Arch Biol Sci. 2021;73(4):457-463 https://doi.org/10.2298/ABS210808039N

# rs2682818/MiR-618 is a novel marker associated with increased risk of breast cancer in the Iranian population

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**Received**: August 8, 2021; **Revised**: August 17, 2021; **Accepted**: August 19, 2021; **Published online**: October 8, 2021

**Abstract:** The presence of single nucleotide variations in the coding region of micro-RNA (miRNA)-encoding genes plays a significant role in the expression and function of these molecules in oncogenesis and cancer. The association of rs2682818 in miR-618 with increased risk of breast cancer was investigated in the Iranian population. rs2682818/miR-618 was genotyped using amplification-refractory mutation system PCR (ARMS-PCR) in 200 healthy individuals and patients with breast cancer. The data revealed the presence of Hardy-Weinberg equilibrium (HWE) for this marker. The frequency of alleles C and A was 70% and 30%, respectively, in healthy individuals; the frequency of alleles C and A was 44% and 56%, respectively, in patients with breast cancer. Analysis of odd ratios showed that the rs2682818/miR-618 polymorphism is associated with increased probability of breast cancer and is statistically significant (OR=2.97, P=0.0003). The data suggest that rs2682818/miR-618 could be considered a novel marker of increased risk of breast cancer.

**Keywords:** breast cancer; miR-618, polymorphism; ARMS-PCR; Iranian population

## INTRODUCTION

Breast cancer (BC) is the most common invasive malignancy in women worldwide and is the second leading cause of cancer death after lung cancer [1]. According to the World Health Organization (WHO), breast cancer kills 609000 people worldwide each year. The total number of breast cancer patients in Iran is 41000, increasing by more than 7000 patients annually. Based on global health statistics, one in every 8 to 10 women develops breast cancer. According to Iranian statistics, out of every 10 to 15 women, one woman is at risk of developing breast cancer, but the age of breast cancer onset in Iranian women is at least a decade earlier than in women in developed countries [2]. Between 2005 and 2014, the incidence of breast cancer showed an increase in the Asian continent by 1.7% each year [3].

Many factors play roles in the development of breast cancer, including genetics and the environment. The risk of cancer could increase by about 35% because of genetic factors. Mutations in genes such as breast cancer type 1 and 2 (BRCA1, BRCA2, respectively) and ataxia-telangiectasia mutated (ATM), have been directly associated with the risk of breast cancer [4].

miRNAs have been considered as one of the diagnostic markers for early detection of breast cancer [5,6]. Because of the importance of miRNAs in gene expression regulation, they have a significant potential for use as biomarkers for cancer detection, diagnosis, classification and treatment [7-10]. miRNAs are small, single-stranded non-coding RNAs that are about 17-22 nucleotides long, which regulate gene expression post-transcriptionally [11]. These molecules bind to complementary sequences that are often located in the 3′UTR region of the target mRNA; studies suggest that these complementary sequences exist in the 5′UTR coding region and even in the promoter [12,13]. miRNAs have an ectopic expression in cancers depending on the gene or regulatory pathways, possessing tumor suppressor or oncogenic effects [14,15]. Increasing evidence suggests that miRNA is involved in complex and diverse processes, including

the cell cycle, apoptosis, cell invasion and migration [12,16,17]. Dysregulation of miRNAs in cancer can occur genetically and epigenetically. Genetic changes such as single-nucleotide polymorphism (SNP), and epigenetic alterations such as inappropriate DNA methylation and histone modifications have been reported to play an essential role in cancer pathology [18,19].

SNPs are the most common variation in the human genome [20,21]. Polymorphisms in miRNAs are in both mature miRNA and pre-miRNA sequences [22,23]. SNPs in miRNA genes could change their regulatory roles and affect miRNA production, alter target affinity and specificity, and miRNA maturation [24]. Studies have shown the association between miRNA polymorphism and cancer risk, and SNPs in miRNAs have been introduced as genetic markers to predict the risk of breast cancer [25, 26]. Several case-control and meta-analyses have linked the association between miRNA gene polymorphisms and breast cancer risk in European [27-29], Asian [30, 31], Arab [32] and Jewish [33] populations. Allele frequency and heterozygosity of polymorphic markers are usually different in different populations [34,35]. Therefore, to determine the suitability of an SNP marker for screening a genome in a population, it is necessary to examine the status of that marker in terms of heterozygosity and allelic diversity [36]. This study aimed to investigate the association of miR-618 C>A (rs2682818) polymorphism with the risk of breast cancer in the Iranian population. This miRNA is located at chromosomal location 12q21.31, suppressing cell proliferation and inducing cell cycle arrest [37]; miR-618 also inhibits the PI3K/AKT signaling pathway, which regulates epithelial-mesenchymal transition (EMT), the cell cycle, angiogenesis and apoptosis [38]. This miRNA participates in different cancers by targeting forkhead box protein P2 (FOXP2) and inhibiting transforming growth factor beta (TGF-β) that inhibits prostate cancer migration, and consequently invasion of prostate cancer [39-41]. miR-618 is also used as a biomarker for lymphogenesis, male breast cancer, neck, head carcinoma and hepatocellular carcinoma [39,42]. A study by Morales et al. in the South American population showed that miR-618 rs2682818 increases the risk of breast cancer [37]. This miRNA can also be used as a cancer biomarker because SNP rs2682818 is a part of the pre-arranged stem-loop miR-618 sequence that can affect the production of this miRNA at different stages. This SNP can alter the stem-loop secondary structure, which can lead to mature miRNA production [37,43]. It was recently reported that rs2682818 is a potential risk biomarker for follicular lymphoma [43,44]; laboratory analysis indicated that the allele A variant in follicular lymphoma reduced mature miRNA production [37,43]. Recent studies have shown an association between miR-618 and various cancers. On the other hand, SNPs in genes encoding miRNAs can affect their expression and function and have different frequencies in diverse populations. Increasingly, studies have shown that SNP rs2682818 is linked to the risk of a range of cancers, including colorectal cancer [45], acute lymphocytic leukemia [46], follicular lymphoma [43] and breast cancer. The association between breast cancer and SNP rs2682818, however, is still controversial.

This study investigates for the first time the association of SNP/rs2682818 in miR-618 with breast cancer in the Iranian population.

## MATERIALS AND METHODS

**Ethics approval:** The research was performed according to the institutional review board (IRB) for research and ethics approval of the University of Isfahan. Written informed consent was obtained from all participants before study participation.

### Bioinformatics studies for SNP selection

The bioinformatics databases dbSNP (http://www.ncbi.nlm.nih.gov/SNP), UCSC Genome Browser (https://genome.ucsc.edu/) and SNPper (http://snpper.chip.org/bio/snpper-enter) were accessed to scan and analyze for markers located in the gene region of miR-618, and the rs2682818 single nucleotide polymorphic marker was selected.

### DNA samples

Blood samples from 100 healthy individuals and 100 patients with breast cancer were collected and used for genomic DNA extraction. Genomic DNAs were extracted from peripheral blood leukocytes using the salting-out method and were stored at -20˚C [48]

### Genotyping

The amplification-refractory mutation system PCR (ARMS PCR) was used as the standard method for determining allele and genotype frequencies of the markers [49]. ARMS PCR is a fast, simple, low-cost and high-performance method for studying allele and genotype frequencies of SNPs. In ARMS PCR, four primers were used for each DNA sample. The large PCR fragment contained the single nucleotide polymorphism and validated the control band, and the two smaller fragments validated each of the two allele-specific products. Two inner primers produced the allele-specific fragments, and specificity was created through a mismatch at the 3' end of the primers corresponding to the site of the SNP. Mismatches were intentionally added at position 3 from the 3' end of the primers to enhance their specificity [50] (Supplementary Fig. S1).

Primer 1 server (LAMP web server at http://primer1.soton.ac.uk/primer1.html) was used to design primers for the ARMS-PCR reaction to detect the selected polymorphism. For all primers, optimization with gene runner software (http://www.generunner.net/, version 6.5.52) was performed, and primer specificity was confirmed using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/, National Center for Biotechnology Information, USA). The optimization condition of PCR for amplification of rs2682818 was as follows: 20 μL total volume containing 50 ng DNA, 1 μL of 10 pmol primers for the forward outer primer, reverse outer primer and reverse inner primers and 10 μL of Master Mix (2 mM $MgCl_2$). Initial denaturation was carried out at 94°C for 5 min, followed by 35 cycles including 1 min denaturation at 94°C; annealing temperature 59°C for 1 min, extension at 72°C for 1 min, followed by a 5 min final extension at 72°C. The sequence of primers is presented in Supplementary Table S1. After the PCR reactions, the products were run on a 2% agarose gel. The bands were visualized using Biometra Gel documentation. Three distinct patterns were detectable on the agarose gels based on the genotypes.

### Statistical analysis

Genotype frequency, allelic frequency and the Hardy-Weinberg equilibrium (HWE) were calculated using PLINK software (http://zzz.bwh.harvard.edu/plink/, ver. 1.07). PICcalc web software (http://w3.georgikon.hu/pic/english/default.aspx) was used to calculate heterozygosity. Genotype and allelic frequencies were calculated based on the number of alleles observed in the population. Then genotype frequency of the polymorphism for investigation of the HWE analysis by chi-square analysis ($P>0.05$) was used. The association of each marker with healthy and unhealthy populations was performed by chi-square analysis with a threshold at $P<0.05$.

## RESULTS

The sequences and structure of the miR-618 gene were analyzed using dbSNP, UCSC Genome Browse, and SNPper, and the SNPs in the miR-618 gene were identified and analyzed using the databases. Based on the characteristics of the markers, including their location and the possible effect on the expression and function of miR-618, the rs2682818 marker was selected. ARMS PCR determined the allelic and genotype frequency of marker rs2682818. In the agarose gel (Fig. 1), each column represents a genotype, the larger band

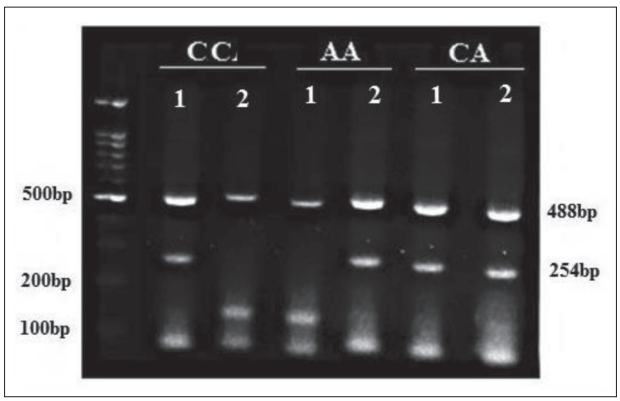


**Fig. 1.** Genotyping of rs2682818 SNP of miR-618 in the Iranian population. Agarose gel electrophoresis (2%) was used to detect the banding pattern of ARMS PCR for selected markers. The genotype of each individual is shown in a separate column. The large fragment containing single nucleotide polymorphism indicates the control band, the two smaller fragments represent each of the two allele-specific products. Wells 1 – PCR product with a primer C-specific allele; wells 2 – PCR product with the primer A-specific allele. Genotype CC – PCR product band in well 1 due to the presence of allele C; well 2 – no product due to the absence of allele A. Genotype AA: well 1 – no band of the PCR product due to the absence of the C allele; well 2 – specific band due to the application of the specific primer A and the presence of allele A. Heterozygous AC genotype: due to the presence of both A and C alleles in the samples, bands of C and A alleles are visible in wells 1 and 2, respectively.

**Table 1.** Genotype frequency and statistical analysis of rs2682818 SNP markers in the Iranian population according to genotyping via ARMS-PCR and gel electrophoresis.

| Genotype | Control (%) | Case (%) | OR | Allele | Control (%) | Case (%) | P | OR |
|---|---|---|---|---|---|---|---|---|
| CC | 54 | 19 | | C | 70 | 44 | | |
| AC | 37 | 50 | 1.70 | A | 30 | 56 | P= 0.0003 | OR= 2.97 |
| AA | 9 | 31 | 4.54 | | | | | |

(488 bp) contains the SNP indicating the control band and the smaller band (254 bp) represents the specific C and A alleles. Based on genotyping data, among 100 women with breast cancer, 50 of them showed AC, 31 AA and 19 CC genotypes (Table 1). The frequency of alleles A and C was calculated in control and patient subjects. The C allele, known as the dominant allele, had a frequency of 70% in the control population and 44% in the patient population. The frequency of allele A was 30% in the control population and 56% in the patient population (Table 1).

Statistical analysis using PLINK software was performed to investigate the correlation of the SNP genotypes with breast cancer. Both patient and control groups were in accordance with HWE (P=0.22 and P=0.14, respectively). The results showed an association between miR-618rs2682818 and breast cancer (P=0.0003). Among the genotypes of the miR-618rs2682818, the frequency of A allele was significantly increased in breast cancer patients in comparison with the controls (P<0.05): the odds ratio was 2.97 (Table 1). According to these results, we suggest that miR-618rs2682818 is associated with breast cancer in the Iranian population.

## DISCUSSION

miRNAs are thought to control at least half of all human genes, including tumor suppressor genes like BRCA1, BRCA2, p53 and phosphatase and tensin homolog (PTEN). Because miRNA targets a number of functionally essential protein-encoding genes, genetic differences in miRNA genes might constitute a novel cancer-predisposition pathway [51,52]. The underpinnings for this investigation were an increasing interest in examining the relationship between the miR-618 SNP and the development of breast cancer. The aim of this study was to see if there was a link between SNPs in the miRNA-618 gene and breast cancer risk in the Iranian population.

The relationship of an SNP in miR-618 with different malignancies, including susceptibility to colorectal cancer [47] and breast cancer [53], has been studied. Polymorphism rs2682818 at miR-618 has been one of the crucial SNPs in case-control studies in different populations based on its association with various cancers [53]. In the present study, we investigated the association and correlation of breast cancer risk with rs2682818 at miR-618 in healthy and unhealthy individuals in the Iranian population. This study showed that the rs2682818 marker had adequate heterozygosity and allele frequency for examining its relationship with breast cancer, and that this marker can be used as a novel breast cancer risk marker.

miR-618 has previously been linked to malignancies such as lymphadenoma, Barrett's esophageal cancer, hepatocellular tumors and breast cancer, suggesting that it might be used as a cancer biomarker or a therapeutic target. Moreover, miR-618 in thyroid cancer targeted XIAP, a protein that inhibits apoptosis, preventing cancer cell proliferation and invasion [54]. These studies confirmed the dual oncogenic and tumor suppressor roles of miR-618 [55].

Because miR-618 controls lymphomagenic pathways, SNP rs2682818, which is found in the hairpin-loop structure of the miR-618 precursor, has previously been shown to be a risk biomarker and therapeutic target in follicular lymphoma. However, the role of miR-618 in breast cancer susceptibility has been controversial. In a case control study published in 2012, no link was found between rs2682818 and the incidence of breast cancer in a Chinese population [30]. In contrast, the study conducted by Morales et al. in 2016 on the South American population revealed the association of rs2682818 miR-618 with increased risk of breast cancer [56]. According to a study in the Chinese population, it was reported that allelic variant A in rs2682818 reduces the production of mature miRNA [31]. The presence of SNPs leads to alteration of the affinity and specificity of target binding and

changes in miRNA maturation [26,57]. Our study of the frequency of alleles and different genotypes of rs2682818 polymorphism in miR-618 in control and patient groups is the first to show that rs2682818 in miR-618 polymorphisms is linked to an elevated risk of BC in an Iranian community. Based on our results, there was a significant association between breast cancer susceptibility and rs2682818 polymorphism in miRNA-618. However, there were some limitations in the current study, for example, the sample size was relatively small, which potentially affects the result. However, as the correlation between polymorphism A and the incidence of breast cancer in the Iranian population was significant, it should be examined further in studies with a larger sample size and in different populations.

**Funding:** This study was supported by the Department of Research at the University of Isfahan through an internal postgraduate research grant (Pajoohaneh).

**Availability of data and material:** The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

**Author contributions:** SV designed the research. AN and NE were involved in the setup of the experimental methods, analysis of data and writing of the manuscript. SV supervised the experimental work and AN performed the experimental work. All authors read and approved the final manuscript.

**Conflicts of interest disclosure:** The authors declare that there is no conflict of interest.

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## Supplementary Material

The Supplementary Material is available at: http://www.serbiosoc.org.rs/NewUploads/Uploads/Najafian-Najafabady%20et%20al_6896_Supplementary%20Material.pdf