

# HER4 rs1595065 3'UTR Variant is a Possible Risk Factor for HER2 Positivity Among Breast Cancer Patients

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Received 2016 September 11; Revised 2016 November 08; Accepted 2016 November 10.

## Abstract

**Background:** Breast cancer (BC) is the most common neoplasia among females worldwide. Single nucleotide polymorphisms (SNPs) located at the 3' untranslated Region (3'UTR) can alter gene expression pattern through increasing/decreasing microRNAs (miRNAs) binding energy. Human epidermal growth factor receptor 4 (*HER4*) can act as either a tumor suppressor or an oncogene in breast cancer.

**Objectives:** We proposed that rs1595065 3'UTR variant of *HER4* with a different target binding site of miRNAs may have a correlation with risk of BC phenotypes. In the current study, we aimed to evaluate the association between *HER4* rs1595065 3'UTR variant and BC pathological features among the Isfahanian population. Moreover, an in-silico prediction was performed to estimate possible function of the rs1595065.

**Methods:** Overall, 156 patients and controls were genotyped using RFLP-PCR. Armitage test for trend was utilized to investigate the association between rs1595065 and susceptibility to BC. The possible change in the interaction between rs1595065 and microRNAs was studied bioinformatically.

**Results:** Bioinformatics analysis using online tools suggest rs1595065 as a polymorphism in the seed region of four miRNAs binding sites including miR-199a-3p, miR-199b-3p, miR-1244 and miR-3129, and C allele can reduce miRNA-mRNA binding occurrence that may increase *HER4* expression. Armitage's trend test showed that C allele of rs1595065 was significantly associated with *HER2* positivity among patients (C allele vs. T allele, OR = 3.111, P = 0.046).

**Conclusions:** rs1595065 could be recommended as a risk factor in regulating *HER4* expression and affecting *HER2* positivity incidence among BC patients.

**Keywords:** Breast Cancer, Single Nucleotide Polymorphism, ErbB4, miRNA

## 1. Background

Breast cancer (BC) is the most frequent malignancy among females (1). Several common genetic BC-associated variants, including single nucleotide polymorphisms (SNP), have been recognized by association studies (2, 3).

Members of epidermal growth factor-related (Her) receptor tyrosine kinases family, *HER1* (ErbB1, EGFR), *HER2* (ErbB2, neu), *HER3* (ErbB3) and *HER4* (ErbB4) showed a critical role in the pathogenesis and tumorigenic processes of BC (4, 5). Controversially, the prognostic and predictive value of *HER4* expression was indefinite, and both favorable and unfavorable impacts of *HER4* expression have been reported (5-12). Therefore, *HER4* can act either as a tumor suppressor or an oncogene in BC.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that hybridize to 3'-Untranslated Regions

(3'-UTRs) and mediate mRNA translational inhibition or cleavage and may consequently contribute to various pathological events (13, 14). Many studies have been designed to illustrate functional genetic polymorphisms that dysregulate miRNA regulation via different molecular mechanisms and could be associated with several pathological events. Functional SNP related to the miRNAs mechanism of action is categorized to two groups, first, polymorphisms in precursor miRNAs (pre-miRNAs), which may disturb miRNA expression possibly through changing pre-miRNA stability, and second, polymorphisms within miRNA target sites (3'-UTR of targets), which may modify miRNA-mRNA binding strength. Bioinformatics tools are useful to predict the effects of SNPs at miRNA loci and targets and offer probable descriptions for the phenotypic associations (15, 16).

*HER4* gene variations in breast cancer have been less

extensively investigated. In a previous study, we reported a possible association between rs1836724 in the 3'-UTR of HER4 gene and BC incidence in the Iranian population (17).

## 2. Objectives

In the present study, we analyzed the frequencies and predictive value of another *HER4* 3'-UTR SNP, rs1595065 (c.\*5699T > C), in the Iranian population upon BC clinico-pathological features. In addition, an *in silico* evaluation was used to estimate the functional influence of the SNP.

## 3. Methods

### 3.1. Study Subjects

This paper originated from a research study, the protocol of which was approved by the Islamic Azad University. All subjects were provided with written informed consents. A total of 82 females with breast cancer and 74 control individuals were included in the present study. All blood samples were collected randomly from Seyedo-Shohada hospital, Isfahan University of Medical Sciences, Isfahan, Iran, over the course of one year, from February 2015 to 2016. Control blood samples were collected randomly from the female subjects, who attended Seyedo-Shohada hospital for general examinations. The patients with other malignancies or bilateral breast cancer, and the control subjects with any breast cancer symptoms were excluded from the study. Ethics approval was provided by the Iranian ministry of health and medical education.

### 3.2. DNA Extraction and rs1595065 Genotyping With RFLP-PCR Analysis

DNA was isolated from whole blood samples using the PrimePrep Genomic DNA Isolation Kit (GeNetBio, Chungnam, South Korea), according to the protocol of the company. The primers used for amplification of the rs1595065 were 5'-GCT AAC TCG TCT CAA ATT CCT-3' and 5'-CCT TTC TTA AGC CAT AGT GGA-3'. Regular cycling was applied in a ASTEC PC-818 thermocycler (ASTEC, Fukuoka, Japan) with the following condition: initial denaturation at 96°C for three minutes followed by 30 cycles at 94°C for 30 seconds, 51°C for 30 seconds, 72°C for 30 seconds, and finally 72°C for seven minutes. Allelic variants were identified by digesting PCR products with restriction of endonuclease AciI (#ER1791, Thermo Fisher Scientific Inc., Waltham, MA, USA). The AciI restriction endonuclease was chosen in order to cut the 233 bp PCR product, containing C allele to two fragments of 140 bp and 93 bp, while the enzyme did not cut PCR products containing the T allele. Electrophoresis of restricted fragments was visualized by 1.5% agarose gel

electrophoresis in 1x Tris-Borate-EDTA buffer at 100V and finally stained with RedSafe™ nucleic acid staining solution (20,000x) (Boca Scientific Inc., Boca Raton, Florida, USA).

### 3.3. Patient Characteristics

Pathology Laboratory of the Seyedo-Shohada Hospital is a reference test center where Immunohistochemistry (IHC) and pathological tests have been assessed centrally by expert operators and a dedicated pathologist, who tracks strict sample handling, processing and reporting protocols, thus ensuring the reliability of results. The pathological and clinical characteristics of the patients are listed in Table 1.

### 3.4. In Silico Analysis

The miRNASNP tool (microRNA-related Single Nucleotide Polymorphisms) (<http://www.bioguo.org/miRNASNP/index.php>) (18) was used in order to predict the effect of 3'-UTR SNP, rs1595065 considering miRNAs interaction.

### 3.5. Statistical Analysis

Deviation from Hardy-Weinberg Equilibrium (HWE), odds ratios (ORs) with 95% confidence intervals (CIs), and Armitage's trend test were completed using the DeFinetti program (<http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>). Markedly, Armitage's trend test reflects the individuals' genotypes rather than just the alleles for association assessment.

Hardy-Weinberg Equilibrium (HWE) and association test P-values were tested by Pearson's chi-square test. Logistic regression models were used to account for odds ratios (OR) and related 95% Confidence Intervals (95% CI). P < 0.05 were considered statistically significant.

## 4. Results

### 4.1. Frequencies of *HER4* 3'-UTR Variant rs1595065 (c.\*5699T > C)

To investigate the prevalence of the *HER4* 3'-UTR SNP, DNA samples from 162 females, either with no tumor or with BC were studied. Genotype frequencies, allele frequencies, and HWE P value were calculated as follows: T/T, 0.68; T/C, 0.21; C/C, 0.12; T, 0.78; C, 0.22; and HWE P value,  $3 \times 10^{-6}$ . As a deviation from HWE was observed, Armitage test for trend, which is a statistical method that does not assume HWE, was used for association test between the SNP and BC phenotypes (19). The age difference between controls and breast cancer cases was significant (independent t test, P < 0.0001). The mean + standard deviation (SD) of controls and cases was  $52.88 \pm 10.07$  and  $37.79 \pm 16.25$ , respectively.

**Table 1.** The Clinicopathological Features of the Patients with Breast Carcinoma

| Characteristic          | Status    | TT Genotype | TC/CC Genotype | Odds Ratio (95%CI)     | P Value <sup>a</sup> |
|-------------------------|-----------|-------------|----------------|------------------------|----------------------|
| <b>HER2<sup>b</sup></b> | Positive  | 42.86       | 57.14          | 3.111 (0.886 - 10.925) | < 0.046              |
|                         | Negative  | 70          | 30             |                        |                      |
| <b>ER<sup>c</sup></b>   | Positive  | 57.14       | 42.86          | -                      | 0.375                |
|                         | Negative  | 83.33       | 16.77          |                        |                      |
| <b>PR<sup>d</sup></b>   | Positive  | 52.63       | 47.37          | -                      | 0.384                |
|                         | Negative  | 87.50       | 12.50          |                        |                      |
| <b>Grade</b>            | Grade I   | 100         | 0              | -                      | 0.114                |
|                         | Other     | 62.96       | 37.04          |                        |                      |
|                         | Grade II  | 60          | 40             | -                      | 0.126                |
|                         | Other     | 76.47       | 23.53          |                        |                      |
|                         | Grade III | 66.66       | 33.33          | -                      | 0.310                |
| <b>Stage</b>            | Other     | 70          | 30             |                        |                      |
|                         | Stage I   | 85.71       | 14.29          | -                      | 0.118                |
|                         | Other     | 68.85       | 31.15          |                        |                      |
|                         | Stage II  | 66.66       | 33.33          | -                      | 0.128                |
|                         | Other     | 73.02       | 26.98          |                        |                      |
|                         | Stage III | 33.33       | 66.66          | -                      | 0.630                |
|                         | Other     | 77.27       | 22.73          |                        |                      |
| <b>Metastasis</b>       | Stage IV  | 75          | 25             | -                      | 0.115                |
|                         | Other     | 68.57       | 31.43          |                        |                      |
|                         | Positive  | 74.07       | 25.93          | -                      | 0.865                |
|                         | Negative  | 71.43       | 28.57          |                        |                      |

<sup>a</sup>Chi-Square test.<sup>b</sup>Human epidermal growth factor receptor 2.<sup>c</sup>Estrogen receptor.<sup>d</sup>Progesterone receptor.

3.2 Association test of the HER4 SNP rs1595065 C allele with breast cancer and clinicopathological features of the patients

Univariate analysis showed that C allele of rs1595065 was significantly associated with *HER2* positivity among patients; odds ratio = 3.111 (95%CI: 0.886 - 10.925),  $P = 0.046$  (Table 1). Noticeably, rs1595065 in *HER4* gene was not significantly associated with BC, ER positivity, PR positivity, stage IV (advanced BC), grade III (poorly differentiated), and metastatic phenotypes incidence.

#### 4.3. In Silico Results

Computational assessment proposed that rs1595065 is located in *HER4* 3'-UTR within the potential target sequence of miR-199a-3p, miR-199b-3p, miR-1244, and miR-3129, and as a result, C allele can reduce miRNA-mRNA binding occurrence (Table 2). Up-regulation of *HER4* gene can be predicted when the mRNA has C allele at rs1595065 position.

## 5. Discussion

The effect of *HER4* expression on the progression and outcome of BC remains mostly unclear. In-vitro and in-vivo

studies demonstrated both good and poor prognostic ability for *HER4* expression (5-12). Most of the published reports have shown an association between high expression level of *HER4* and positivity status of ER, lower grades of breast malignancy and lower rate of proliferation (20). Moreover, Fujiwara, et al. determined that *HER4* overexpression is associated with a better prognosis (5).

In 2011, Zhu et al. (21) showed the significant association of rs1595066, located within *HER4* 3'UTR, with the risk of breast cancer. According to this report, AG and AA genotypes in rs1595066 position depicted significantly lower risk of breast cancer. Another study conducted to analyze rs11895168 SNP, located on *HER4* gene, noticeably showed that breast cancer patients carrying rs11895168 C allele were significantly associated with elevated breast cancer risk and ER/PR positivity (22). Furthermore, Zabihi et al. (23) reported that harboring G allele in rs1972820 position, located in 3'UTR of *HER4* gene, is significantly associated with decreased risk of breast cancer. Altogether, these data support the importance of *HER4* gene SNPs, especially the miRNA-related ones.

In the current study and with regards to the associations with clinicopathological parameters of BC, we found

**Table 2.** In Silico Examination of the SNP-miRNA Binding

| miRNA       | miRNA Sequence            | miRNA Site on ErbB4 3'-UTR With rs1595065 | Energy Change (kcal/mol) C vs. U Allele |
|-------------|---------------------------|---|---|
| miR-199a-3p | AUUGGUUACACGUCUGAUGACA    | CAAACUAC[U/C]G                            | +17.4                                   |
| miR-199b-3p | AUUGGUUACACGUCUGAUGACA    | CAAACUAC[U/C]G                            | +17.4                                   |
| miR-1244    | UUUGUAGAGAUUGUUUGUUGAUGAA | CUGUUUAGUGAACUAUCAAAUAC[U/C]              | +19.5                                   |
| miR-3129    | UUUGGUUAGAGAUUGAUGACG     | UAAGUGAACUAUCAAAUAC[U/C]G                 | +22.3                                   |

that C variant of rs1595065 in *HER4* gene is associated with enhanced risk of *HER2* positivity incidence, odds ratio = 3.111, 95% CI: 0.886-10.925 ( $P = 0.046$ ). As compared to other studies, here we showed the importance of a single nucleotide polymorphism in *HER4* gene in terms of its association with *HER2* positivity status. In addition, the functional consequence of the C allele was investigated bioinformatically and a possible association between C allele and decreasing miRNA interaction and the following up-regulation of *HER4* was suggested. However, more biochemical studies, such luciferase reporter assay, are required to validate this potential interaction. In contrast to our results, *ErbB4* expression is typically linked to estrogen receptor (ER) and progesterone receptor (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 (10).

This study had several limitations. First was depart from HWE observed in our sample cohort; more holistic investigations should be implemented on a new independent sample set with a larger size to verify the outcomes of this study. Next, this study did not evaluate the expression of *HER4* gene along with rs1595065 genotyping analysis; as a result, we could not discuss the connection between *HER4* gene expression and rs1595065 genotypes.

## Footnote

**Authors' Contribution:** The experiments were conceived and designed by Bahareh Moradi, Hossein Tabatabaieian and Kamran Ghaedi. The experiments were performed by Bahareh Moradi, Hossein Tabatabaieian and Samira Sadeghi. The data was analyzed by Hossein Tabatabaieian and Kamran Ghaedi. The manuscript was written by Bahareh Moradi, Hossein Tabatabaieian, Samira Sadeghi and Kamran Ghaedi.

## References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin*. 2015;65(1):5-29. doi: [10.3322/caac.21254](https://doi.org/10.3322/caac.21254). [PubMed: 25559415].

2. Easton DF, Pooley KA, Dunning AM, Pharoah PD, Thompson D, Ballinger DG, et al. Genome-wide association study identifies novel breast cancer susceptibility loci. *Nature*. 2007;447:1087-93. doi: [10.1038/nature05887](https://doi.org/10.1038/nature05887).
3. Allman R, Dite GS, Hopper JL, Gordon O, Starlard-Davenport A, Chlebowski R, et al. SNPs and breast cancer risk prediction for African American and Hispanic women. *Breast Cancer Res Treat*. 2015;154(3):583-9. doi: [10.1007/s10549-015-3641-7](https://doi.org/10.1007/s10549-015-3641-7). [PubMed: 26589314].
4. Hynes NE, MacDonald G. ErbB receptors and signaling pathways in cancer. *Curr Opin Cell Biol*. 2009;21(2):177-84. doi: [10.1016/j.ccb.2008.12.010](https://doi.org/10.1016/j.ccb.2008.12.010). [PubMed: 19208461].
5. Fujiwara S, Ibusuki M, Yamamoto S, Yamamoto Y, Iwase H. Association of ErbB1-4 expression in invasive breast cancer with clinicopathological characteristics and prognosis. *Breast Cancer*. 2014;21(4):472-81. doi: [10.1007/s12282-012-0415-5](https://doi.org/10.1007/s12282-012-0415-5). [PubMed: 23100016].
6. Bacus SS, Chin D, Yarden Y, Zelnick CR, Stern DF. Type 1 receptor tyrosine kinases are differentially phosphorylated in mammary carcinoma and differentially associated with steroid receptors. *Am J Pathol*. 1996;148(2):549-58. [PubMed: 8579117].
7. Kew TY, Bell JA, Pinder SE, Denley H, Srinivasan R, Gullick WJ, et al. c-erbB-4 protein expression in human breast cancer. *Br J Cancer*. 2000;82(6):1163-70. doi: [10.1054/bjoc.1999.1057](https://doi.org/10.1054/bjoc.1999.1057). [PubMed: 10735500].
8. Sassen A, Rochon J, Wild P, Hartmann A, Hofstaedter F, Schwarz S, et al. Cytogenetic analysis of HER1/EGFR, HER2, HER3 and HER4 in 278 breast cancer patients. *Breast Cancer Res*. 2008;10(1):2. doi: [10.1186/bcr1843](https://doi.org/10.1186/bcr1843). [PubMed: 18182100].
9. Koutras AK, Kalogeris KT, Dimopoulos MA, Wirtz RM, Dafni U, Briasoulis E, et al. Evaluation of the prognostic and predictive value of HER family mRNA expression in high-risk early breast cancer: a Hellenic Cooperative Oncology Group (HeCOG) study. *Br J Cancer*. 2008;99(11):1775-85. doi: [10.1038/sj.bjc.6604769](https://doi.org/10.1038/sj.bjc.6604769). [PubMed: 18985033].
10. Koutras A, Kalogeris KT, Wirtz RM, Alexopoulou Z, Bobos M, Zagouri F, et al. Evaluation of the prognostic significance of HER family mRNA expression in high-risk early breast cancer: a Hellenic Cooperative Oncology Group (HeCOG) validation study. *J Transl Med*. 2015;13:171. doi: [10.1186/s12967-015-0530-0](https://doi.org/10.1186/s12967-015-0530-0). [PubMed: 26021752].
11. Bieche I, Onody P, Tozlu S, Driouch K, Vidaud M, Lidereau R. Prognostic value of ERBB family mRNA expression in breast carcinomas. *Int J Cancer*. 2003;106(5):758-65. doi: [10.1002/ijc.11273](https://doi.org/10.1002/ijc.11273). [PubMed: 12866037].
12. Lodge AJ, Anderson JJ, Gullick WJ, Haugk B, Leonard RC, Angus B. Type 1 growth factor receptor expression in node positive breast cancer: adverse prognostic significance of c-erbB-4. *J Clin Pathol*. 2003;56(4):300-4. doi: [10.1136/jcp.56.4.300](https://doi.org/10.1136/jcp.56.4.300). [PubMed: 12663644].
13. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116(2):281-97. doi: [10.1016/S0092-8674\(04\)00045-5](https://doi.org/10.1016/S0092-8674(04)00045-5). [PubMed: 14744438].
14. Mesrian Tanha H, Mojtavabi Naeini M, Rahgozar S, Moafi A, Honardoost MA. Integrative computational in-depth analysis of dysregulated miRNA-mRNA interactions in drug-resistant pediatric acute lymphoblastic leukemia cells: an attempt to obtain new potential gene-miRNA pathways involved in response to treatment. *Tumour Biol*. 2016;37(6):7861-72. doi: [10.1007/s13277-015-4553-1](https://doi.org/10.1007/s13277-015-4553-1). [PubMed: 26700663].

15. Jin Y, Lee CG. Single Nucleotide Polymorphisms Associated with MicroRNA Regulation. *Biomolecules*. 2013;**3**(2):287-302. doi: [10.3390/biom3020287](https://doi.org/10.3390/biom3020287). [PubMed: [24970168](https://pubmed.ncbi.nlm.nih.gov/24970168/)].
16. Meshkat M, Tanha HM, Naeini MM, Ghaedi K, Sanati MH, Meshkat M, et al. Functional SNP in stem of mir-146a affects Her2 status and breast cancer survival. *Cancer Biomark*. 2016;**17**(2):213-22. doi: [10.3233/CBM-160633](https://doi.org/10.3233/CBM-160633). [PubMed: [27434289](https://pubmed.ncbi.nlm.nih.gov/27434289/)].
17. Bagheri F, Mesrian Tanha H, Mojtavavi Naeini M, Ghaedi K, Azadeh M. 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. *Molecular Medicine Reports*. 2016;**13**:4494-8. doi: [10.3892/mmr.2016.5078](https://doi.org/10.3892/mmr.2016.5078).
18. Gong J, Tong Y, Zhang HM, Wang K, Hu T, Shan G, et al. Genome-wide identification of SNPs in microRNA genes and the SNP effects on microRNA target binding and biogenesis. *Hum Mutat*. 2012;**33**(1):254-63. doi: [10.1002/humu.21641](https://doi.org/10.1002/humu.21641). [PubMed: [22045659](https://pubmed.ncbi.nlm.nih.gov/22045659/)].
19. Sasieni PD. From genotypes to genes: doubling the sample size. *Biometrics*. 1997;**53**(4):1253-61. doi: [0.2307/2533494](https://doi.org/10.2307/2533494). [PubMed: [9423247](https://pubmed.ncbi.nlm.nih.gov/9423247/)].
20. Sundvall M, Iljin K, Kilpinen S, Sara H, Kallioniemi OP, Elenius K. Role of ErbB4 in breast cancer. *J Mammary Gland Biol Neoplasia*. 2008;**13**(2):259-68. doi: [10.1007/s10911-008-9079-3](https://doi.org/10.1007/s10911-008-9079-3). [PubMed: [18454307](https://pubmed.ncbi.nlm.nih.gov/18454307/)].
21. Zhu XI, Song FJ, Zheng H, Zhang LN, Zhao YR, Chen KX. Relationship between polymorphism of ErbB4 gene in mirco-RNA binding site and the risk for breast cancer. *Tumor*. 2011;**31**:233-8.
22. Salimi Z, Sadeghi S, Tabatabaeian H, Ghaedi K, Fazilati M. rs11895168 C allele and the increased risk of breast cancer in Isfahan population. *Breast*. 2016;**28**:89-94. doi: [10.1016/j.breast.2016.05.007](https://doi.org/10.1016/j.breast.2016.05.007). [PubMed: [27262100](https://pubmed.ncbi.nlm.nih.gov/27262100/)].
23. Zabihi N, Sadeghi S, Tabatabaeian H, Ghaedi K, Azadeh M, Fazilati M. The association between rs1972820 and the risk of breast cancer in Isfahan population. *J Cancer Ther Res*.