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Tumor-promoting function of single nucleotide polymorphism rs1836724 (C3388T) alters multiple potential legitimate microRNA binding sites at the 3'-untranslated region of *ErbB4* in breast cancer

FATEMEH BAGHERI[1], HAMZEH MESRIAN TANHA[2], MARJAN MOJTABAVI NAEINI[3], KAMRAN GHAEDI[2] and MANSOUREH AZADEH[4]

[1]Biochemistry Division, Department of Biology, Faculty of Science, Payame Noor University of Taft, Yazd 47416-95447; [2]Cellular and Molecular Biology Division and [3]Genetics Division, Department of Biology, Faculty of Science, University of Isfahan, Isfahan 81746-73441; [4]Zist Fanavary Novin Institute, Isfahan 14115-111, Iran

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Abstract. *ErbB4* can act as either a tumor-suppressor gene or an oncogene in breast cancer. Multiple genetic factors including single nucleotide polymorphisms (SNPs) affect gene expression patterns. Multiple 3'-untranslated region (3'-UTR) SNPs reside within the target binding site of microRNAs, which can strengthen or weaken binding to target genes. The present study aimed to predict potential 3'-UTR variants of *ErbB4* that alter the target binding site of microRNAs (miRNAs) and to clarify the association of the potential variant with the risk of developing breast cancer. *In silico* prediction was performed to identify potential functional SNPs within miRNA target binding sites in the 3'-UTR of *ErbB4*. Thus, 146 patients and controls were genotyped using restriction fragment length polymorphism-polymerase chain reaction. In addition to the Cochran-Armitage test for trend, allele and genotype frequency differences were determined to investigate the association between rs1836724 and the susceptibility to breast cancer. Bioinformatics analysis identified rs1836724 to be a polymorphism in the seed region of four miRNA binding sites (hsa-miR335-5p, hsa-miR-28-5p, has-miR-708-5p and has-miR-665), which may participate in the development of breast cancer. Logistic regression data indicated that the T allele of the polymorphism [OR (95% CI)=1.72 (1.056-2.808), P=0.029] is associated with the risk of breast cancer. Using bioinformatics tools, a correlation was indicated between the presence of the T allele and a reduction in *ErbB4* RNA silencing based on miRNA interaction. Furthermore, case subgroup data analysis revealed an association between the C/T genotype and an ER positive phenotype [OR (95% CI)=6.00 (1.082-33.274), P=0.028] compared with the T/T genotype. ErbB4 and estrogen receptor 1 (ESR1) are regulated by identical miRNAs thus there may be a competition for binding sites. Due to this pattern, if the interaction between miRNAs with one gene is reduced, it may be consistent with the increase in interaction with another one. Therefore, more interaction with rs1836724 C variant within ErbB4 may be associated with higher expression of ESR1 (ER-positive phenotype). miRNAs interact with ErbB4 mRNA more frequently when it carries C allele at the rs1836724 position compared with the T carriers. Therefore, the identical miRNA interacts with ESR1 less frequently when ErbB4 mRNA has a C allele. Therefore, ESR1 expression may be higher when ErbB4 mRNA has a C allele.

Correspondence to: Professor Kamran Ghaedi, Cellular and Molecular Biology Division, Department of Biology, Faculty of Science, University of Isfahan, Hezar Jarib Street, Isfahan 81746-73441, Iran
E-mail: kamranghaedi@yahoo.com; kamranghaedi@sci.ui.ac.ir

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Introduction

Breast cancer is one of the most common types of malignancies among women worldwide (1). Hereditary genetic factors are responsible for ~25% of breast cancer according to twin studies (2). Despite a study that involved variants associated with a risk of breast cancer (3), less is known regarding the significance of these prognostic genetic variants.

Receptor tyrosine-protein kinase *ErbB4* is a member of the epidermal growth factor receptor (*EGFR*) subfamily as well as *EGFR* (*ErbB1*), *ErbB2* (*HER2*, *neu*), *ErbB3* (*HER3*) and *ErbB4* (*HER4*) (4). Experimental studies have demonstrated oncogenic and tumor suppressive functions of *ErbB4* in breast cancer (5). In breast cancer, *ErbB4* expression is associated with a favorable outcome; however, *ErbB4* expression combined with *ErbB1* and *ErbB2* upregulation may lead to an unfavorable outcome (6). Additionally, overexpression of *ErbB4* in conjunction with *ESR1* expression may also promote favorable outcome in breast cancer patients (7). Conversely,

the pro-apoptotic BH3 domain of *ErbB4* has been suggested to enhance the apoptosis of breast cancer cells when overexpressed (8). MicroRNAs (miRNAs) are key in the regulation of the expression of numerous genes, including *ErbB4*. A number of miRNAs, such as miR-193a-3p, target specific sites in the 3'-untranslated region (3'-UTR) of *ErbB4* mRNA in order to downregulate its expression (9).

Single nucleotide polymorphisms (SNPs) in growth regulatory genes, such as *ErbB4,* may affect tumor growth in breast cancer. Association between SNPs at the promoter and within the intronic region of *ErbB4* and the development of breast cancer has been shown in previous studies (10,11). In addition, functional SNPs (12), located in the 3'-UTR could directly weaken or strengthen the interaction of miRNA with 3'-UTR consensus sites (13), that functions in RNA silencing. Numerous databases and online tools, including miRNASNP (13), dbSMR (14) and PolymiRTS (15), have been developed to predict the effect of SNPs in miRNA target sites, which may aid in clarifying the role of SNPs in the development of certain types of cancer.

In the present study, *in silico* investigation was used to predict the effect of SNPs in the 3'-UTR on *ErbB4* expression. According to predictions, SNP rs1836724 is a putative functional polymorphism that alters the interaction of miRNAs targeting *ErbB4* mRNA. To the best of our knowledge, this study is the first to investigate whether rs1836724 influences susceptibility to breast cancer in the Iranian population. Moreover, computational analysis was enhanced in order to interpret experimental observations and achieve a molecular insight into breast cancer.

Materials and methods

In silico analysis. miRNASNP (version 2.0) bioinformatics online tools (bioguo.org/miRNASNP/) (13) were used in order to predict putative SNPs in the 3'-UTR of the *ErbB4* gene that could alter miRNA interactions. All possible miRNA/SNP-variant mRNA interactions and ΔΔG (the difference between the mRNA-miRNA hybrid free energy connected to each allele), were provided by miRNASNP. Results were categorized by the gain or loss of miRNA/target-interaction ability, which was calculated based on binding energy change between the two SNP variants. Additional bioinformatics analysis was performed using the miRWalk (version 2.0) database (16) to determine potential common miRNAs which target *ESR1* and *ErbB4* mRNAs.

Sampling, DNA extraction and genotyping. Peripheral blood samples were retrieved from 70 patients who were recently diagnosed and histologically confirmed to have breast cancer between August 2013 and August 2014 at the Sayed-ol-Shohada Hospital (Isfahan, Iran). In total, 76 control blood samples were obtained from female individuals undergoing a regular health check at the hospital. In this study, control samples with any history of cancer were excluded. The clinical and pathological characteristics of the patients were collected from the hospital and are summarized in Table I. Age ranges of case and control subjects were 31-72 and 30-85 years old, respectively. Written informed consent was collected from all involved participants. The current study was approved by the ethics committee of Sayed-ol-Shohada Hospital (Isfahan, Iran).

Table I. Clinicopathological characteristics of the patients with breast carcinoma.

Characteristic	No. of patients
Early metastasis	
Positive	26
Negative	44
Histological grade	
I	10
II	26
III	18
Unknown	16
Stage	
I	10
II	12
III	2
IV	40
Unknown	6
Estrogen receptor status	
Positive	32
Negative	12
Unknown	26
Progesterone receptor status	
Positive	30
Negative	14
Unknown	26
HER2 status	
Positive	16
Negative	28
Unknown	26

HER2, human epidermal growth receptor 2.

Genomic DNA was extracted using the PrimePrep Genomic DNA Isolation kit (GeNetBio, Chungnam, South Korea), according to the manufacturer's instructions. DNA purity and concentration was determined using a spectrophotometer (NanoDrop 1000; Thermo Fisher Scientific Inc., Wilmington, DE, USA).

DNA fragments were amplified using the following primers for rs1836724: Forward: 5'-TTAATAGAAATTTGA GTTTTGCGTT-3' and reverse: 5'-TATCAGATTCCAGAG GCCAAT-3'. Standard cycling was performed in a thermocycler (ASTEC PC-818; ASTEC, Fukuoka, Japan) under the following conditions: Initial denaturation at 96˚C for 2 min followed by 35 cycles of 94˚C for 30 sec, 56.5˚C for 30 sec, 65˚C for 30 sec, and finally 65˚C for 7 min. It should be noted that flanking region of rs1836724 is AT-rich and despite alternative genotyping tools (17), the PCR program with reduced extension temperature (65˚C) and following restriction fragment length polymorphism was the best strategy for AT-rich DNA genotyping (18). The PCR products were

Table II. Estimation of allele frequency, observed genotypes, expected genotypes and HWE P-values of rs1836724 in control, case groups, and ER-positive, and ER-negative subgroups.

	Allele frequency		Observed genotypes			Expected genotypes			HWE P-value		
Group	T	C	T/T	T/C	C/C	T/T	T/C	C/C	Pearson's	Llr	Exact
Control	0.59	0.41	26	38	12	26.64	36.71	12.64	0.759440	0.759217	0.816842
Case	0.71	0.29	36	28	6	35.71	28.57	5.71	0.867109	0.867489	1.000000
ER negative	0.75	0.25	8	2	2	6.75	4.50	0.75	0.054292	0.065276	0.089899
ER positive	0.66	0.34	12	18	2	13.78	14.44	3.78	0.162761	0.150196	0.252820

HWE, Hardy-Weinberg equilibrium; ER, estrogen receptor.

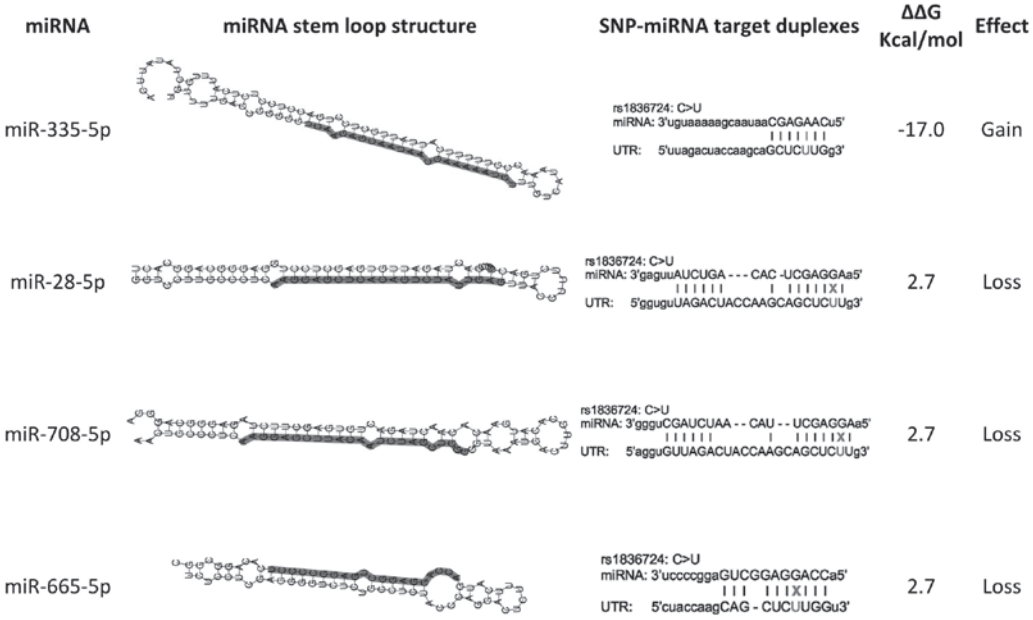

Figure 1. *In silico* analysis of SNP-miRNA binding. Shaded boxes on the miRNA secondary structure indicate a mature form of miRNAs. The effect is a description as to whether the miRNA had a weakened (loss) or strengthened (gain) binding site as the result of the SNP. ΔΔG shows the difference between the free energy of two alleles. SNP, single nucleotide polymorphism; miRNA, microRNA; UTR untranslated region.

electrophoresed by 2.5% agarose gel electrophoresis in 1X Tris-Borate-EDTA buffer at 100 V and stained with RedSafe Nucleic Acid Staining solution (Boca Scientific, Inc., Boca Raton, FL, USA) for visualization. Detection of allelic variations was enhanced by digesting polymerase chain reaction products with the restriction enzyme *Alw*NI (Thermo Fisher Scientific Inc.). The *AlwNI* restriction enzyme does not cut PCR product containing a T allele (band is 383 bp); furthermore, it yielded two fragments of 272 bp and 111 bp, as there is a C allele in the original PCR product. The accuracy of the genotypes was confirmed by randomly performed Sanger sequencing using the Bioneer Sequencing Service (Bioneer Corporation, Daejeon, South Korea).

Statistical analysis. Statistical analysis was assessed by comparing case and control samples, and ER positive and ER negative samples. SNPStats online tools (bioinfo.iconcologia.net/snpstats/) (17), was used to calculate allele frequency, and genotype frequency.

Table III. Association analysis of rs1836724 and risk of breast cancer.

	Allele frequency comparison		Armitage's trend test	
Risk allele	Odds ratio (95% CI)	P-value	Common odds ratio	P-value
C	0.581 (0.356-0.947)	0.02869	0.586	0.02913
T	1.722 (1.056-2.808)		1.697	

CI, confidence interval.

Deviation from Hardy-Weinberg equilibrium (HWE), odds ratios (ORs) with 95% confidence intervals (CIs), and the Cochran-Armitage (CA) test for trend were executed using

Table IV. Suggested miRNAs with increased binding possibility to the 3'-UTR *ErbB4* by C allele may target *ESR1* mRNA according to the aforementioned algorithms.

Putative miRNA binding site	Database used
hsa-miR-28-5p	miRWalk Microt4 miRMap RNA22 RNAhybrid
hsa-miR-665	miRMap RNA22 RNAhybrid
hsa-miR-708-5p	miRWalk Microt4 miRMap RNA22 RNAhybrid

miRNA, microRNA; ESR1, estrogen receptor 1.

the DeFinetti program (ihg.gsf.de/cgi-bin/hw/hwa1.pl) to analyze the association between rs1836724 and breast cancer. The CA test considers individuals' genotypes, as opposed to solely the alleles for association assessment, using the guidelines provided by the DeFinetti program. Consistency with Hardy-Weinberg equilibrium was investigated using Pearson's χ^2, Log likelihood ratio (Llr) χ^2, and exact tests. In addition, the association test was evaluated using χ^2 test. Logistic regression models were used to determine if odds ratios (OR) are associated with 95% confidence intervals (95% CI). P<0.05 was considered to indicate a statistically significant difference. Additional bioinformatics investigation was conducted to acquire estrogen receptor (*ESR1*) targeted miRNAs using miRWalk V.2.0 database (19).

Results

In silico analysis. Computational predictions suggested that rs1836724 is located in *ErbB4* 3'-UTR within the potential target sequence of has-miR-335-5p, hsa-miR-28-5p, has-miR-708-5p and has-miR-665 (Fig. 1).

Statistical analysis. Allele frequencies, observed genotypes, expected genotypes and HWE P-values are shown in Table II. No deviation from HWE was observed in the groups. According to the allele frequency comparison [OR (95% CI)=1.722 (1.056-2.808), P=0.02869) and the CA test for trend (OR=1.697, P=0.2913), T allele of rs1836724 was found to be associated with a risk of breast cancer (Table III). Moreover, the C/T genotype of rs1836724 was significantly associated with an ER-positive phenotype among patients [OR (95% CI)=6.000 (1.082-33.274), P=0.02846] compared with the T/T genotype. Finally, *in silico* algorithms demonstrated that *ESR1*, the gene responsible for the ER phenotype in the case group, may be targeted by similar miRNAs as *ErbB4*, including has-miR-28-5p, has-miR-708-5p and has-miR-665 (Table IV).

Discussion

Thus far, altered expression of *ErbB4* has been reported in various studies of breast cancer (20,21). miRNAs are important in regulating *ErbB4* expression (22). It was demonstrated that polymorphisms in the 3'-UTR of genes could affect miRNA binding sites, resulting in post-translational dysregulation of mRNA and a predisposition to cancer (23,24). Computational analysis scrutinized the 3'-UTR region of *ErbB4* for its cancer risk variants. Noticeably, the rs1836724 SNP was identified within the 3'-UTR of *ErbB4* and target binding site of four miRNAs. The presence of SNP rs1836724T/T would weaken the target sites of has-miR-28-5p, has-miR-708-5p and has-miR-665, and strengthen the target site of has-miR-335-5p (Fig. 1). A significant calculated $\Delta\Delta G$ suggested rs1836724T/T as a possible causative genetic factor in the development of breast tumor cells. To the best of our knowledge, this is the first case-control study conducted in an Iranian population attempting to examine the correlation between rs1836724T/T and the risk of breast cancer.

In this case-control study, all female participants selected belonged to the same ethnicity in order to eliminate variation of alleles and genotype frequencies, as certain ones may only occur in specific ethnic groups, thus skewing the results. Analysis of allele frequencies suggested that the C allele is the minor allele (allele frequency, 0.35) when observing all subjects of the study, the same as that demonstrated in the NCBI SNP databank reports (ncbi.nlm.nih.gov/snp/). However, comparing allele frequencies of the T allele in the control group (0.65) and case group (0.71), indicated that it is a risk factor for breast cancer. The genotype distribution data suggested that C/C is the minor genotype in the control and case groups (genotype frequency, 0.16 and 0.09, respectively). Notably, the case subgroup analysis highlighted allele frequency differences between ER-negative (T, 0.75; C, 0.25) and ER-positive (T, 0.66; C, 0.34) that may lead to the different effect of alleles on this phenotype. HWE P-value data (Pearson, Llr, and exact test) were all >0.05 and no deviation from HWE was identified.

This study determined an association between rs1836724 and the susceptibility to breast cancer using allele frequency comparison and the CA test for trend. Together, the association between the T allele and the risk of breast cancer was confirmed (1.722 OR and P=0.02869). The computational data obtained in the current study suggested that the miR-335-5p binding site may be strengthened in T allele carriers; however, miR-28-5p, miR-665, and miR-708-5p were presumed to have weaker binding site in the T allele variant (Fig. 1). Therefore, downregulation of *ErbB4* expression levels in individuals carrying the T allele in rs1836724 may result in greater susceptibility to breast cancer.

Conversely, the present study demonstrated an association between the C/T and perhaps C/C genotypes of rs1836724 and an ER-positive phenotype. As was predicted, the C allele was associated with more post-translational suppression corresponding to stronger mRNA binding sites within the 3'-UTR *ErbB4*. Consequently, further bioinformatics analysis was executed to reveal identical regulatory miRNAs of *ErbB4* and

ESR1. As shown in Table IV, the miRNAs that regulate *ErbB4* and *ESR1* were similar. According to previously demonstrated association between *ErbB4* and *ESR1* expression and breast cancer outcome (25), and the identical miRNAs, association between the C allele and an ER-positive phenotype can be explained. The present study hypothesized that stronger target binding sites at the 3'-UTR *ErbB4* due to the presence of the C allele may reduce the possibility of ESR1 being targeted by the same miRNAs. In other words, the C allele leads to more preferable sequences at 3'-UTR *ErbB4*, which may in turn recruit miRNAs able to target 3'-UTR *ESR1*. Therefore, observation of ER positive phenotype in the state of C/T genotype 6 times (OR=6, P=0.028) more than T/T genotype seemed completely logical. It is notable that, further discussion of miR-335-5p in the ER phenotype has not been conducted as it was not listed as one of the *ESR1*/*ErbB4* common regulatory miRNAs.

In conclusion, to the best of our knowledge, this population based case-control study demonstrated for the first time a correlation between the SNP rs1836724 located at the 3'-UTR *ErbB4* and susceptibility to breast cancer. The data suggested a significant association between the T allele and a risk of breast cancer. Computational data correlated the experimental observations with altered target binding sites of three *ErbB4* regulatory miRNAs, hsa-miR-28-5p, hsa-miR-7085p and hsa-miR-665. Furthermore, the ER-positive phenotype was shown to be associated with the C carriers of rs1836724. miRNAs that regulate both *ESR1* and *ErbB4* explained a competitive correlation between these genes, which leads to less *ESR1* downregulation in the C allele state due to stronger target binding in the 3'-UTR of *ErbB4*. Together, this investigation suggests that the rs1836724T/T SNP is a potential risk factor for the development of breast cancer.

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