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Research Article

Elif Ulu, İlhan Yaylım*, Soykan Arıkan and Canan Cacına*



The evaluation of *PIK3CA* gene variation and serum PI3K level in breast cancer risk and prognosis in Turkish population

[Meme kanseri risk ve prognozunda *PIK3CA* gen varyasyonu ve serum PI3K düzeyinin Türk toplumunda değerlendirilmesi]

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Abstract

Objectives: The PI3K (Phosphatidylinositol 3-kinase) is the member of lipid kinase family that plays important roles in tumorigenesis, cancer development and cell proliferation. In our study, we aimed to investigate the relationships between breast cancer risk and prognosis with *PIK3CA* rs6443624 (C>A) intron region gene polymorphism and serum PI3K levels.

Methods: A total of 61-patients with breast cancer and 101 controls were included to the study. *PIK3CA* polymorphism was detected by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique. Serum PI3K levels were measured by Enzyme-Linked Immuno Sorbent Assay (ELISA).

Conclusions: We suggest that serum PI3K levels may play role in breast cancer risk and larger patient groups may have clinical value in assessment of the genetic risk and tumor progression of breast cancer. **Keywords:** apoptosis; breast cancer; phosphatidylinositol-

Results: PIK3CA (C>A) gene polymorphism genotype and

allele distributions were no significant in cases and con-

trols (p>0.05). The serum PI3K levels of breast cancer patients were found significantly higher than the control

groups (p=0.033). There were not significant association between *PIK3CA* (C>A) gene polymorphism and clinic and

prognostic parameters in our study group. We also evalu-

ated serum PI3K levels in the term of tumor progression,

but we did not observe any significant data.

Keywords: apoptosis; breast cancer; phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*); serum level; single nucleotide polymorphism (SNP).

ÖZ

Amaç: PI3K (Fosfatidilinozitol 3-kinaz); tümör gelişimi, kanser ve hücre proliferasyonunda önemli rol oynayan lipid kinaz ailesinin üyesidir. Bu çalışmada, *PIK3CA* rs6443624 (C>A) gen polimorfizmi ve serum PI3K düzeylerinin meme kanseri risk ve prognozundaki etkilerinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Çalışmamıza 61-meme kanserli hasta ve herhangi bir benign veya malign tümörü olmayan 101-kontrol bireyi dahil edilmiştir. Polimeraz Zincir Reaksiyon- Restriksiyon Parça Uzunluk Polimorfizmi (PCR-RFLP) tekniği ile *PIK3CA* polimorfizmi, ve Enzim Bağlı İmmünosorbent analizi (ELISA) ile serum PI3K seviyeleri belirlenmiştir.

Bulgular: Çalışmamız sonucunda *PIK3CA* (C>A) gen polimorfizm genotipi ve allel dağılımları açısından hasta ve

Elif Ulu, Department of Molecular Medicine, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey. https://orcid.org/0000-0002-5167-588X

Soykan Arıkan, General Surgery Clinics, Istanbul Education and Research Hospital, Istanbul, Turkey. https://orcid.org/0000-0001-7132-6161

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^{*}Corresponding authors: Dr. İlhan Yaylım and Asst. Prof. Dr. Canan Cacına, Professor, Department of Molecular Medicine, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Çapa-Istanbul, Istanbul, Turkey, Phone: +90 212 414 2000 33326, Fax: +90 212 542 4171, E-mail: ilhanyaylim@gmail.com (İ. Yaylım), canan.cacina@istanbul.edu.tr (C. Cacına). https://orcid.org/0000-0001-8998-2028 (İ. Yaylım). https://orcid.org/0000-0001-7911-4390 (C. Cacına)

kontrol grubu arasında anlamlılık saptanmamıştır (p>0.05). Meme kanseri hastalarının, serum PI3K düzeyleri kontrol gruplarına göre anlamlı olarak yüksek oranda tespit edilmiştir (p=0.033). Çalışma grubumuzda PIK3CA (C>A) gen polimorfizmi, serum PI3K düzeyleri ile klinik ve prognostik parametreler arasında anlamlı bir iliski gözlenmemiştir.

Sonuç: Araştırmamız sonucunda serum PI3K düzeylerinin, meme kanseri riskinde rol oynayabileceğini ve meme kanserinin genetik risk ve tümör ilerlemesinin değerlendirilmesinde daha büyük hasta gruplarında klinik değeri olabileceğini düşünmekteyiz.

Anahtar Kelimeler: apoptoz; Meme kanseri; Fosfatidilinozitol-4,5-bifosfat 3-kinaz katalitik alt birim alfa (PIK3CA); serum düzeyi; tek nükleotid polimorfizmi (SNP).

Introduction

Breast cancer (BC) underlying mechanism is related with various growth factors and receptors that activate cell proliferation and it is also known as the most common malignant tumor that causes death among women [1]. It has been reported that many signaling pathways and their interactions also dysregulation in their functions play a key role in the progression of BC [2-5]. Phosphatidylinositol 3-kinases (PI3Ks) are family of intracellular lipid kinases phosphorylate the 3'-hydroxyl group of phosphatidylinositols and phosphoinositides that regulate many cellular processes including protein synthesis, cell survival, proliferation, differentiation, angiogenesis and apoptosis [3-9]. Furthermore (PI3Ks) pathway is one of the most frequently disrupted mechanisms in human cancers and shown to be responsible for resistance to anticancer therapies by provoking tumor development [3–9]. To our best knowledge PI3K isoforms induce the signal transduction trafficking that activated by cell surface receptors including receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCRs) and trigger other effector pathways such as serine/threonine kinase AKT and mammalian target of rapamycin (mTOR) [7–11]. Tumor suppressor PTEN (The Phosphatase and Tensin homolog deleted on chromosome 10) which is a negative regulator of the PI3K/AKT pathway, acts as an antagonist of the PI3K effects [12]. It has been previously reported that the loss of PTEN functions may cause excessive activation of the PI3K pathway and consequently promote cell proliferation [9, 13].

There is a broadening characterization for each of the three categories PI3K classes (I, II and III) and further level of their complexity is related with eight PI3K isoforms [14, 15]. Previous studies reported that Class I; PI3Ks primarily phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP2) to generate the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3) and they are related with (RTKs) and (GPCRs) thus they play important roles in several pathways such as metabolism, proliferation, autophagy, chemotaxis also described as the prominent class that associated with cancer [9–11, 16–18]. PI3K; Class I members are heterodimeric molecules consist of catalytic and regulatory subunit and activated through the inhibitory action of the p85 subunit on the p110 catalytic subunit (or p110α protein) [11, 19, 20]. p110 alpha (p110α) protein is a member of Class IA; PI3K and it was known encoded by the PIK3CA gene [21].

It has been that reported breast cancer tumorigenesis is closely related with PI3K pathway due to the majority of cases of this disease harbor at least one molecular mechanism that potentially enhances the pathway [22]. Further scientific advances have led to the discovery novel biomarkers associated with BC risk and prognosis and these mutations of PI3K specifically PIK3CA gene mutations have the potential to become a clinically useful biomarkers, because they are located on the important signaling pathway related with several biological molecules and found at high frequency also easy to measure (present or absent) [22-26].

PIK3CA gene is 34 kb length and located in the 3q26.3 chromosome region, it has 20 exons, encodes 1,068 amino acids [19, 21]. Somatic missense mutations and polymorphisms have been identified along the PIK3CA gene p110α sequence [24-27]. PIK3CA; rs6443624 (C>A) gene polymorphism is located in intron region [27]. Although the effects of several PIK3CA polymorphisms have been investigated in different types of malignancy [27, 28] there are limited number of studies that subjected directly rs6443624 gene variation in the literature. The available literatures regarding the significance of PIK3CA rs6443624 report conflicting results [27, 28]. In several studies researchers suggested that variation might affect the binding of transcription factors and change the splicing patterns or transcription of the PIK3CA gene while other group of researchers reported PIK3CA rs6443624 variation could be an independent predictor and prognostic factor [27, 28]. However studies concluded that the association between *PIK3CA* rs6443624 and BC risk and patient survival remains still unclear [27, 28].

Several analysis that related with PIK3CA gene mutations and polymorphisms have been conducted in different populations [26-28] but we did not observe directly associated data with PIK3CA rs6443624 gene variation and serum PI3K levels in Turkish population. In this purpose, the present study was designed to investigate the possible relationship between the PIK3CA rs6443624 genetic variation and serum PI3K expression in BC risk and prognosis.

Materials and methods

Study design

This case control study was approved by the Istanbul University Faculty of Medicine Ethics Committee [Project No. 35782]. The clinical and histopathological evaluations of 61 breast cancer patients were performed by the Istanbul Education and Research Hospital General Surgery Clinic. The control group consists of 101 healthy individuals with no signs of malignancy and preferably no family history of cancer. We used the Power and Sample Size Program software to calculate power and the effective sample size. The study protocol was consistent with the World Medical Association Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects). After obtaining informed consent, the blood specimens were collected from the patients before any treatment had been started (chemotherapy or radiotherapy). Data on age, family history, smoking status and alcohol consumption were obtained from the study questionnaire and classified in cases and control groups. Histological grade for individual tumors was determined through the assessment of differentiation. Questionnaires, medical records, and pathological reports were received to confirm the diagnosis and cancer status.

Polymorphism analysis

Genomic DNA was obtained from 10 mL of fresh peripheral blood samples which collected in EDTA tubes and extracted by salt out precipitation method based on isolation of leukocytes from anticoagulated blood, digestion with proteinase K [29] and stored at $-80\,^{\circ}\text{C}$ until assayed. To assess quantity and quality of DNA, we measured samples at 260/280 nm ratio absorbance spectrum by using the Thermo Scientific NanoDrop ND 1000 Spectrophotometer (Thermo Fisher Scientific Inc.). The PIK3CA rs6443624 (C>A) polymorphism genotyping was performed by polymerase chain reaction (PCR) and restriction fragment length analysis (RFLP) methods. Rigorous quality control procedures were applied throughout the genotyping process. Reagents for PCR were carefully aliquoted, and each aliquot was used no more than three times to avoid contamination. A negative control (no DNA template) was added to monitor PCR contamination for each assay. Approximately 10–15% of the samples in each genotype group were randomly selected for repeated assays by PCR following RFLP. A 355 bp PCR product of the gene was amplified by using Forward 5'-TAAGATGTGCAGAGTTCGTTGTATG-3' and Reverse 5'-TTGCCTTTG-TAAATATGCTCCATAATC-3' primers [30]. The reaction mixture was prepared as 12.9 µL distilled water, 3 µL 10× PCR Buffer, 1.2 µL MgCl₂, 3 μL dNTP, 1.2 μL from each primer and 0.5 U Taq polymerase (i-StarTag™ DNA Polymerase, Korea). 23 µL of reaction mix was dispensed into the PCR tubes as the number of samples. Afterwards $2\,\mu\text{L}$ of DNA, 50–100 ng, was added to each tube immediately and the reaction volume reached 25 μL . The PCR reactions were started with an initial denaturation the DNA at 95 °C for 5 min, followed by 35 cycles at $94\,^{\circ}\text{C}$ for $45\,\text{s}$, $60\,^{\circ}\text{C}$ for $45\,\text{s}$, $72\,^{\circ}\text{C}$ for $45\,\text{s}$; the final extension step was at 72 °C for 4 min. The PCR products were digested with AluI restriction

enzyme (Thermo Fisher Scientific™, Vilnius, Lithuania) overnight at 37 °C and analyzed on 2% agarose gel containing ethidium bromide. After the digestion by the restriction enzyme, the homozygous CC genotype produced two fragments (26,293 bp), heterozygous CA genotype produced three fragments (355,262 and 93 bp) and the AA genotype produced one fragments (355 bp) [30] (Figure 1).

PI3K assay

Peripheral blood samples of 43 patients and 62 control groups were collected in serum-separating tubes without anticoagulant. The samples were centrifuged at 10,062×g for 5 min for individual serum collection. The eluted sera were aliquoted into portions and preserved at -80 °C until analysis under laboratory conditions. Serum PI3K levels were determined with the Sandwich Enzyme-Linked Immuno Sorbent Assay (ELISA) kit (SunRed, China, Cat No. 201-12-0897, Lot No. 201910). Reference ranges for PI3K were 0.25-70 ng/mL.

Statistical analysis

All statistical analyzes were performed using the SPSS (version 15.0 SPSS Inc., Chicago, IL, USA) software package. Data are expressed as means ± SD. Categorical data were evaluated with Student's t-test, and chi-square (χ^2) . The differences in the distribution of PIK3CA rs6443624 (C>A) polymorphism genotypes or alleles between cases and controls were tested using the chi-square (χ^2) test. Significance was accepted as p<0.05. Hardy-Weinberg (HWE) balance was checked with the chisquare test. The genotype frequency distribution was consistent with the Hardy-Weinberg Equilibrium in control group (p>0.05). Odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated to estimate breast cancer risk. Student's t-test or ANOVA and tests based on data distribution with Mann-Whitney U or Kruskal-Wallis tests were used to assess both the prevalence of the genotype and allele frequencies and the levels of PI3K between groups.

Results

A total of 61 breast cancer cases and 101 healthy controls were included in the current study. All participants were female. Characteristics of patients with BC and healthy control groups are shown in (Table 1). The mean age of BC cases were 47.53 ± 12 years and the mean age of 101 healthy individuals were 44 ± 9.49 years. There were no significant differences in the distribution of age (p=0.183), family history (p=1.00), smoking status (p=1.00) among the studied groups. We evaluated the cases in terms of tumor type, invasive ductal carcinomas were observed with a rate of 82.6% and other classifications (in situ ductal and mucinous) in 17.3% of BC cases. The tumor stage rates were determined as 29.2% T1, 41.7% T2, 12.5% T3 and 16.7% T4. Also, we observed that the 63.6% of cases were in premenopausal and 36.4% were in postmenopausal period.

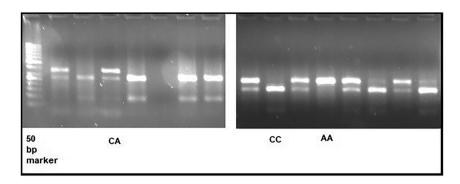


Figure 1: Agarose gel electrophoresis images of the PIK3CA (C>A) fragments.

When our study group was evaluated by the terms of genotype distribution, the PIK3CA rs6443624 (CC, CA and AA) genotypes frequencies were detected as (47.5, 45.5, 6.9; 59, 39.3, and 1.6%) in controls and patients groups, respectively. We did not observe statistically significant

Table 1: Clinical features of control individuals and BC cases (%).

	Controls (n=101)	Cases (n=61)
Mean ± SD age, years	44 ± 9.49	47.53 ± 12.56
p>0.05		
Smoking status		
Smoker	0	8.3
Non smoker	100	91.7
p>0.05		
Family history of cancer		
Yes	0	33.3
No	100	66.7
p>0.05		
Tumor stage		
T1 and T2		70.8
T3 and T4		29.2
Lymph node status		
NO NO		42.9
N+		57.1
Histopathology		
Invasive ductal		82.6
İn situ ductal		4.3
Mucinous		13.0
Distant metastasis		
(+)		27.0
(-)		73.0
Menopausal status		
Premenopause		63.6
Postmenopause		36.4
ER status		
Negative		9.1
Positive		90.9
PR status		
Negative		22.2
Positive		77.8
cerb B		
(-)		72.4
(+++)		27.6

differences between the two groups in terms of the genotype distribution and allele frequency (Table 2). In addition, the PIK3CA (C>A); alleles frequencies were analyzed according to the histopathological features of breast cancer patients such as tumor stage, presence of nodal metastasis, distant metastasis, estrogen receptor, progesterone receptor, tumor necrosis. We couldn't find any significant relationship between the allele frequency and these prognostic parameters (p>0.05) (Table 3).

We also evaluated the serum PI3K levels in our study groups and found that the mean serum levels of the cases were significantly increased (1.188 \pm 0.743) compared to 62 the control groups (0.910 \pm 0.574) (p=0.033) (Table 4). Furthermore we investigated the possible association between PIK3CA (C>A); CC, CA, AA genotypes and serum PI3K levels in cases and control groups but there were no significant relation between PIK3CA polymorphism genotype distribution and serum PI3K levels (data not shown). When we analyzed clinicopathological parameters and PI3K levels according to PIK3CA allele distributions in breast cancer patients, the allele frequencies are not associated with neither serum PI3K levels nor prognostic parameters (p>0.05) (Table 5).

Discussion

Single nucleotide polymorphisms (SNPs) can affect susceptibility to various diseases by altering protein structure

Table 2: Genotypes and allele frequencies for PIK3CA C>A gene polymorphism in BC cases and controls.

Controls, n (%)	Cases, n (%)	p-Value
48 (47.5)	24 (59)	0.129
46 (45.5)	36 (39.3)	
7 (6.9)	1 (1.6)	
142 (70.29)	84 (68.85)	X^2 =0.075, p=0.783
60 (29.70)	38 (31.14)	
	48 (47.5) 46 (45.5) 7 (6.9) 142 (70.29)	46 (45.5) 36 (39.3) 7 (6.9) 1 (1.6) 142 (70.29) 84 (68.85)

p-Value obtained by chi-square test.

Table 3: Association of PIK3CA C>A polymorphism with clinicopathological features of BC.

Clinicopathological features		C allele, %			A allele, %				
		CC + CA	AA	p-Value	Odd ratio (OR) (%95 Confidence interval)	AA + CA	СС	p-Value	Odd ratio (OR) (%95 Confidence interval)
T stage	T1 + T2	69.6	100	1.00	1.438	68.8	75.0	1.00	1.091
	T3 + T4	30.4	-		(1.097-1.884)	31.3	25.0		(0.649-1.833)
Distant metastasis	(-)	72.2	100	1.00	1.385	85.0	58.8	0.136	0.692
	(+)	27.8	_		(1.131-1.696)	15.0	41.2		(0.446-1.073)
Lymph node status	NO	41.2	100	0.429	2.429	45.0	40.0	0.767	0.889
	N+	58.8	_		(1.625-3.629)	55.0	60.0		(0.405-1.952)
Progesterone receptor	(-)	23.1	-	1.00	1.30	7.7	35.7	0.165	0.696
	(+)	76.9	100		(1.053-1.605)	92.3	64.3		(0.457-1.061)
Estrogen receptor	(-)	9.1	_	-	-	5.3	14.3	0.561	0.905
	(+)	90.9	_			94.7	85.7		(0.713-1.149)
Tumor necrosis	(-)	68.2	100	1.00	1.467	75.0	57.1	0.626	0.762
	(+)	31.8	-		(1.102-1.951)	25.0	42.9		(0.378-1.536)

Table 4: The serum PI3K levels in BC patients and control group.

Study groups	n	Serum PI3K, ng/mL	p-Value	
BC patients	43	1.188 ± 0.743	0.033	
Control	62	0.910 ± 0.574		

Values are given as mean \pm SD (standard deviation).

and function [31]. Genetic modifications in several signaling pathways such as PI3K/AKT/mTOR induce the cancer risk by promoting critical cellular functions [30]. According to the studies PIK3CA gene mutations in breast cancer are commonly studied worldwide [23, 25, 32, 33]. For instance. Dirican et al. have conducted comprehensive studies about PIK3CA mutations and clinical correlations of PIK3CA gene mutations in breast cancer and they reported 31% PIK3CA mutation in Turkish BC patients [26].

In current study we focused on PIK3CA rs6443624 gene polymorphism which is the located in intronic region and to the best of our knowledge, this is the first study that analyzed the PIK3CA rs6443624 gene polymorphism in Turkish population. Also we aimed to investigate the possible correlation between serum PI3K levels and clinical, pathological features of the patients according to importance of the PI3K signaling pathway in different types of cancer. There were no significant association between the PIK3CA rs6443624 polymorphism genotype distributions and allele frequency in cases and control groups in our study. Furthermore we did not observe any correlation between PIK3CA rs6443624 genotype distributions, allele frequencies and clinical parameters such as age, family history, smoking status and alcohol consumption also histological parameters (p>0.05). On the other hand, we found the serum PI3K levels significantly higher in the BC patients compared to the control groups (p<0.05). Moreover the clinicopathological parameters were analyzed according to PIK3CA rs6443624 allele distributions and PI3K serum levels in cases but the allele frequencies are not associated with neither serum PI3K levels nor prognostic parameters (p>0.05) (Table 5).

There are limited number of studies that subjected directly rs6443624 gene variation in the literature. In a study conducted by Wang et al., they observed the statistically differences in PIK3CA gene variant rs6443624 genotype frequency and allele distributions between BC patients and control groups in the Chinese population. They found the CC, AC and AA genotypes distribution as

Table 5: PIK3CA:rs6443624 (C>A) polymorphism allele distribution and serum PI3K levels (ng/mL) in BC patients and healthy control groups.

PI3K genotype		Cases (n=43)			Controls (n=62)			
	n	Serum level, ng/mL	p-Value	n	Serum level, ng/mL	p-Value		
AA	_	=	_	3	0.801 ± 0.072	0.739		
CA + CC	43	1.18 ± 0.74		59	0.91 ± 0.588			
CC	22	1.22 ± 0.94	0.736	34	0.858 ± 0.55	0.440		
CA + AA	21	1.14 ± 0.46		28	$\textbf{0.97} \pm \textbf{0.60}$			

Values are given as mean \pm SD (standard deviation).

(24.9, 48.6, and 26.5%) in healthy controls and (17.7, 45.7, and 36.6%) in breast cancer cases, respectively [27]. In our study, CC, AC and AA genotypes distribution in control groups were (47.5, 45.5, and 6.9%) and in breast cancer patients were (59, 39.3, and 1.6%) respectively and we did not observe statistically differences. There are conflicting results in the available literature in terms of prognostic significance of PIK3CA rs6443624 polymorphism. When we performed stratification analyses by regarding prognostic parameters; we did not detect significant correlation between the allele frequencies and histopathological features of patients. However, Wang et al. reported that the PIK3CA polymorphism AC/AA alleles were associated with advanced tumor stage in cases [27]. Bodnar et al. noticed a two-fold increased risk of death in the carriers of the PIK3CA variant A allele compared to individuals with CC genotype in renal cell carcinoma (RCC) [28]. In another study which was performed by Wang LE et al. [34] the risk and clinical outcomes of endometrial cancer were investigated. Their research was included non-Hispanic whites (76.2%), African Americans (8.7%), and Mexican Americans (14.8%) individuals of endometrial cancer patients. They examined 48 SNPs in their study and followed all cases in terms of death and recurrence after surgical treatment. They found that three SNPs (rs6443624, rs9838411, and rs2699887) in the intron of PIK3CA, were significantly associated with susceptibility and survival or recurrence of endometrial cancer. They concluded that the PIK3CA gene has different effects on the risk and recurrence of endometrial cancer due to its different biological roles in the initiation and progression of endometrial cancer [34]. In contrast Lacey et al., reported no significant relationship between the rs6443624 variant and endometrial cancer in their study which was conducted in Polish population (p=0.66) [35].

In another study Bizhani et al. investigated PIK3CA, AKT1 and mTOR polymorphisms and they reported a statistical significance between the PIK3CA rs6443624 (C>A) gene variation and the risk of bladder cancer in the Iranian population. According to their result carriers of PIK3CA variant genotype was shown reduced risk of bladder cancer [30].

Wan et al., investigated PIK3CA rs6443624 polymorphism in oral squamous cell carcinoma. They found that the PIK3CA gene expression was significantly higher in tumor tissues, but they did not observe a link between PIK3CA rs6443624 polymorphism and OSCC risk. Furthermore they did not detect differences in the distribution of clinical parameters such as gender, age, and smoking status [36].

Recent studies have reported that PIK3CA can stimulate PIP3 to activate phosphoinositide-dependent kinases PDK1 (4, 9, 37). AKT is activated after phosphorylation by the *PDK1* that contributes the proliferation, survival, metastasis, inhibition of cell apoptosis, and even oncogenic transformation of tumor cells (4, 9, 37). Hildebrandt et al. investigated the AKT2 rs892119, PIK3CA rs6443624 and PTEN rs12357281 polymorphisms in esophageal cancer patients treated with chemoradiotherapy, and reported that the different recurrence-free survival times might be related with gene-gene interactions [37].

Slattery et al.; investigated several gene (NFxB1, NFκB1A, PTEN, TSC1, TSC2, STK11, RPS6KA2, IκBKB, mTOR, PDK2, PIK3CA, PRKAA1, PRKAG2) variations which play important roles in the regulation of signaling pathways associated with breast cancer pathogenesis. They also analyzed the association between the molecular mechanism of these genes and ethnic differences in cancer risk, they concluded that the differences of breast cancer incidence in various populations might be associated with changes in biological factors [38]. In present study we did not observe any significances between PIK3CA rs6443624 polymorphism and BC risk and prognosis and our data was consistent with the results of different cancer types in studies that conducted in Chinese [36], Polish [35] populations.

However, our study has limitations for instance the pathogenesis of the disease is complex; this was a preliminary study and the number of the study group was relatively small. Although our research provided a valuable clue for the serum PI3K levels may play a role in BC risk. Larger patient groups data evaluation may have better value in assessment of genetic risk and tumor progression.

Conclusions

We have concluded that *PIK3CA* rs6443624 (C>A) gene polymorphism may not have an effective role in the development and tumor progression of BC in the Turkish population but the PI3K serum levels may related with BC risk.

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Conflicts of interest: The authors declare no conflicts of interest.

Informed consent: Informed consent was obtained. **Ethical approval**: Ethical approval (Date: 02.09.2020 No: 147435) was obtained from Istanbul University Faculty of Medicine Ethics Committee.

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