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rs3798577 polymorphism located in a putative miRNAs target site of estrogen receptor 1 reduced breast cancer risk in an Iranian population

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Summary. *Purpose:* In the current case-control study, the possible association between rs3798577, a microRNA-related SNP located on *ESR1* 3'-untranslated regions (3'-UTR), and breast cancer was evaluated in Iranian women for the first time. *Materials and Methods:* 126 breast cancer patients and 141 hospital healthy controls were enrolled in this study. Genotyping of the selected SNP in *ESR1* was disclosed using the allele-specific primer polymerase chain reaction (ASP-PCR) assay. Odds ratio, 95% confidence interval, and p value were calculated to examine the association between SNP and breast cancer related clinical features. In addition, an in silico prediction was performed to identify potential functionality of the SNP within miRNA binding sites in the 3'-UTR of *ESR1*. *Results:* The T allele carriers of the SNP had significantly inverse association with BC incidence (T/T and C/T vs C/C; OR, 0.50; 95% C.I., 0.27-0.92; P value, 0.025). In addition, T allele carriers conferring decreased risk of metastasis, ER/PR negativity, HER2 positivity, and stage IV incidence and also increased risk of BC death and grade III incidences but these results did not reach statistical significance. Bioinformatically, rs3798577 is located on *ESR1* 3'-UTR within the potential target sequence of miR-1278 and miR-125b-2-3p. Hence, the T allele may increase miRNA-mRNA binding strength. *Conclusion:* The results showed that the *ESR1* rs3798577 T allele significantly reduced breast cancer risk, in agreement with bioinformatical results. The association between rs3798577 genotypes and breast cancer has been reported contradictorily in different studies. Furthermore, the bioinformatical results need to verify. Therefore, further studies with large sample size and functional assessment are strongly suggested.

Key words: breast cancer, *ESR1*, functional SNP, 3'-UTR

1. Introduction

Breast cancer (BC) is the most commonly diagnosed malignancy among women with heterogeneous clinical, genetic, and biochemical features (1, 2). Estrogen induces proliferation of mammary epithelial tissue by interacting with nuclear receptors, estrogen receptors estrogen receptors (ER α and β), acting as a transcription factor and can be used for therapeutic purposes (2). ER α expression has been used for predicting responsiveness to hormone therapy, and loss of its expression is associated with poorer BC outcomes (3, 4). As previous studies have shown (5, 6), *ESR1*, encodes estrogen receptor α, is a strong candidate gene for BC association studies.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that bind to 3'-untranslated regions (3'-UTRs) of target mRNAs and mediate trans-

lational inhibition or cleavage; thus, it may participate in various pathological events (7, 8). Many studies have aimed to characterize functional single nucleotide polymorphisms (F-SNPs) related to miRNA regulation process. These variants are categorized into the two main groups. Firstly, precursor miRNAs (pre-miRNAs) polymorphisms may cause miRNA aberrant expression possibly via altering pre-miRNA stability. Secondly, miRNA target sites (3'-UTR of targets) polymorphisms may modify miRNA-mRNA binding strength. Bioinformatics tools are useful to predict the effects of SNPs at miRNA loci and targets and offer possible descriptions for the phenotype associations (9, 10).

Here, we hypothesized that the *ESR1* 3'-UTR SNP, rs3798577 (c.*1029C>T) genetic variation can alter the expression of *ESR1* and its downstream signaling through miRNA interactions; hence, may affect BC susceptibility. Briefly, we genotyped the rs3798577 SNP in a cohort of Iranian BC patients in order to search for associations between the polymorphism and BC and its clinicopathological characteristics, including stage, grade, early metastasis status, hormone receptors (ER and PR), and HER2/neu overexpression. In addition, an in silico assessment was performed to investigate possible function of the selected SNP.

2. Materials and methods

2.1 Ethics Statement

Written informed consent for the genetic study was obtained from all participants. This study was approved by the institutional review board of Islamic Azad University, Shahrekord Branch, Shahrekord, Iran.

2.2 Patients and healthy controls

Genomic DNA was isolated from blood samples of 126 unrelated Iranian patients with BC and 141 women without family history of any type of cancers using the PrimePrep Genomic DNA Isolation Kit (GeNetBio, Chungnam, South Korea) according to the manufacturer's protocol.

2.3 Pathological diagnosis and grading

Clinicopathological characteristics and follow-up data were obtained from the files of reference pathology Laboratories where immunohistochemistry (IHC) and pathological tests are performed centrally by experienced operators and a dedicated pathologist who tracks strict sample handling, processing and reporting protocols, thus ensuring the reliability of results. The pathological and clinical attributes of the patients are listed in Table 1.

2.4 SNP genotyping

Allele-specific primer polymerase chain reaction (ASP-PCR) assay was applied for SNP genotyping (11). SNP genotyping was performed by using the C allele specific forward (5'GGC ATG GAG CTG AAC AGT AAC3') T allele specific forward (5'GGC ATG GAG CTG AAC AGT AAT3'), and common reverse (5'AAT GAA GAA GAG CTG GAC TAC CC3') primers. Standard cycling was performed in a thermocycler (ASTEC PC-818; ASTEC, Fukuoka, Japan) under the following conditions: Initial denaturation at 94°C for 5 min followed by 33 cycles of 94°C for 30 sec, 57°C for 30 sec, 72°C for 40 sec, and finally 72°C for 7 min. PCR reaction with C allele specific forward and T allele specific forward primers are performed separately (two reactions are needed for each sample). The allele specific PCR product was electrophoresed by 1.5% agarose gel electrophoresis in 1X

Table 1. The clinicopathologic characteristics of the patients with breast carcinoma

Characteristics	Number
Histological grade	I: 12, II: 48, III: 36, Unknown: 30
Stage	I: 18, II: 15, III: 15, IV: 66, Unknown: 12
Estrogen receptor (ER) status	Positive: 63, Negative: 18, Unknown: 45
Progesterone receptor (PR) status	Positive: 57, Negative: 24, Unknown: 45
HER2 status	Positive: 24, Negative: 57, Unknown: 45

Tris-Borate-EDTA buffer at 100 V and stained with RedSafe Nucleic Acid Staining solution (Boca Scientific, Inc., Boca Raton, FL, USA) for visualization. The amplicon sizes for *ESR1* 3'-UTR variant (rs3798577) was 121 bp for both alleles (Figure 1).

2.5 Bioinformatic analysis

miRNASNP version 2.0 (12) was used to predict impact of the rs3798577 *ESR1* 3'-UTR SNP based on miRNA binding free energy comparison of the two SNP alleles. To illustrate, this database was utilized to computationally estimate the impact of rs3798577 in modifying the affinity between miRNAs and 3'-UTR of *ESR1* mRNAs (either gain or loss) based on alterations in Gibbs free energy of binding reaction.

2.6 Statistical analysis

Deviation from Hardy-Weinberg equilibrium (HWE), odds ratios (ORs) with 95% confidence intervals (CIs), and Armitage's trend test were achieved using DeFinetti program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Noticeably, Armitage's trend test performs association assessment with considering the individuals' genotypes rather than just the alleles for and also without relying on the assumption of HWE, following the guidelines provided by Sasieni (13).

Consistency with HWE was examined by the exact test. In addition, association tests were evaluated using chi-square test. Logistic regression models were used to account odds ratios (OR) and related 95% confidence intervals (95% CI). P-value of <0.05 was considered statistically significant.

3. Results

*3.1 Frequencies of ESR1 3'-UTR variant rs3798577 (c.*1029C>T)*

Among all individuals, including patients and healthy controls, alleles and genotypes were distributed as following frequencies: C, 0.63; T, 0.37; C/C,

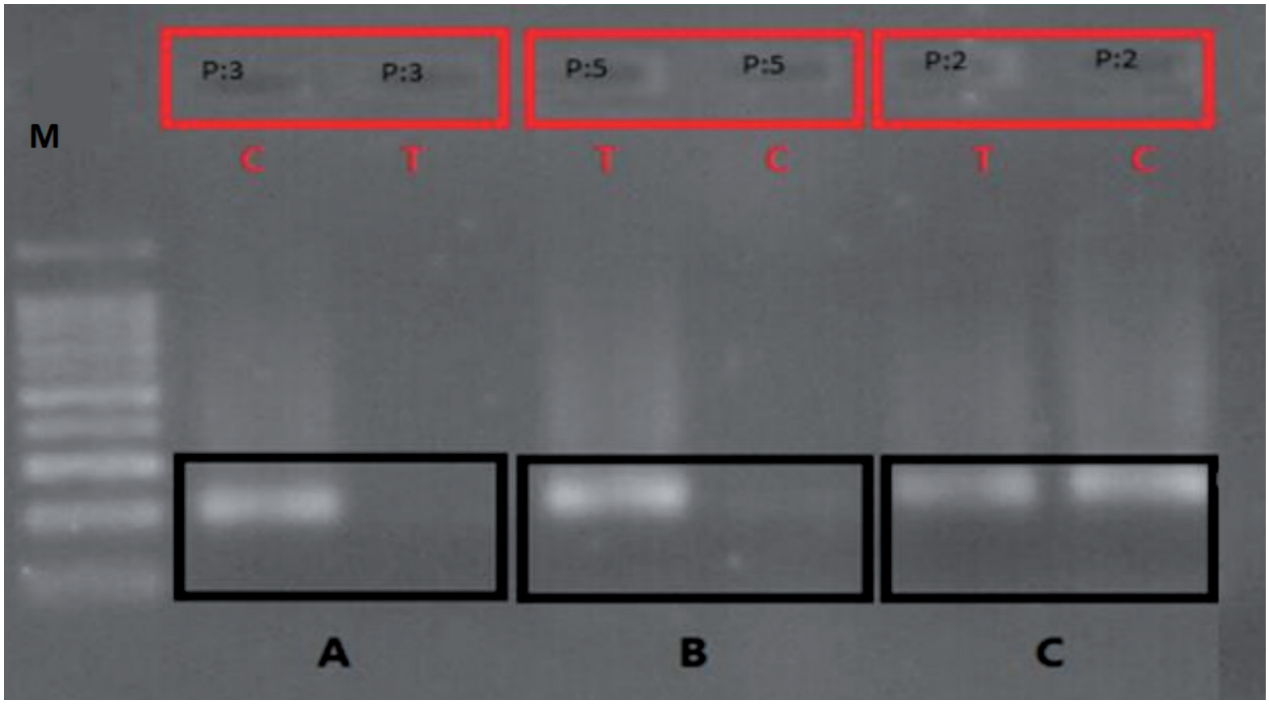

Figure 1. Representative PCR amplicons of allele-specific primer PCR resolved by 1.5% agarose gel electrophoresis to detect the ESR1 3'-UTR variant rs3798577 polymorphism genotyping. Lanes 1 and 2: C/C; lanes 3 and 4: T/T; lanes 5 and 6: C/T; M: DNA marker

0.41; C/T, 0.44; T/T, 0.15. The observed genotype frequencies showed that rs3798577 SNP was in HWE proportions in the population of the study (P value, 0.517).

3.2 Associations with BC and tumor phenotypes

To test the relationships between the SNP and prognostic tumor phenotypes, allele frequency differences, Armitage's trend test, and genotypic association (assuming a dominant model of inheritance for T allele) were performed by Pearson's chi test and OR calculation (Table 2). From the all association tests with various events, T allele carriers (T/T and C/T) were inversely associated with BC compared with C/C genotype (OR, 0.50; 95% C.I., 0.27-0.92; P value, 0.025). In addition, T allele carriers conferring decreased risk of metastasis, ER/PR negativity, HER2 positivity, and stage IV incidence and also increased risk of BC death and grade III incidences but these did not reach statistical significance (P value >0.05).

3.3 Bioinformatical results

Computational predictions proposed that rs3798577 is located on *ESR1* 3'-UTR within the potential target sequence of miR-1278 and miR-125b-2-3p. As a result, the T allele may increase miRNA-mRNA binding occurrence (Table 3).

4. Discussion

Associations between variants in *ESR1* and BC risk have evaluated by previous studies (6, 14) and were extensively reviewed by Herynk et al. (15). Estrogen induced ERα can directly bind to estrogen response elements or indirectly interacts with chromatin via binding to other transcription factors, such as coactivators or corepressors (16). Dysregulation of ERα can affect BC progression and susceptibility (17).

Rs3798577 is located on 3'-UTR of *ESR1* and predicted target sequence of miR-1278 and miR-

Table 2. Allelic, Armitage's trend, and genotypic association tests between rs3798577 and characteristics of patients

Variable comparison	Allelic association (T *vs* C)		Armitage's trend test (T *vs* C)		Genotypic association (T/T + C/T *vs* C/C)	
	OR (95% CI)	*P*	OR	*P*	OR (95% CI)	*P*
BC vs Control	0.81 (0.52-1.26)	0.357	0.91	0.373	**0.50 (0.27-0.92)**	**0.025**
Metastatic BC vs Non-metastatic BC	0.62 (0.31-1.24)	0.172	0.70	0.241	0.62 (0.25-1.53)	0.297
BC death vs Survival	1.64 (0.71-3.75)	0.241	1.45	0.315	1.58 (0.50-5.04)	0.434
ER- vs ER+	0.74 (0.26-2.09)	0.573	0.87	0.620	0.55 (0.14-2.11)	0.380
PR- vs PR+	0.72 (0.28-1.84)	0.494	0.79	0.547	0.67 (0.20-2.20)	0.505
HER2+ vs HER2-	0.72 (0.28-1.84)	0.494	0.79	0.547	0.67 (0.20-2.20)	0.505
Stage IV vs Stage I/II/III	0.61 (031-1.22)	0.162	0.65	0.224	0.78 (0.32-1.92)	0.587
Grade III vs Grade I/II	1.72 (0.78-3.82)	0.179	1.50	0.235	1.86 (0.66-5.21)	0.237

Table 3. An in silico analysis of the SNP-miRNA binding

miR	miR sequence	miR site on ESR1 3'-UTR with rs3798577	Effect (T allele *vs* C allele)
miR-1278	UAGUACUGUGCAUAUCAUCUAU	AUGGAGCUGAACAGUAC[C/U]	Gain
miR-125b-2-3p	UCACAAGUCAGGCUCUUGGGAC	CUGAACAGUAC[C/U]UGUG	Gain

125b-2-3p. Therefore, in the present study, *ESR1* 3'-UTR SNP, rs3798577 (c.*1029C>T) was selected and genotyped in an Iranian population comprising 126 BC cases and 141 cancer-free controls. Although, the C allele was introduced as a minor allele in the rs3798577 location in various populations at dbSNP database (http://www.ncbi.nlm.nih.gov/SNP), we observed lower frequency for the C allele compare with the T allele in the Iranian population. In our cases, we found inverse significant association between the T allele carriers and BC incidence (OR, 0.50; 95% C.I., 0.27-0.92; P value, 0.025). In accordance with our results, the C allele was strongly associated with the risk of BC in Caucasian population (18). Possible functional mechanism of this SNP bioinformatically attributed to changes in ERα expression by enhancing the target sequence of miR-1278 and miR-125b-2-3p due to C>T substitution. In addition, the C allele has been reported that to be associated with surviaval and distant metastasis (18, 19) which is in agreement with our nonsignificant observations (Table 2). Moreover, additional associations between the SNP and other BC characteristics were not statistical significant in the present study.

Inconsistent with our finding, this SNP was not statistically associated with BC risk in other studies (6, 14, 20). In addition, converse with our and others observation, the T allele frequency of rs3798577 was significantly higher in BC cases than controls in Chinese and Korean populations (21, 22). This supports the fact that associations between polymorphisms and complex phenotypes can be varied mostly due to ethnicity background and variant allele frequencies of polymorphisms; hence, association results should not be generalized between different populations.

To our knowledge, this is the first investigation which was aimed to evaluate the possible association between rs3798577 and BC phenotypes among Iranian women. This case–control study showed an inverse association between the T allele carriers and BC incidence (T/T and C/T are protective genotypes) which is interestingly consistent with the bioinformatical results. It can be concluded that the T allele may strengthen *ESR1* mRNA-miRNA binding; thus, it may lead to *ESR1* downregulation and better BC outcomes. However, limitations of the current study, including the small sample size and unvalidated bioinformatics results need to ameliorate in further investigations. All in all, the rs3798577 polymorphism functionality in BC is still inconclusive and more examinations are required to test the association of this polymorphism in BC and also its biological importance.

5. Compliance with Ethical Standards

Written informed consent for the genetic study was obtained from all participants and also this study was approved by the institutional review board of Islamic Azad University, Shahrekord Branch, Shahrekord, Iran.

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