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ORIGINAL RESEARCH ARTICLE

ErbB4 3′-UTR Variant (c.*3622A>G) is Associated with ER/PR Negativity and Advanced Breast Cancer

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Abstract A genetic variant may alter a gene expression level and as a result be associated with pathological characteristics in breast cancer. In this research, the frequency and association of the ErbB4 3′-untranslated region (3′-UTR) variant, rs12471583 (c.*3622A>G) was studied in an Iranian breast cancer patients. *In silico* assessment was performed to predict the function of the rs12471583 variant located on the 3′-UTR of ErbB4. Furthermore, as a case–control study, this polymorphism was genotyped in 243 breast cancer patients and non-cancerous controls using the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) technique. The Armitage's trend test and regular association tests were performed to analyze a possible association between the rs12471583 and risk of breast cancer and its relevant pathological traits. The bioinformatics analysis predicted that the rs12471583 SNP is located on the four miRNA binding sites, including miR-511-5p, miR-4659a-5p, miR-4659b-5p, and miR-6830-3p. According to logistic regression tests, the G allele is negatively associated with ER− (OR = 0.20, 95% C.I. = 0.04–0.93, p = 0.026), PR− (OR = 0.31, 95% C.I. = 0.10–0.98, p = 0.039), ER−/PR− (OR = 0.20, 95% C.I. = 0.04–0.93, p = 0.026), and advanced breast cancer (OR = 0.40, 95% C.I. = 0.18–0.85, p = 0.016). It has been found that ErbB4 expression may be linked to unfavorable outcomes in breast cancer. Likewise, our results suggest that the G allele may strengthen miRNA-ErbB4 binding efficiency and as a result reduce expression of ErbB4. This is a possible explanation for the observed association.

Keywords Estrogen receptor · Functional SNP · HER4 · Progesterone receptor · Stage IV

Introduction

Breast cancer is the most common malignancy type and the cause of cancer death among women [1]. Epidemiological studies have provided numerous evidence to show the connection between genetic factors, including single nucleotide polymorphisms (SNPs) and susceptibility to cancer [2–5].

Along with important genes such as BRCA1/BRCA2/EpCAM/c-Myc [6], members of the EGF receptor (EGFR) subfamily of receptor tyrosine kinases, ErbB1 (EGFR, HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4) play a critical role in the pathogenesis and tumorigenic processes of breast cancer [7–10]. ErbB4 expression is typically correlated with estrogen receptor (ER) and progesterone receptor (PR) positivity, HER2 receptor-negativity, well-differentiated phenotype (lower tumor grade), smaller tumor size, lower risk for relapse,

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longer overall survival, and also favorable outcome [8, 11–15]. On the other hand, ErbB4 upregulation is correlated with worse relapse-free survival in woman with node-negative [16] and also with reduced survival in node-positive breast cancer patients [17]. Hence, the ErbB4 expression has been recognized as a double-edged sword in breast cancer.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that hybridize to 3′-untranslated regions (3′-UTRs) and regulate post-transcriptionally mRNA expression; therefore, may participate in various pathological events [18–24]. Many association studies have aimed to evaluate genetic polymorphisms that potentially modify gene expression [25]. Similarly, a polymorphism may alter the function of miRNAs through two mechanisms. First, a variant in a precursor miRNA (pre-miRNA) may change pre-miRNA stability; hence, affect miRNA expression. Second, a polymorphism within a miRNA target site (3′-UTR of a gene) can alter miRNA-mRNA binding strength. Bioinformatics tools are highly useful to predict the effect of SNPs at miRNA loci and mRNA targets and offer possible descriptions for the phenotype associations [26].

We have been investigating potential associations between miRNA-related polymorphisms and breast cancer in the Iranian population [22, 27–33]. Herein, we focused on the frequencies and predictive value of another ErbB4 3′-UTR SNP, rs12471583 (c.*3622A>G) in Iranian breast cancer patients. In addition, *in silico* examination was used to predict the functional impact of this SNP.

Materials and Methods

Study Subjects, DNA Isolation, and Genotyping

Genotyping of the rs12471583 SNP was conducted in 243 Iranian women, including 123 breast cancer and 120 cancer-free samples using a well-established assay, polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP). Patients with other malignancies or bilateral breast cancer were excluded from the present study. This study was permitted by the Ethics Committee of the Islamic Azad University. Written informed consent was collected from all participants.

DNA was extracted from whole blood samples using the PrimePrep Genomic DNA Isolation Kit (GeNetBio, Chungnam, South Korea) [34], following the provided structure. The sequence of primers used in this study is 5′-ACA TCC TTC CCT GTC AGG CT-3′ and 5′-CCA CAA AGC ATC TGC ACC AC-3′. Standard cycling was implemented in the ASTEC PC-818 thermocycler (ASTEC, Fukuoka, Japan) as following condition: (1) initial denaturation at 96 °C for 2 min (2) 30 cycles of 94 °C for 30 s, 59.3 °C for 30 s, and 72 °C for 50 s (3) 72 °C for 7 min. The genotype of individuals was detected by digesting PCR products using TaiI (#ER1141, Thermo Fisher Scientific Inc., Waltham, MA, USA) as a restriction enzyme. TaiI cuts the 445 bp PCR product containing the G allele in two fragments 278 bp and 167 bp. Otherwise, it does not cut the PCR product containing the A allele. Electrophoresis of restricted fragments was performed by 1.5% agarose gel electrophoresis in 1 × Tris–Borate–EDTA buffer at 100 V and final staining with the Red-Safe™ Nucleic Acid Staining Solution (20,000 ×) (Boca Scientific Inc., Boca Raton, Florida, USA). Genotyping validity was examined by an accidental assortment of preparation of 5 samples for Bioneer sequencing service (Bioneer Inc., Daejeon, Korea).

ER, PR, HER2 Immunohistochemistry, and Other Pathological Features

Pathology Laboratory in the Sayed-ol-Shohada Hospital is a reference laboratory in which immunohistochemistry (IHC) and pathological tests were done centrally by experienced operators and an expert pathologist who monitored sample handling and examining; hence, ensuring the correctness of results. The pathological and clinical attributes of the patients are listed in Table 1.

In Silico Analysis

The PolymiRTS Database 3.0 tool [35] was used to predict an impact of the rs12471583 SNP on the miRNA-ErbB4 mRNA duplex and compare the binding score between the two SNP alleles.

Statistical Analysis

Hardy–Weinberg equilibrium (HWE) deviation, 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 studies the individuals' genotypes rather than just the alleles for an association test.

Consistency with HWE was tested by Pearson's Chi square, Log-likelihood ratio (Llr) Chi square, and exact tests. Moreover, association tests were performed using the Chi square test. Logistic regression models were employed to account odds ratios (OR) and related 95% confidence intervals (95% CI). p value of < 0.05 was considered statistically significant.

Table 1 The clinicopathological characteristics of the patients with breast carcinoma

Characteristics	Number
Histological grade	I: 15, II: 48, III: 30, unknown: 30
Stage	I: 21, II: 18, III: 12, IV: 66, unknown: 6
Estrogen receptor (ER) status	Positive: 63, negative: 18, unknown: 42
Progesterone receptor (PR) status	Positive: 57, negative: 24, unknown: 42
HER2 status	Positive: 24, negative: 45, unknown: 42

Results

Frequencies of ErbB4 3′-UTR Variant rs12471583 (c.*3622A>G)

RFLP–PCR and agarose gel electrophoresis were used in order to genotype the samples. The genotyping was determined as mentioned in the "Materials and Methods" section (Fig. 1). To investigate the prevalence of the ErbB4 3′-UTR SNP, DNA samples from 243 women with breast cancer or no cancer were analyzed. Allele frequencies, genotype frequencies, and HWE p values in breast cancer and cancer-free groups are listed in Table 2. No deviation from HWE was found in the defined groups and this may endorse the accuracy of genotyping results.

Associations of the ErbB4 SNP rs12471583 Alleles with Breast Cancer and Clinicopathological Features of the Patients

Compared with ER+, PR+, ER/PR+ (ER+/PR+, ER−/PR+, and ER+/PR−), and non-advanced breast cancer (stage I/II/III), the G allele has a negative (inverse) association ($0 < \text{OR} < 1$) with ER−, PR−, ER−/PR−, and advanced breast cancer (stage IV). Notably, alleles of the candidate SNP was not significantly associated with breast cancer, grade 3 (poorly differentiated), HER2 expression, and cancer death phenotypes (data are not shown). Significant results are shown in Table 3.

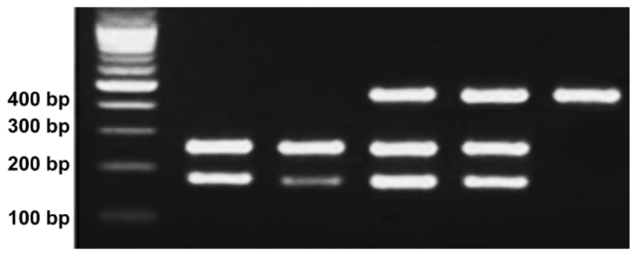

Fig. 1 The agarose gel reflecting the results of RFLP–PCR. Lane 1 is 100 bp ladder. Lanes 2 and 3 show G/G homozygotes, Lanes 4 and 5 show G/A heterozygotes and lane 6 shows A/A homozygote genotype

In Silico Results

Bioinformatics analysis showed that the rs12471583 is located on ErbB4 3′-UTR within the potential binding site of miR-511-5p, miR-4659a-5p, miR-4659b-5p, and miR-6830-3p. Therefore, the G allele may alter miRNA-mRNA binding energy (Table 4).

Discussion

ErbB4 expression may associate with breast cancer development [12, 13, 36]. Conversely, ErbB4 has been reported to induce cell apoptosis, reduce cell proliferation, and promote the differentiation and inhibition of growth in breast cancer cells [37–39]. Hence, it can be introduced as a good prognostic marker in breast cancer [8, 13]. Furthermore, ErbB4 expression is classically linked to ER and PR positivity, HER2 receptor-negativity, well-differentiated phenotype (lower tumor grade), smaller tumor size, lower risk for relapse, longer overall survival, and better clinical outcome. [15]. Similarly, it has been demonstrated that ErbB4 expression both at the mRNA and the protein level has a positive prognostic ability in patients with breast cancer [40]. In addition, ErbB4 expression level can be linked to the expression of ErbB3, a breast cancer favorable biomarker [8, 15, 36]. One of the possible explanation for this inconsistency is that ErbB4 may function erratically in different breast cancer development stages.

ErbB4 downregulation was reported to be related with promoter hypermethylation [37]. A functional SNP located in ErbB4, 815A>T, has been demonstrated to be associated with poorer prognosis. This SNP may have effect on the expression of ErbB4 gene, as it is located in the promoter region [41]. However, little is known about the impact of functional SNPs within ErbB4 3′-UTR on ErbB4 expression. These types of SNPs may modify the structure of ErbB4 mRNA which leads to its stability alteration; and therefore, the protein expression can be affected. It has been found that SNPs in the 3′-UTR of genes can alter miRNA-mRNA binding energy, resulting in post-translational dysregulation of mRNA and susceptibility to cancer [26]. To date, eight miRNAs, including miR-372-3p, miR-

Table 2 Estimation of allele frequency, genotype frequency, and HWE *p* values of rs12471583 in healthy and breast cancer groups

Group name	Allele frequency		Genotype frequency			HWE *p* value		
	A	G	A/A	A/G	G/G	Pearson	Llr	Exact
Control	0.79	0.21	0.62	0.32	0.05	0.796	0.798	0.744
Breast cancer	0.74	0.26	0.56	0.37	0.07	0.718	0.721	0.772

Table 3 Allelic association and Armitage's trend tests of rs12471583G allele with ER−, PR−, ER−/PR−, and stage IV incidence

Variable comparison	ER− vs ER+		PR− vs PR+		ER−/PR− vs ER/PR+		Stage IV vs Stage I/II/III	
Allele comparison	G allele vs A allele		G allele vs A allele		G allele vs A allele		G allele vs A allele	
Association result	Odds ratio	*p*	Odds ratio	*p*	Odds ratio	*p*	Odds ratio	*p*
Allele frequency difference (95% C.I.)	0.20 (0.04–0.93)	0.026	0.31 (0.10–0.98)	0.039	0.20 (0.04–0.93)	0.026	0.40 (0.18–0.85)	0.016
Armitage's trend test	0.16	0.015	0.25	0.024	0.16	0.015	0.45	0.015

Table 4 *In silico* analysis of the SNP-miRNA binding

miRNA	Sequence	miRNA site on ErbB4 3′-UTR with rs12471583	Score change (G allele vs A allele)
miR-511-5p	GUGUCUUUUGCUCUGCAGUCA	AAAGAC[A/G]	+ 0.137
miR-4659a-5p	CUGCCAUGUCUAAGAAGAAAAC	C[A/G]TGGCA	− 0.059
miR-4659b-5p	UUGCCAUGUCUAAGAAGAA	C[A/G]TGGCA	− 0.059
miR-6830-3p	UGUCUUUCUUCUCUCCCUUGCAG	AAAGAC[A/G]	+ 0.015

19a-3p, miR-302d-3p, miR-146a-5p, miR-145-5p, miR-221-3p, miR-302b-3p, and miR-146a-5p have been experimentally recognized as ErbB4 regulators [42].

To our knowledge, this is the first evaluation of the frequencies and association significance of the ErbB4 3′-UTR rs12471583 variant (c.*3622A>G) in breast cancer patients. Bioinformatically, this SNP is located on the 3′-UTR of ErbB4, target binding site of four miRNAs, including miR-511-5p, miR-4659a-5p, miR-4659b-5p, and miR-6830-3p. The presence of 3622G allele potentially strengthens the binding site for miR-511-5p and miR-6830-3p, and at the same time weakens the target site for miR-4659a-5p, miR-4659b-5p (Table 4).

Regarding the association results of the rs12471583 SNP alleles with breast cancer clinicopathological features, the G allele was negatively (inversely) associated with ER− (ER− vs ER+, OR = 0.20, 95% C.I. = 0.04–0.93, $p = 0.026$), PR− (PR− vs PR+, OR = 0.31, 95% C.I. = 0.10–0.98, $p = 0.039$), ER−/PR− (ER−/PR− vs ER+/PR+, ER−/PR+, and ER+/PR−, OR = 0.20, 95% C.I. = 0.04–0.93, $p = 0.026$), and stage IV (stage IV vs stage I/II/III, OR = 0.40, 95% C.I. = 0.18–0.85, $p = 0.016$). ER−/PR− breast cancer patients have poorer survival compared with ER/PR+ ones [43]; thus, the G allele is a potential protective factor in breast cancer.

The bioinformatics result suggests a correlation between the G allele and the miRNA-ErbB4 binding energy alteration. Moreover, our case–control study identified that the rs12471583 SNP reduces ER and PR negativity among Iranian breast cancer patients. Interestingly, ErbB4 expression was indicated to be positively correlated with ER and PR positivity [8]. Therefore, the present study hypothesized that the G allele may totally strengthen miRNA-ErbB4 binding efficiency and result in a reduction of ErbB4 expression and ER and PR negativity status.

It is highly recommended using in vitro studies in order to fully understand the function of SNP rs12471583 in breast cancer. To this aim, proliferation, colony formation and further in vitro assays, along with ErbB4 expression profiling can be conducted in CRISPR-Cas9 isogenic cell lines with AA, AG and GG genotypes. This will reveal the

potential capacity of rs12471583 A allele in enhancing viability and cell growth.

Taken together, this study presents rs12471583 G allele at ErbB4 3′-UTR as a novel protective marker for ER and PR negativity and stage IV incidence in breast cancer. Hence, the G allele has potentially a favorable predictive role in breast cancer.

Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interest.

Research Involving Human Participants and/or Animals All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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