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miR-559 polymorphism *rs58450758* is linked to breast cancer

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ABSTRACT

Background: MicroRNAs (miRNAs) participate in gene regulation and the control of cancer-related mechanisms such as apoptosis, invasion and differentiation. Single nucleotide polymorphisms (SNP) of the miRNA encoding genes may influence the development of cancer. We hypothesized a link between *miR-559* SNP *rs58450758* and breast cancer.

Materials & methods: Bioinformatics analyses were performed to predict the miR-559 target genes and the effect of the *rs58450758* SNP on the stem-loop structure. A total of 129 breast cancer cases and 153 controls were genotyped using PCR-RFLP.

Results: The recessive genotype (TT) was more common among breast cancer patients (23.3%) than among controls (2%). The non-dominant genotypes (CT+TT) were associated with breast cancer in patients (OR 3.62; 95% CI, 1.95–6.69; $p < 0.0001$). Bioinformatics analyses suggested that *rs58450758* changes miR-559 secondary structure and forms new DICER sites in the pre-miRNA.

Conclusion: The miR-559 *rs58450758* variant is linked to breast cancer.

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Breast cancer; miR-559; SNP; rs58450758

Introduction

Breast cancer, is the most frequent non-skin cancer in women and leads to half a million deaths in the world each year [1–3]. Despite the well-known environmental risk factors, genetic defects can significantly contribute to predisposition to breast cancer [4,5].

MicroRNAs (miRNAs) are single-stranded molecules ~22 nucleotides in length. Biogenesis begins with the synthesis of pri-miRNA, stem-loop structure being cleaved by the complex composed of DROSHA and DGCR8 [6]. This miRNA precursor (pre-miRNA) is exported to the cytoplasm and is further processed by DICER into an miRNA duplex. Translational repression or degradation of mRNA occurs when miRNA binds to the RNA-induced silencing complex (RISC) [7]. They can inhibit the expression of mRNA by binding to the 3′ UTR region [8] (Figure 1). miRNAs control several cancer-related mechanisms, including differentiation, apoptosis, migration and invasion [9,10]. Several studies suggest that miRNAs play key roles in many types of cancers, including breast cancer [11].

A single nucleotide polymorphism (SNP) is the most frequent genetic variation and may occur every 100–300 bases [12]. Some studies have revealed that SNPs could increase the incidence of breast cancer [13,14]. Since miRNAs are small functional units, single base changes (i.e. SNPs) in both the precursor elements and mature miRNA sequences may drive the evolution of new miRNAs by changing their biological function and affecting their interaction with their target mRNA [15,16]. A connection between structural changes and ectopic expressions has been indicated for miRNA and the diagnosis and incidence of breast cancer [17]. One study demonstrated that miR-559 interacts with a target sequence in the 3′-UTR of *ERBB2* and plays a major role in carcinogenesis [18]. In light of these studies, we hypothesized a link between *miR-559 rs58450758* and the incidence of breast cancer using both *in silico* and molecular genetic techniques.

Methods and materials

In an *in-silico* analysis, breast cancer microarray profiles of miR-559 were acquired from the Gene Expression Omnibus (GEO) database [19]. The GSE31309 dataset of 48 patients with early stage breast cancer and 57 disease-free individuals served to compare *miR-559* expression [20]. Molecular interaction networks of miR-559 and its targets were visualized by 'miRTargetLinkHuman' which represents experimentally validated interactions [21]. The effect of this variation on stem-loop structure was analysed using the 'ViennaRNA' Web Services. This online server assesses RNA secondary structures and minimum free energy (MFE) using the partition function (pf) algorithm of McCaskill [22]. The 'PHDcleave' website was employed to predict DICER processing sites in pre-miRNA of human, based on the pre-miRNA sequence using Support Vector Machine (SVM) model [23].

In a clinical study we recruited 129 newly diagnosed patients with breast cancer and no previous history of

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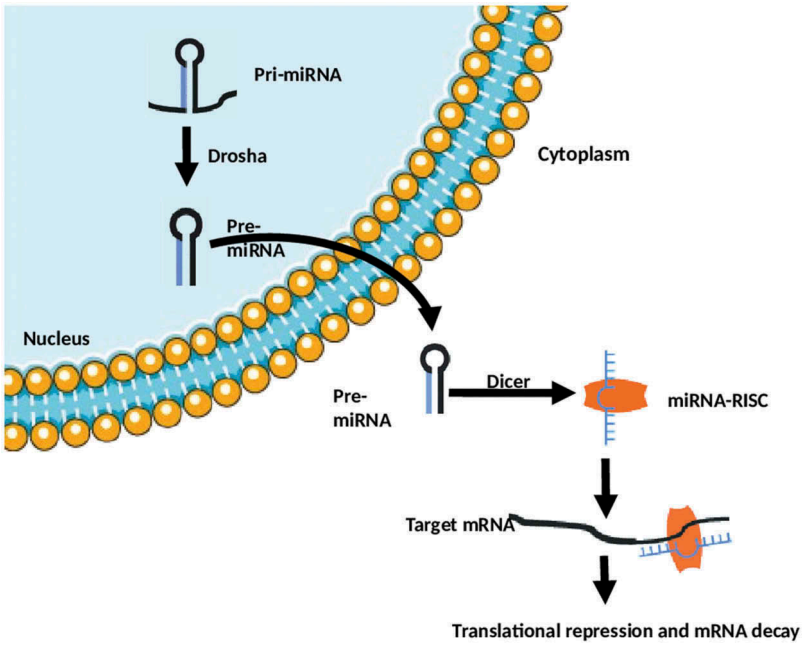


Figure 1. The biogenesis of miRNA. (see text for details)

cancer or prior radiation therapy or chemotherapy. Prognostic factors and clinic-pathological variables, including lymph node involvement, histology, epidermal growth factor receptor status and hormone receptors (progesterone and oestrogen receptors) were obtained from medical records. The 153 control subjects were chosen randomly from healthy women who underwent regular health check-ups at hospitals and clinics and who had a negative personal or family history of cancer. Women with liver, cardiovascular, kidney or metabolic disease and participants who reported other types of malignancy were excluded. The two groups were matched for age: mean [SD] 48.9 [15.8] in the cases and 50.1 [16.4] in the controls (P = 0.59). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

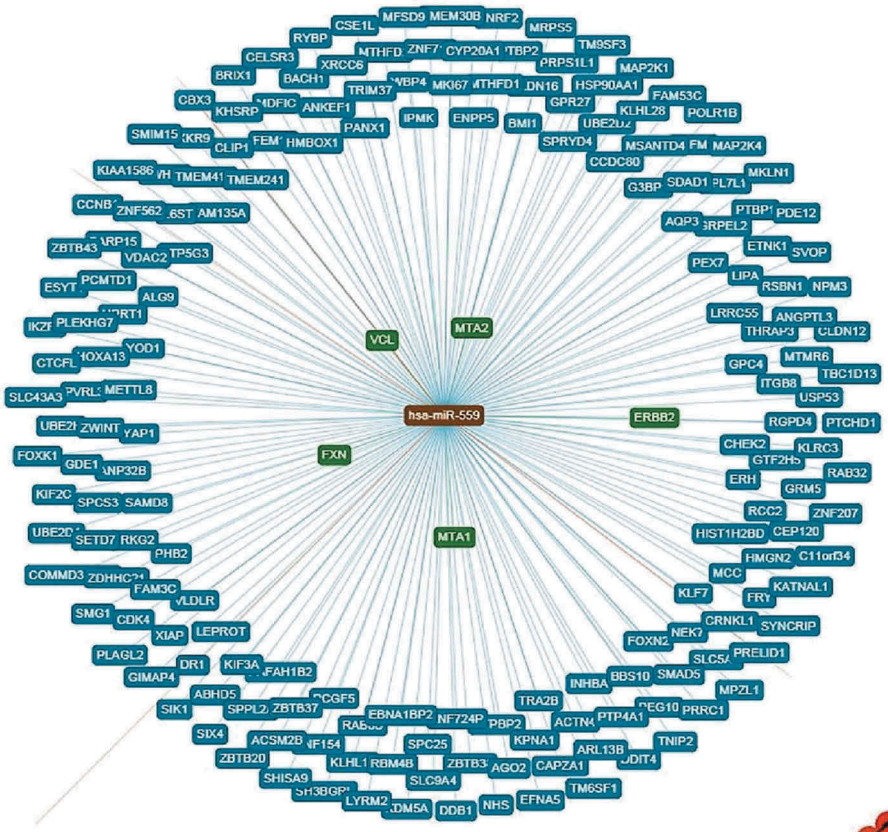
Genomic DNA was extracted from 500µl blood samples using Triton X-100. DNA concentration and purity were evaluated by NanoDrop 1000 spectrophotometry (NanoDrop Technologies; USA). The extracted DNA was stored at −20°C until use. For genotyping *rs58450758*, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used. Amplification of the fragment of this SNP used F-5′ TATTGCTCTCTTCCCCAG 3′ and R-5′ GTTCCGTCACACTATT CA3′ primers. Each reaction mixture comprised 2.5µl of extracted DNA, 5µl of Taq DNA polymerase Master Mix Red (Ampliqon; Denmark), 0.5 µM of each primer (forward and reverse) and 1.5µl of purified water (Kimia Tehran; Iran). After an initial denaturation step (94 °C for 5 min), samples were subjected to 30 rounds of PCR at 94 °C for 30 sec, 59 °C for 40 sec, 72 °C for 40 sec with a final extension time of 5 min for 72 °C followed by a 4 °C hold cycle amplifying a 610bp fragment. The PCR products were analysed by 1% agarose gel electrophoresis in 1X Tris-Borate-EDTA buffer at 100V and stained using RedSafe Nucleic Acid Staining Solution (20,000×; Boca Scientific; USA). PCR products were digested by 2µl of restriction enzyme TaaI (HpyCH4III; #ER1361, ThermoFisher Scientific; USA) for 3h at 65 °C in order to detect allelic variations. After digestion with TaaI, the amplicon was cut into 464 and 146 bp fragments in the presence of the C allele, but the T allele remained undigested (610bp). Digested PCR products were separated on 2% agarose gel, stained using RedSafe and visualized under UV illumination. All assays were blind and conducted by two researchers without knowledge of the control or case status. For quality control, 5% of samples were randomly repeated with 100% concurrence.

MedCalc statistical software (version 14.8.1; Ostend, Belgium) was used for statistical analysis. The statistical significance of differences between patients and control cases was calculated by the Pearson's χ^2 test. The odds ratio (OR) and 95% confidence interval (CI) were also calculated. A value of $p < 0.05$ was considered statistically significant.

Results

The expression of genomic blood *miR-559* in patients with breast cancer were significantly lower than in the healthy controls in the GSE31309 dataset ($p < 0.001$). The associations between miR-559 and its target genes were explored using the 'mirTargetLink' software. Among 189 target genes

a



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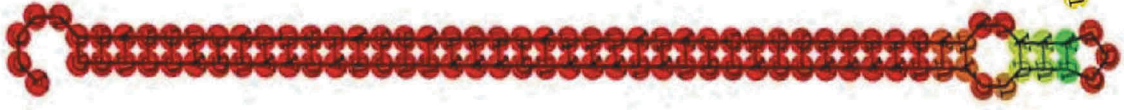

d

Name	Sequence length	Structure pattern	Cleavage position (nt)	Score
hairpin_1	96	CACCAAA AUUACUU GUGGU UUUGAUG--	35	0.5261816
hairpin_1	96	ACCAAAA UUACUUU UGGUU UUGAUG---	36	0.65013327
hairpin_1	96	CCAAAAU UACUUUU GGUUU UGAUG----	37	1.1911605
hairpin_1	96	CAAAAUU ACUUUUG GUUUU GAUG-----	38	1.5727875
hairpin_1	96	AAAUUAC UUUUGGU UUUGA UG-----CA	40	0.70338076

e

Name	Sequence length	Structure pattern	Cleavage position (nt)	Score
hairpin_1	96	GCACCAA AAUUACU CGUGG UUUUGAU--	34	0.053049651
hairpin_1	96	CACCAAA AUUACUU GUGGU UUUGAU--A	35	0.14010504
hairpin_1	96	CAAAAUU ACUUUUG GUUUU GAU--AAA-	38	0.18026722
hairpin_1	96	AAAUUAC UUUUGGU UUUGA U--AAA---	40	1.3169196
hairpin_1	96	AAUUACU UUUGGUA UUGAU --AAA---U	41	0.69321699
hairpin_1	96	AUUACUU UUGGUAA UGAU- -AAA---UU	42	0.85042691
hairpin_1	96	UUACUUU UGGUAAA GAU-- AAA---UUU	43	0.4466708
hairpin_1	96	UACUUUU GGUAAAU AU--A AA---UUU-	44	1.7769434

Figure 2. (a) Network interaction between miR-559 and genes; 5 interactions with strong evidence (green) and 184 interactions with weak evidence (blue) which are associated with the breast cancer incidence. Predicted secondary structure of human mir-559 with either (b) C allele and (c) T allele (n.79C >T), Minimum free energy (MFE) for rs58450758-C and rs58450758-T was calculated −60.19 and −61.73 kcal/mol, respectively. Predicted DICER processing sites in pri-mir-559 with either (d) allele C and (e) allele T.

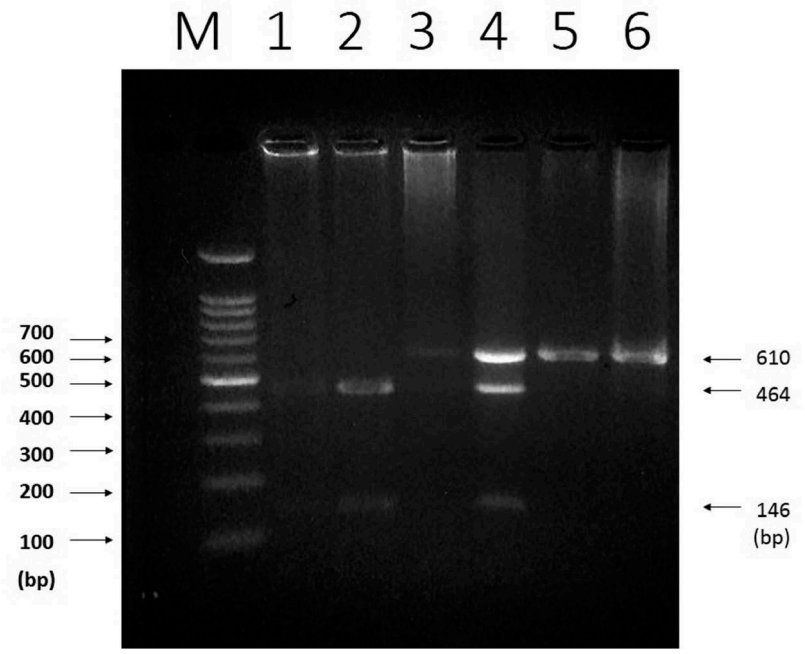

Figure 3. RFLP analysis of the *miR-559 rs58450758* polymorphism. Lanes 1 and 2: two fragments of 464 and 146 bp for CC (homozygous). Lanes 3 and 4: three fragments of 146, 464 and 610 bp for CT (heterozygous). Lanes 5 and 6: undigested PCR products of 610 bp for TT (homozygous). M: molecular size marker.

Table 1. Genetics of miR-559 SNP in cases and controls.

Model	Patients N(%)	Controls N(%)	OR (95%CI)
Codominant genotype			
C/C	87(67.4%)	135(88.2%)	1.00
C/T	12(9.3%)	15(9.8%)	1.24 (0.55–2.77)[a]
T/T	30(23.3%)	3(2.0%)	15.51 (4.59–52.40)[b]
Dominant genotype			
C/C	87(67.4%)	135(88.2%)	1.00
C/T + T/T	42(32.6%)	18(11.8%)	3.62 (1.95–6.69)[b]
Recessive genotype			
C/C + C/T	99(76.7%)	150(98.0%)	1.00
T/T	30(23.2%)	3(2.0%)	15.15 (4.50–50.99)[b]
Over-dominant genotype			
C/C + T/T	117(90.7%)	138(90.2%)	1.00
C/T	12 (9.3%)	15(9.8%)	0.94 (0.42–2.09)[c]
Alleles			
C	198(76.7%)	300(98%)	1.00
T	30(23.3%)	6(2%)	5.23 (3.12–8.83)[b]

[a]p = 0.59, [b]p<0.0001, [c]p = 0.88. OR = odds ratio, CI = confidence interval.

of miR-559, ERBB2, MTA1, MTA2, CCND1 and ULK1 were found to have strong association with breast cancer. Bioinformatics predictions using (ensemble.org) web server confirmed that *rs58450758* is located in the stem-loop structure of miR-559. It also showed that the frequency of this SNP is 0.21. The rna.tbi.univire.ac.at/cgi.bin server was used to predict the effect of this polymorphism on secondary structural changes in miR-559. This webserver represented that this polymorphism changes the secondary structure of miR-559 (Figure 2(a,b)). 'PHD CLEAV' showed that this SNP creates new DICER sites in a sequence of pre-miR599 (Figure 2(c,d)). Altogether, bioinformatics analyses provide supporting evidence that miR-559 polymorphism (rs58450758) could be associated with breast cancer.

We evaluated the genotype data of 153 female controls and 129 female patients with breast cancer (Figure 3). In the cases, 91 (70.5%) had ductal carcinoma, 38 (29.5%) had lobular disease. Tumour size was <5 cm in 41 (31.8%), ≥5 cm in 88 (68.2%) whilst tumour stage was 0-II in 44 (34.1%) and II-IV in 85 (65.9). Lymph nodes were involved in 87 (67.4%) women. HER2 status was positive in 35 (27.1%), negative in 94 (72.9%), oestrogen receptor status was positive in 82 (63.6%) and negative in 47 (36.4%), whilst progesterone receptor status was positive in 92 (71.3%) and negative in 37 (28.75).

The observed genotypes and allele frequencies with their estimated ORs are summarized in Table 1. In a codominant model, the T/T genotype was found more frequently among breast cancer patients than among controls, and in a dominant model, the C/T + T/T

genotypes were associated breast cancer. The homozygous T/T was significantly more common among women with breast cancer. In a recessive model, a significant difference in the T/T genotype frequency was found among patients. The T allele was more frequent in breast cancer.

Discussion

miR-559 (*rs58450758*) SNP was determined in 129 breast cancer patients and 153 population-matched controls. We found a significant association for the *miR-559* rs58450758 TT genotype with an increased risk of breast cancer. Studies on multiple SNPs and the incidence of breast cancer have shown the association between these two phenomena. These findings are similar to the result of this study; however, there have been studies showing no relation between this SNP and breast cancer [24–27].

MiRNAs are known to regulate several genes and down-regulation of their expression has been shown in many cancers, including breast cancer [28–30]. Studies have demonstrated the links between over-expression of *ERBB2* (human epidermal growth factor receptor 2; HER2) and invasion of tumour cells, the development of breast cancer through several intracellular signalling pathways, including the Janus kinase/signal transducer [31–33].

Studies have also demonstrated that structural changes in miRNA caused by a SNP or other mutations alter their designated function and bring about defects in the translation of targeted transcripts [34]. Several studies have reported significant associations of SNPs in miRNAs, including miR-27a, miR-196a2 and miR146a, and the risk or incidence of breast cancer [35,36]. Meshkat et al. suggested that functional SNP in miR-146a would lead to breast cancer survival by affecting the HER2 status [37]. Yang et al. suggested that the decreased expression of miR-559 correlated with tumour size in patients with glioblastoma multiforme [38]. Bioinformatics analysis suggests that miR-559 may play a relevant biological role in the regulation of *HER2* expression by interacting with the 3′-UTR of *HER2* mRNA [39].

The results of the present study also predict the effect of *rs58450758* on miR-559 molecular structure. The miR-559 *rs58450758* is located in the coding region of the pre-miR-559 hairpin in the stem-loop structure. This variant is located in miR-559 3p site, but since the miR-559 3p strand is unstable, this strand acts as the passenger strand after the DICER cut-off activity and then degrades. *In silico* analysis indicates that *rs58450758* changes the secondary structure of miR-559 and, in the secondary structure, creates new cleavage sites for DICER. As shown in Figure 2d, one of the new cleavage positions is in the 34th nucleotide of the pre-miRNA. If DICER cleaves the pre-miRNA in this position, the produced mature miRNA consists of 19 nucleotides and the miR-559 is made up of 21 nucleotides. The truncated miR-559 likely has less stability and, therefore, a shorter half-life; thus, probably for people with this mutation, miR-559 probably does not function properly. 'GEO' analysis showed that *miR-559* is down-regulated in breast cancer patients. Based on our bioinformatics analysis, *miR-559* down-regulation could increase the risk of breast cancer.

Some limitations need to be considered when the results are analysed. Firstly, this study was restricted to a single racial/ethnic group, and we acknowledge that there may be differences in this SNP in the breast cancer characteristics of different populations which should be further investigated. Secondly, this study included 129 breast cancer patients and 153 control subjects, and so an expanded study using a larger patient and control population is required. Finally, just one potentially functional SNP of miRNA was researched, which did not cover all variants. To verify the effect of *rs58450758* SNP on the secondary structure of miR-559, further studies are needed.

This work represents an advance in biomedical science because it shows a link between the incidence of breast cancer and the *miR-559 (rs58450758)* SNP.

Summary Table

What is known about this subject:

- MiR-559 interacts with a target sequence in the 3′-UTR of *ERBB2* and plays a major role in carcinogenesis.
- There is an association between alteration in miRNA expression, and breast cancer prevalence.
- Structural changes of miRNAs in response to structural miR-SNPs could result in abnormalities in their function.

What this paper adds:

- Bioinformatics analysis showed that *rs58450758* polymorphism changes the secondary structure of miR-559
- 'PHD CLEAV' web server showed that this SNP creates new DICER sites in a sequence of pre-miR599.
- C/T + T/T genotypes of the variant *rs58450758*was significantly associated with breast cancer.

Disclosure statement

No potential conflict of interest was reported by the authors.

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