
<td>TGAATAATCTGCTTATTTAATCCT</td>
</tr>
<tr>
<td>PIK3CA-9-R sequencint</td>
<td>TGTGTTAATTTGTTCTTTTGT</td>
</tr>
<tr>
<td>PIK3CA-20.1-F sequencing</td>
<td>TGGTCTCAAAACTGCAACCA</td>
</tr>
<tr>
<td>PIK3CA-20.1-R sequencing</td>
<td>GCATGCTTATTATGTGTGGG</td>
</tr>
<tr>
<td>PIK3CA-9-F sequencint</td>
<td>GCAAGAGGCTTTGGAGTATTTCCA</td>
</tr>
<tr>
<td>PIK3CA-9-R sequencint</td>
<td>AGCGTGTATTAGTGGAAAGATC</td>
</tr>
</tbody>
</table>

List of Primer Sets Used for High-Resolution Melting and Sanger Sequencing

Table 1. List of Primer Sets Used for High-Resolution Melting and Sanger Sequencing

displayed as a difference plot. Either genomic wild-type DNA isolated from cell line (MCF-7) or healthy sample DNA was used for normalization.

Cycling and melting conditions were as follows: an initial denaturation at 95°C for 10 minutes followed by 45 cycles of 20 seconds at 60°C, 25 seconds at 72°C, and 5 seconds at 95°C, followed by melting from 65°C to 97°C with 25 acquisitions/°C and a 10 minute cooling to 40°C with a ramp rate of 2.2°C/second (for PIK3CA exon 9 and exon 20).

**PCR for sequencing**

DNA of tumor samples were isolated according to the standard protocol. Primers (Table 2) were ordered as described by Ney et al. (2012). Different reaction conditions were used for two exons. For exon 20, after incubation at 94°C for 1 min, amplification was performed at the initial annealing temperature of 53°C, with a subsequent annealing temperature at 72°C for 5 min. Thirty nine amplification cycles were then performed. On the other hand, for exon 9, after incubation at 94°C for 1 min, amplification was performed at the initial annealing temperature of 56°C, with a subsequent annealing temperature at 72°C for 5 min. Thirty nine amplification cycles were then performed. Finally, we confirmed PCR amplification by 2% agarose gel electrophoresis and photographed using UV light trans illuminator. Then, PCR products were sent to DNA sequencing (BGI Tech).

**Sanger sequencing**

We sent samples to BGI (BGI Tech Solutions Co., Ltd., Build 11, Beishan Industrial Zone, Yantian District, Shenzhen, 518083, China) for Sanger sequencing. Analyses were performed from all samples to determine the mutation status and to verify the HRM results. Depending on the HRM assay design, verification of results was done either with conventionally amplified DNA fragments with Taq DNA Polymerase (Roche) or with fragments amplified by real-time PCR for HRM analysis. The later PCR products could be directly used for sequencing analyses, whereas conventionally amplified PCR products were checked for the right fragment length by agarose gel electrophoresis. Sequencing analysis results are shown in Figure 1 A-I.

**IGFBP-5 gene expression analysis**

Ten mg breast cancer frozen tissue was used to isolate RNA with High Pure RNA Tissue Kit Version 09 (Roche, Germany). Transcriptor High Fidelity cDNA synthesis kit (Roche, Germany) was used for cDNA synthesis with 500 ng of total RNA in a reaction volume of 20μL. Real time quantitative PCR for IGFBP5 and beta actin were performed using LightCycler 480 Probes Master (Roche, Germany). 2.5μL of cDNA was used in a reaction volume of 10. All reactions were performed in duplicate for reference housekeeping gene, beta actin and IGFBP5. Analysis and quantification was performed using LightCycler 480 software. Relative quantification was calculated by delta delta Ct method, subsequent to IGFBP5 expression normalization to beta actin.

**Statistics**

Variables that were deemed statistically significant by the univariate analysis were included in the final multivariate Cox regression analysis. P-values of less than 0.05 were considered statistically significant for single testing, whereas statistical significance was set at lower threshold (p<0.01 or p<0.025) for multiple testing. Statistical data were obtained by using Statistical Package for the Social Sciences (SPSS) for Windows version 16.0 (SPSS Inc., Chicago, Illinois, USA). The associations between mutations in PIK3CA and age, ER/PR status, stage of disease, and histologic grades were determined by the Fisher exact test, the x² test, and the x² test for trends. Association analysis between the mutational status in PIK3CA and survival was performed using the Kaplan-Meier method and the log-rank test. All tests were 2-tailed, and results were considered significant when p value was <0.05.

**Results**

**HRM and Sanger sequencing**

With the use of HRM assay we tried to distinguish the wild-type and the mutant (DNA). Samples with possible mutations (n=37) were further analyzed by direct Sanger sequencing to define the exact mutational status. HRM results of tumor DNA are depicted in Figure 2 and 3, for PIK3CA exon 20, and in Figure 4 for PIK3CA exon 9. The difference in the melting behavior of heterozygous mutations results from heteroduplex formation. Besides, heteroduplexes melt earlier and are therefore peaked above the wild-type baseline. On the other hand, both DNA strands are fitted to each other when the mutation is homozygous; depending on the nucleotide composition, binding characteristics may either differ or may be similar to those of the wild type sequence.

We analysed exon 9 and exon 20 in 101 breast cancer tumors (as shown in Table 2). Totally 32 mutations were...

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**Figure 1. Examples of PIK3CA mutations in breast cancer tissue samples determined by automated sequence analysis.**

A) A H1047L mutation in exon 20
B) A G1049R mutation in exon 20
C) A H1047R mutation in exon 20
D) A T1025T mutation in exon 20
E) An E542K mutation in exon 9
F) An E542K mutation in exon 9
G) An E542K mutation in exon 9
H) An E542Q mutation in exon 9
I) An E542Q mutation in exon 9
identified (in breast cancer samples) (31%), 22 of the mutations were in exon 20 and 10 mutations in exon 9. Exon 20 mutations were identified as H1047L (13.6%; 3/22), H1047R (77.2%; 17/22), T1025T (4.5%; 1/22) and G1049R (4.5%; 1/22) by Sanger sequencing (Fig. 4A-D).

Survival analysis
An overall survival analysis was performed on 101 patients with long term follow-up information (2010-2014), but no statistically significant association was found between survival and the PIK3CA mutation status. At the time of the last follow-up visit, 99 (98.01%) patients were alive. Two (1.98%) patients without PIK3CA mutation have died, one from cirrhosis and the other one from breast cancer.

Relation between PIK3CA mutations and IGFBP-5 gene expression in breast cancer
Randomly selected 37 breast cancer samples within our study group were involved in IGFBP5 expression analysis. The median of IGFBP-5 expression were determined and the results higher than the median were considered highly expressed, the results below the median were considered lowly expressed. The Table 3. Frequency of PIK3CA Mutations in Samples with Higher/ lower Expression of IGFBP-5.

<table>
<thead>
<tr>
<th>PIK3CA mutation</th>
<th>Higher expression of IGFBP-5</th>
<th>Lower expression of IGFBP-5</th>
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<tbody>
<tr>
<td>(+/−)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>(+)</td>
<td>7 (35)</td>
<td>5 (29.41)</td>
</tr>
<tr>
<td>(−)</td>
<td>13 (65)</td>
<td>12 (70.59)</td>
</tr>
<tr>
<td>Total n (%)</td>
<td>20 (100)</td>
<td>17 (100)</td>
</tr>
</tbody>
</table>

*p<0.0024*
were considered lowly expressed. When we compare the IGFBP-5 expression with PIK3CA mutations in breast cancer samples, we detected 35% of the samples with high IGFBP-5 expression have PIK3CA mutations. On the contrary, 29.41% of the samples with low IGFBP-5 expression have PIK3CA mutations. According to these results, there is no significant correlation between IGFBP-5 expression and PIK3CA mutations (p>0.05) (see Table 3).

Relation between PIK3CA mutation and clinicopathological characteristics and molecular subtypes in breast cancer

Several studies reported a correlation between presence of PIK3CA mutation and clinicopathological characteristics in breast cancer. PIK3CA mutations most often present in tumors with intact, expressed PTEN genes, in tumors that had metastasized to lymph nodes and in tumors with positive ER, PR, and ERBB2 (HER2). However, we have not found any significant correlation in our study. We then compared breast cancer subtypes (Luminal A, Luminal B, HER2, Luminal-HER2 and basal type) according to PIK3CA mutation presence, we have not found any significant correlation.

Discussion

Breast cancer is one of the most frequent and severe cancer types in women worldwide and its recurrence rate is very high (Bos et al., 2009). Breast carcinoma encompasses a heterogeneous group of tumors with great variability, both at the molecular and the morphological levels and also clinical outcome is variable.

After the initial discovery of PIK3CA mutations in breast cancer, many attempts were carried out to correlate them with clinicopathological features/characteristics of breast cancer cases.

In one of the earliest studies on PIK3CA mutations in breast cancer, mutations were identified in 1/12 breast cancer patients (Samuels et al., 2004). Yamaguchi (2001) suggested that PI3K signaling, via p110α, regulates invadopodia-mediated invasion and migration of breast cancer cells. Besides, several groups have initiated more comprehensive mutational analysis of PIK3CA in breast cancer (Campbell et al., 2004; Bachman et al., 2004; Lee et al., 2005; Levine et al., 2005; Saal et al., 2005; Wu et al., 2005). Scientists have reported that PIK3CA was the most commonly mutated oncogene yet discovered in breast cancer. PIK3CA mutations are valuable and most commonly mutated oncogene yet discovered in breast cancer, followed by exon 9 mutations. Similar to this study, we found exon 20 mutations in 22% and exon 9 mutations in 10% of our patient population. Moreover, we detected 14.3% of the mutations in their breast cancer population were outside the mutation “hot spots”. Li et al., (2006) reported that PIK3CA mutations were detected in 1/12 breast cancer patients. This is the first study which shows PIK3CA mutation screening in Turkish breast cancer population. Our results were consistent with other population. Bachman et al. (2004) detected that the PIK3CA mutation rate is 22% in a smaller set of breast cancer samples. In a set of 292 breast tumors, the reported mutation rate of PIK3CA mutations was 26% (Saal et al., 2005). In another study, 21% in 92 breast tumors (Wu et al., 2005). The vast majority of PIK3CA mutations (89%) occurs in exon 9 (helical) and exon 20 (kinase) (Campbell et al., 2004). Campbell et al., (2004) reported 28 (40%) of to cases harbored somatic mutations of PIK3CA, %53.6 and 32.1% of these mutations are in exon 9 and 20 respectively. Bachman et al. (2004) reported 14.3% of the mutations in their breast cancer population outside the mutation “hot spots”. Li et al., (2006) reported that exon 20 mutations predominate in breast cancer, followed by exon 9 mutations. Similar to this study, we found exon 20 mutations in 22% and exon 9 mutations in 10% of our patient population. Moreover, we detected H1047R mutation (77.3%) in exon 20 and E545K mutation (60%) in exon 9 to be the two most frequent mutations in our breast cancer samples.

Many methods are available for mutation detection and in this study we showed that the developed methodology HRM is a rapid and cost-effective screening tool for point mutations in breast cancer patients and it is possible to detect all the PIK3CA gene mutations occurring in this group. We determined that Sanger sequencing confirmed 87% of the HRM results and this shows us that HRM is a very effective method for the PIK3CA mutation detection.

We aimed to investigate the association between PIK3CA gene mutations and IGFBP-5 expressions in breast cancer patients. This is the first study done by HRM assay to reveal PIK3CA mutations and to compare IGFBP-5 expression in breast cancer. PIK3CA mutations were present in 35% of samples with high IGFBP-5 expression, and of 29.41% of samples with low IGFBP-5 expression. The distribution of mutations according to IGFBP-5 expression is not significant. Increasing the number of patients would be necessary to determine a significant association.

PIK3CA is the most common mutant a gene and play
crucial functions in breast cancer. The majority mutation changes after chemotherapy were from mutant status to wild-type, suggesting that cancer cells harboring PIK3CA mutations might be more sensitive to chemotherapy than those without mutation (Jiang et al., 2014). More studies are required to elucidate the mechanism by which loss of mutations in PIK3CA regulates chemosensitivity. For this reason sometimes patients was a combination of taxanes and platinum for the mutation loss (Bachman et al., 2004).

So that, chemotherapy may affect somatic mutation status in patients with breast tumor, lowering the number of PIK3CA mutations.

On the other hand, IGFBP5 expression has been found to correlate with prognosis, clinical outcome (Daigeler et al., 2010), survival (Ahn et al., 2010), response to therapy (Yamashita et al., 2009), and drug resistance (Taylor et al., 2010), either negatively or positively. IGFBP5 is one of the genes that has been shown to predict the prognosis of primary breast tumors in reverse tamoxifen resistance (Ahn et al., 2010). Moreover, lower expression levels of IGFBP5 are reported to be associated with shorter overall survival after tamoxifen (TAM) therapy.

In conclusion, detection of IGFBP-5 expression within the patients with PIK3CA mutations could be substantial in risk assessment of breast cancer patients, early prediction of metastasis or proliferation. Moreover, if such studies are carried out with increased number of populations that have PIK3CA mutations in the PI3K pathway, it will provide a stronger rationale evidence for development of new therapeutic and diagnostic approaches for breast cancer patients. PIK3CA mutations may be a potential diagnostic biomarker for breast cancer patients and may be used in process of choosing a breast cancer treatment in the future. Because these mutations are important for adjuvant chemotherapy and prognosis. PIK3CA driver mutations might be resistant to chemotherapeutic agents.

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