RESEARCH ARTICLE

Evaluation of Genetic Variations in miRNA-Binding Sites of BRCA1 and BRCA2 Genes as Risk Factors for the Development of Early-Onset and/or Familial Breast Cancer

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Abstract

Although genetic markers identifying women at an increased risk of developing breast cancer exist, the majority of inherited risk factors remain elusive. Mutations in the BRCA1/BRCA2 gene confer a substantial increase in breast cancer risk, yet routine clinical genetic screening is limited to the coding regions and intron-exon boundaries, precluding the identification of mutations in noncoding and untranslated regions. Because 3' untranslated region (3'UTR) polymorphisms disrupting microRNA (miRNA) binding can be functional and can act as genetic markers of cancer risk, we aimed to determine genetic variation in the 3'UTR of BRCA1/BRCA2 in familial and early-onset breast cancer patients with and without mutations in the coding regions of BRCA1/BRCA2 and to identify specific 3'UTR variants that may be risk factors for cancer development. The 3'UTRs of the BRCA1 and BRCA2 genes were screened by heteroduplex analysis and DNA sequencing in 100 patients from 46 BRCA1/2 families, 54 non-BRCA1/2 families, and 47 geographically matched controls. Two polymorphisms were identified. SNPs c.*1287C>T (rs12516) (BRCA1) and c.*105A>C (rs15869) (BRCA2) were identified in 27% and 24% of patients, respectively. These 2 variants were also identified in controls with no family history of cancer (23.4% and 23.4%, respectively). In comparison to variations in the 3'UTR region of the BRCA1/2 genes and the BRCA1/2 mutational status in patients, there was a statistically significant relationship between the BRCA1 gene polymorphism c.*1287C>T (rs12516) and BRCA1 mutations (p=0.035) by Fisher's Exact Test. SNP c.*1287C>T (rs12516) of the BRCA1 gene may have potential use as a genetic marker of an increased risk of developing breast cancer and likely represents a non-coding sequence variation in BRCA1 that impacts BRCA1 function and leads to increased early-onset and/or familial breast cancer risk in the Turkish population.

Keywords: Breast cancer - BRCA1 - BRCA2 - 3'UTR - SNP - early onset - Turkey

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Introduction

Breast cancer (BC) is the most frequently diagnosed cancer among women and is the leading cause of cancer death in females worldwide (Jemal et al., 2011). At present, approximately 1 in 8 women (12%) will develop breast cancer in her lifetime (Downs-Holmes and Silverman, 2011). Additionally, 5-10% of all breast cancer cases are believed to be associated with a genetic predisposition, and this frequency increases to 25% in cases diagnosed before age 30 (Claus et al., 1991).

The majority of cases of a genetic predisposition to breast cancer have been linked to inherited mutations in BRCA1 (BRCA1-OMIM: 113705; GenBank: NM_007294.3) on chromosome 17q12-21 and BRCA2 (BRCA2-OMIM: 600185; GenBank: NM_000059.1) on chromosome 13q12-13 (Szabo and King, 1997; Ferla and Calò, 2007). Mutations in these genes account for 2-3% of all breast cancers and approximately 30-40% of all familial breast cancers (Kenemans et al., 2004; Kooshyar et al., 2013). Indeed, the identification of mutations in these genes is extremely beneficial for patients pursuing a risk reduction strategy and for their cancer treatment (Hennessy et al., 2010). However, although no variation was determined via BRCA1/2 screening in some patients, they did not respond to the available therapy (Ford et al., 1998). To date, the genetic screening of the BRCA1 and BRCA2 genes has been performed only for coding regions; nonetheless, the importance of miRNAs in the regulation of BRCA genes was recently identified (Sehl et al., 2009; Pelletier et al., 2011). As the majority of these miRNAs binds to the 3'UTR region of the genes (Sehl et al., 2009), variations in the 3'UTR regions of BRCA1/2 may also affect the regulation of related proteins and thus response to the applied therapy (Nilsen, 2007). In addition, the importance of miRNA-binding site single-nucleotide

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polymorphisms (SNPs) of the BRCA1/2 genes in breast and ovarian cancer has been emphasized in recent studies of certain individual populations (Barroso et al., 2009; Pongsavee et al., 2009; Sehl et al., 2009; Kontorovich et al., 2010; Nicoloso et al., 2010; Joseph et al., 2011). However, knowledge about the effect of 3'UTR site variations of BRCA1/2 remains elusive. Furthermore, there is no study focusing on these variations in the Turkish population.

The aim of this study was to analyze the 3'UTR regions of the BRCA1/BRCA2 genes in familial and early-onset breast cancer patients with and without mutation in the coding regions of BRCA1/BRCA2 and to identify the possible miRNA-binding sites of the specific 3'UTR variations in the Turkish population using a computer-aided analysis.

Materials and Methods

Subjects

The study utilized case information from 100 female patients with breast cancer who were treated in the Department of Breast Surgery at Uludag University in Bursa, Turkey. The study was approved by the local Ethics Committee (2012-5/11) and conformed to the ethical standards of the Helsinki Declaration. The patients involved in the study were de novo diagnosed and had either been diagnosed with early-onset BC or had family members with BC. Of the selected patients, 46 had a familial breast cancer history, and 78 were younger than 50 years of age. Additionally, 27 of the patients who were without a first-degree familial breast cancer history had another type of cancer in their family. The patients were between 21 and 65 years of age (median, 41.67± 1.17 years). Histopathological (tumor localization, tumor grade, tumor type, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER-2), proliferation index (Kİ67), sentinel lymph node biopsy, benign reactive hyperplasia, metastatic status, extracapsular tumor extension, lymphatic invasion and perineural invasion) and oncological (first-line neoadjuvant therapy, first-line adjuvant therapy, herceptin therapy, hormone therapy, radiotherapy, metastatic breast cancer first-line treatment and second-line treatment) features were included in the clinical data.

All patients were fully screened for coding mutations in BRCA1 and BRCA2 by sequence analysis. The BRCA molecular screening of patients as routine analysis at the Department of Medical Biology was conducted, as reported in our previous study (Cecener et al., 2014). In addition, genomic DNA from the blood samples of 47 unaffected healthy women who had no family history of cancer was also analyzed for DNA variations in the 3'UTR of the BRCA1 and BRCA2 genes after informed consent was given.

PCR and heteroduplex analysis

Blood samples were obtained from the 100 patients and 47 healthy controls, and genomic DNA was extracted using the phenol/chloroform extraction method. Primer pairs were designed for PCR amplification and DNA sequencing reactions; 4 pairs of primer sets were designed for the 3'UTR region of the BRCA1 gene (1383 bp), and 2 pairs of primer sets were designed for the 3'UTR region of the BRCA2 gene (902 bp) (Table 1). A polymerase chain reaction (PCR) analysis was performed for each fragment of the 3'UTR of BRCA1 and BRCA2. A 15-µl reaction mixture was used for the PCR, and this volume contained 0.15 mM of each deoxyribonucleoside triphosphate (dNTP, Promega-U1515, USA), 10 pmol of each primer, 5 u/µl of Taq DNA polymerase (Promega-M8305, USA), and 150 ng of genomic DNA. The PCR program for the amplification of each coding exon was as follows: 35 cycles of denaturation (94°C for 2 minutes), annealing (55°C for 30 seconds), and extension (72°C for 3 to 7 minutes according to the primer base composition). The PCR products were separated on a 2% agarose gel and stained with ethidium bromide. The high-quality amplified products were assessed using a heteroduplex analysis (HDA). For this analysis, 11 µl of each PCR product was heat-denatured at 96°C for 6 minutes and then cooled to 50°C for 10 minutes, 37°C for 15 minutes, and 20°C for 30 minutes. Next, 2 µL of stop solution (95 percent formamide, 20 mM EDTA, 0.05 percent bromophenol blue and 0.05 percent xylene cyanol) was added, and the tube was immediately stored on ice until the sample was analyzed. The processed PCR products (3.5 μL each) were electrophoresed on 1X MDE gels (BMA Rocland, Maine, USA) at 900 V for 9 to 16 hours at room temperature in 0.5X TBE buffer (89 mM tris-base, 89 mM boric acid, 2 mM EDTA, pH 8.0). The gels were stained using a non-radioactive silver staining method, and the results were visualized. Samples that had one or two bands separated from the wild-type bands were identified as HDA-positive. All samples that contained 3'UTR SNPs were subjected to the HDA analysis procedure at least twice to rule out contamination.

Sequencing analysis

The HDA-positive DNA fragments were re-amplified for sequencing using a PCR-product sequencing kit (DTCS, Quick Start Mix- M010812, USA), and samples that had different band features were sequenced using a Beckman Coulter Automated Sequencer, with the base sequences analyzed using a CEQ-8000 Automated DNA Sequencing System (Beckman Coulter, Inc., Fullerton, CA, USA). The results of the sequencing analysis were compared with wild-type samples of the 3'UTR of the BRCA1/BRCA2 genes. The relationships between the defined variations and the risk of breast/ovarian cancer were verified using the Ensemble Genome Browser (Ensemle; http://www.ensembl.org/), the human gene mutation database (HGMD; http://www.hgmd.cf.ac.uk/ac/all.php), Leiden Open Variation Database (LOVD; (http://www.lovd.nl/3.0/home) and Human Genome Variation Society (HGVS; http://www.hgvs.org/dblist/dblist.html).

Statistical analysis

To study the potential influence of clinical features, a statistical analysis was carried out. All analyses were conducted using SPSS version 16.0 and were considered statistically significant when $p<0.05$ (SPSS, Chicago, IL,

USA); 95% confidence intervals were calculated using the associated estimated standard errors. Fisher's exact test and independent sample t test were used to determine the association of the SNPs with clinical-histopathological tumor variables and mutational status.

Results

Study population

Two polymorphisms in the 3'UTRs of the BRCA1 and BRCA2 genes were identified. SNPs c.*1287C>T (rs12516) (BRCA1) and c.*105A>C (rs15869) (BRCA2) in the 3'UTR regions were identified in 27% (27/100) and 24% (24/100) patients, respectively (Table 2) (Figure 1). Ten percent of the patients with the c.*1287C>T (rs12516) (BRCA1) polymorphism and eight percent of the patients with the c.*105A>C (rs15869) (BRCA2) polymorphism tested positive for BRCA1/BRCA2 mutations.

Control population

The 3'UTRs of the BRCA1/BRCA2 genes were sequenced in the age and geographically matched control population. Two variations were also determined, with no family history of cancer (BRCA1 c.*1287C>T (rs12516), 23.4% (11/47); BRCA2 c.*105A>C (rs15869), 23.4% (11/47)) (Figure 1).

Bioanalysis

Analyses of the miRNA-binding sites in the BRCA1/BRCA2 3'UTR were performed using microRNA.org (http://www.microrna.org/microrna/home.do), microinspector (http://www.imbb.forth.gr/microinspector), and microRNA database (http://www.mirbase.org/). We used the NCBI database of single-nucleotide polymorphisms (dbSNP) (http://www.ncbi.nlm.nih/gov/SNP) to obtain information about genetic variations. We also developed a publicly available online database, mirSNP (http://cmbi.bjmu.edu.cn/mirsnp), which is a collection of human SNPs in predicted miRNA-mRNA binding sites. According to these analyses, no human miRNA-binding site was identified in the zone of interest for the c.*105A>C (BRCA2) variant. For the c.*1287C>T (BRCA1) variant, the base substitution slightly increased the binding of several miRNAs (Table 3).

Clinical association

The relationship of genomic variations in the 3'UTR of BRCA1/2 genes between patients and controls was evaluated using Fisher's exact test; no significant association between the patients and controls was found ($p>0.05$). However, based on fisher's exact test, although no significant association was determined

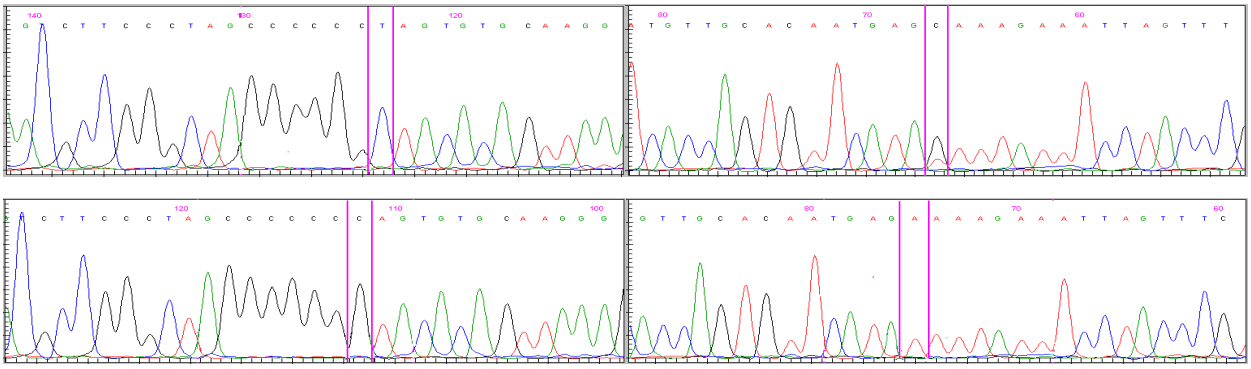

Figure 1. Images of DNA Sequence Analysis From Patients and Controls. A) BRCA1 rs12516 C→T; **B**) BRCA2 rs15869 A→C; **C**) Normal (wt) Sequence in BRCA1 rs12516 C→T; **D**) Normal (wt) Sequence in BRCA2 rs15869 A→C

Table 1. Primers for the Amplification of the 3'UTR of the BRCA1/BRCA2 Genes

Gene	Primer sequences	Annealing	Base pair (bp)
BRCA1 3'UTR 1 Forward	5'- AGCACTCTACCAGTGCCAG- 3'	60	645
BRCA1 3'UTR 1 Reverse	5'- AGGTTTCAAGTTTCCTTTTCA- 3'	56	645
BRCA1 3'UTR 2 Forward	5'- GAGTGCTTGGGATCGATTATGTGACTT- 3'	78	522
BRCA1 3'UTR 2 Reverse	5'- ACCGGTTCTTGAAAATCTTCTGCTG- 3'	72	522
BRCA1 3'UTR 3 Forward	5'- GCAGGAGAATCACTTCAGCCCGGGA- 3'	70	368
BRCA1 3'UTR 3 Reverse	5'- GCAACAGCTTCCTTCCTGGTGGG- 3'	74	368
BRCA1 3'UTR 4 Forward	5'- GGAAAATGAAACTAGAAGAGATTT- 3'	62	560
BRCA1 3'UTR 4 Reverse	5'- AGGCTCTGAGAAAGTCGGCT- 3'	62	560
BRCA2 3'UTR 1 Forward	5'- GTTCAGAAGATTATCTCAGACT- 3'	60	338
BRCA2 3'UTR 1 Reverse	5'- CTATTTCATAGTGAGTTACCTC- 3'	60	338
BRCA2 3'UTR 2 Forward	5'- TAGTTTCAAATTTACCTCAGCG- 3'	60	472
BRCA2 3'UTR 2 Reverse	5'- AGATATGCAACTGAAGCAAAAG- 3'	60	472

Table 2. Determined BRCA1/2 3'UTR Polymorphisms

ID	Gene	Variant type	Genomic location	Preferred name	Alleles	Ancestral allele	Class	MAF
rs12516	BRCA1	3'UTR	Chr17:43044391	BRCA1:c.*1287C>T	C/T	C	SNP	A=0.3118/679
rs15869	BRCA2	3'UTR	Chr13:32973012	BRCA2:c.*105A>C	A/C	A	SNP	C=0.1556/338

*MAF Global minor allele frequency (dbSNP)

Table 3. Prediction Results of the MicroRNA Targeting 3'UTR SNPs

Gene	miRNA	SNP	mirSVR	Effect	Allele	Score	Energy	Conservation
BRCA1	hsa-miR-1264	rs12516	-0.494	decrease	C	147	-22.42	0.256
					T	146	-18.06	0.256
BRCA1	hsa-miR-4278	rs12516		create	C			
					T	144	-18.54	0.046
BRCA1	hsa-miR-4704-5p	rs12516		create	C			
					T	156	-13.37	0.119
BRCA1	hsa-miR-637	rs12516	-0.12	break	C, T	152	-24.19	0.059

*mirSVR: The mirSVR score (Betel et al., 1994) of this binding site, Effect: The impact of different alleles, Allele: The considered allele; Score: The predicted score of miRNA-mRNA binding by miRanda software. The higher the score, the more stable the binding, Energy: The free energy of miRNA-mRNA duplex, Conservation: Conservative information of phastCons 46way vertebrates from UCSC (average value of 7-nt seed motif)

between BRCA2 gene polymorphism [c.*105A>C (rs15869)] and BRCA2 mutation (p=0.443), there was a statistically significant relationship between BRCA1 gene polymorphism [c.*1287C>T (rs12516)] and patients with mutations in the BRCA1 gene (p=0.035). Nonetheless, based on the independent sample t test, no relevance was determined among the clinical parameters of these patients (p>0.05) (Table 4).

Table 4. The Relationship of Clinical Parameters between BRCA1 Mutations and 3'UTR Variation Carriers

	p value*	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
				Lower	Upper
Age	0.405	3.833	4.405	-5.982	13.649
Tumor Size	0.584	12.992	22.989	-38.232	64.215
Grade	0.207	0.667	0.494	-0.435	1.768
Ki67	0.752	-39.833	122.708	-313.245	233.5784

*p values were evaluated with Independent Sample T Test

Discussion

Mutations in a number of genes, including BRCA1, BRCA2, PALB2, TP53 and ATM, have been shown to be breast cancer risk factors, and 30%-40% of familial breast cancer cases have been reported to be associated with a mutation in the BRCA1 and BRCA2 genes (Miki et al., 1994; Wooster et al., 1995; Chen et al., 1999; Peto et al., 1999; Khana and Jackson, 2001; Keen and Davidson, 2003). Cancer-related mutations have usually been reported within the coding sequences of BRCA1 and BRCA2, which can directly influence protein structure and affect their various biological functions (Scully and Livingston, 2000; Venkitaraman, 2002). However, nucleotide changes within the noncoding region of genes can also lead to various diseases (Cazzola and Skoda, 2000), and cumulative evidence strongly suggests that the 3'UTR of mRNA is involved in the control of gene expression. mRNA stability is believed to be predominantly regulated through the interaction of cis-acting elements in the 3'UTR of a gene with trans-acting factors. Goto et al., (2001) reported that changes in the 3'UTR of the human dihydrofolate reductase gene could enhance gene expression. Interestingly, mutations within the 3'UTR of an mRNA transcript arising from one allele can have a dominant negative effect by decreasing trans-acting regulatory protein products that could induce gene expression (Conne et al., 2000). miRNAs play critical roles in many different cellular processes, including metabolism, apoptosis, proliferation, differentiation, and development (Hayashita et al., 2005; Jovanovic and Hengartner, 2006; Zhang et al., 2006; Kayani et al., 2011). In addition, close correlations between altered miRNA expression and tumorigenesis have been reported (He et al., 2005; Lu et al., 2005; O'Donnell et al., 2005; Li et al., 2013), and the expectation of up to 1872 miRNAs in the human cell was proposed. Recently, microRNA (miRNA) expression profiles and miRNA signaling pathways have been intensively studied for their potential role in human disease development, including breast cancer (Esquela-Kerscher and Slack, 2006; Wu et al., 2007; Hafez et al., 2012; Erturk et al., 2014). Our previous reports of familial and early-onset breast cancer patients in Bursa, Turkey, showed that mutations in either BRCA1 or BRCA2 coding exons are present in approximately one third of cases (Egeli et al., 2006; Cecener et al., 2014). To determine whether genetic changes outside of the coding regions could also be related to breast tumorigenesis, we screened for nucleotide variations in the 3'UTR of BRCA1/BRCA2 and further established the functional significance of the variations detected.

In the present study in Turkish breast cancer patients, two SNPs were detected in the 3'UTR of BRCA1 and BRCA2 at positions c.*1287C>T (rs12516) (BRCA1) and c.*105A>C (rs15869) (BRCA2). In the literature, these polymorphisms are associated with an increased risk of breast/ovarian cancer among familial and early-onset cancer groups, particularly in patients without mutations in the coding sequences of BRCA1 and BRCA2 (Pongsavee et al., 2009; Nicoloso et al., 2010). One study (Sehl et al., 2009) reported that SNPs within or near several double-stranded break repair DNA repair pathway genes are associated with breast cancer in individuals from a high-risk population. The authors investigated 104 SNPs in 17 genes with protein products that are involved in double-stranded break repair and found that 12 of the polymorphisms are associated with breast or breast and ovarian cancers. Most notably, rs16888927, rs16888997, and rs16889040 in introns of RAD21, rs12516 in introns of BRCA1 and rs15869 in introns of BRCA2 were reported, suggesting that SNPs in other genes in the DSBR pathway may affect breast cancer risk. In another study, Pelletier et al., (2011) reported numerous known BRCA1 3'UTR SNPs. To identify the frequency of these known polymorphisms and/or to identify novel SNPs

in 221 sporadic European American (EU) and African American (AA) breast cancer patients, the entire 3'UTR of BRCA1 in breast cancer patients with different breast cancer subtypes was sequenced. The initial screen of the entire BRCA1 3'UTR in these patients identified variation at only the three previously reported functional SNPs: rs12516, rs8176318 and rs3092995. Additionally, a novel SNP was identified in the BRCA1 3'UTR: 6824G>A or 5711+1113 G>A. This study provided evidence that the 3'UTR variant rs8176318 is a marker for breast cancer risk for AA women and a marker for triple-negative breast cancer; the potential of other 3'UTR variants (rs12516 and rs3092995) to serve as genetic markers for an increased risk of developing breast cancer was also reported.

In addition, Brewster et al., (2012) found two common heterozygous polymorphisms, rs8176318 (c.*421G>T) and rs12516 (c.*1287C>T), in the 3'UTR of BRCA1. To determine whether any of the combinations of common BRCA1 3'UTR SNPs identified in the Fox Chase Cancer Center breast cancer cases displaying differential allelic expression of BRCA1 were likely to affect gene expression, each of the alternate BRCA1 3'UTR alleles were assayed for regulatory activity. The BRCA1 3'UTR containing both c.*421G>T and c.*1287C>T (421T/1287T) was shown to have a statistically significant decrease in activity (~67%), whereas c.*1287C>T (1287T) alone increased expression (185%). The presence of c.*421G>T (421T) had no effect, showing a luciferase activity similar to the wild-type BRCA1 3'UTR (421G/1287C).

In the present study, c.*1287C>T (rs12516) (BRCA1) and c.*105A>C (rs15869) (BRCA2) variations in the 3'UTR regions were identified in 27% (27/100) and 24% (24/100) patients, respectively. In addition, 10% of patients with the c.*1287C>T (rs12516) (BRCA1) polymorphism and 8% of patients with the c.*105A>C (rs15869) (BRCA2) polymorphism tested positive for BRCA1/BRCA2 mutations. When we analyzed these polymorphisms in the control group, BRCA1 c.*1287C>T (rs12516) and BRCA2 c.*105A>C (rs15869) were determined in 23.4% (11/47) and 23.4% (11/47) of the samples, respectively. According to our findings, no significant association was determined between BRCA2 gene polymorphism [c.*105A>C (rs15869)] and BRCA2 mutation carrier cases (p=0.443); in contrast, there was a statistically significant relationship between BRCA1 gene polymorphism [c.*1287C>T (rs12516)] and BRCA1 gene mutation carriers (p=0.035). However, the clinical relevance of these alterations could not be determined.

This study also provides preliminary data for investigating miRNAs related to BRCA1 and BRCA2 3'UTR regions. miRNAs comprise one of the most abundant classes of regulatory genes, with 30% of human genes harboring miRNA target sites. The ability of miRNAs to bind to the 3'UTR of mRNA is critical for regulating mRNA levels and protein expression, and this binding can be affected by single-nucleotide polymorphisms. Data from our group and others indicate that BRCA1/2 3'UTR variants may be potential genetic markers of breast cancer risk (Pongsavee et al., 2009; Pelletier et al., 2011). It is possible that the c.*1287C>T (rs12516) SNP in the 3'UTR region of the BRCA1 gene reported here could decrease the affinity of binding or create or eliminate new binding sites for some miRNAs. According to microRNA.org (http://www.microrna.org/microrna/home.do), microinspector (http://www.imbb.forth.gr/microinspector), and microRNA database (http://www.mirbase.org/), four miRNAs (hsa-miR-1264, hsa-miR-4278, hsa-miR-4704-5p and hsa-miR-637) would bind to the 3'UTR of BRCA1. Thus, we suggest that increased miRNA binding to the BRCA1 3'UTR could explain an increase breast cancer risk. This work provides a greater insight into the molecular mechanisms of breast cancer initiation. However, further studies are required to clarify the effect of the 3'UTR region of BRCA2.

In summary, our findings support the hypothesis that c.*1287C>T (rs12516) of BRCA1 may be a candidate as a genetic marker for an increased risk of developing breast cancer and likely represents a non-coding sequence variation in BRCA1 that impacts BRCA1 function and leads to increased early-onset and/or familial breast cancer risk in the Turkish population.

In conclusion, this study reveals new information of the approximate 3'UTR region of BRCA1 and BRCA2 polymorphism types and frequencies in the Turkish population. A significant association was determined between BRCA1 gene mutation and c.*1287C>T (rs12516) the polymorphism. However, the significance of these polymorphisms in Turkish families with breast cancer is not clarified, which will require more detailed studies of large patient/control groups. Additionally, the functions of the miRNAs binding to these polymorphic regions remain to be determined. Thus, further studies should be performed to evaluate the miRNA-binding features of these sites to promote the development of miRNA-based treatment models in the future.

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