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Effects of miR-27a, miR-196a2 and miR-146a polymorphisms on the risk of breast cancer

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ABSTRACT

Background: microRNAs (miRNAs) are potentially involved in many physiopathological processes, including regulation of cell growth, differentiation, apoptosis and cancer. Single nucleotide polymorphisms of the genes encoding miRNAs can alter their expression and may influence cancer risks. This case-control study explored the relationship between three microRNA polymorphisms (*miR-27a*, *miR-196a2* and *miR -146a)* and breast cancer (BC).

Methods: A total of 353 breast cancer cases and 353 controls were genotyped for *miR-27a* (rs895819), *miR-196a2* (rs11614913) and *miR -146a* (rs2910164). The miR-27a and miR-146a variants were discriminated using a PCR–restriction fragment length polymorphism method, while miR-196a2 were analysed by tetra-primers amplification refractory mutation system PCR. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were used to estimate associations.

Results: The CC homozygous genotype of *miR-146a* (rs2910164) was seen in 45(12.7%) patients with breast cancer and 18(5.1%) controls (OR 4.09 [95%CI 2.19–7.67] $p < 0.001$). The minor allele G of *miR-27a* was associated with a decreased risk of breast cancer (OR 0.24 [95% CI 0.14–0.42] $p < 0.001$). The *miR-196a2* (rs11614913) was not related to breast cancer ($p > 0.05$).

Conclusion: Our data indicate that *miR-146a* (rs2910164) and *miR-27a* (rs895819) variants contribute to breast cancer. Further studies in larger populations including other genetic and environmental factors are required to achieve a definitive conclusion.

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miRNA; microRNA; genetic polymorphism; breast cancer

Introduction

Breast cancer (BC) is the leading cancer in women both in the developed and the developing world, and has a multi-factorial and multistep etiology that involves complex interplay among exogenous environmental and endogenous genetic factors. The known risk factors are lower age of menarche, late age of first pregnancy, fewer pregnancies, shorter or no periods of breastfeeding, later menopause, obesity, alcohol consumption, inactivity, and hormone replacement therapy (HRT) [1]. The incidence of breast cancer is increasing in the developing world due to increase life expectancy, increase urbanization and adoption of western lifestyles [2]. In Iran, breast cancer has become the most common primary female cancer, with an estimated prevalence rate of 23.65 per 100,000 [3]. It seems that in Iran, like in other middle-income countries, breast cancer appears in women at least one decade younger than their counterparts in high-income countries [4].

MicroRNAs (miRNAs) are a 21–25 long class of small non-protein coding RNA that function as gene regulators by inhibiting the degradation of their target mRNAs and inhibiting translation. As each miRNA has hundreds or thousands of targets, a broad segment of the protein coding genome is under their control [5]. Approximately half of human microRNAs are found in intergenic regions, 40% located within intronic regions of genes, and the final 8% are exonic [6]. miRNAs might serve as onco- and/or tumour suppressors in cancer [7]. Amplification or over-expression of oncogenic miRNAs can downregulate tumour suppressor proteins. *miR-27a*, that promotes angiogenesis by mediating endothelial differentiation of breast cancer stem like cells, has been shown to be related with poor overall survival of breast cancer patients and may be a valuable marker for tumour progression [8]. miR-196a2 is over-expressed in head-neck, oral and cervical cancers [9–11]. miR-196a2 suppresses metastasis in skin and breast cancers [12,13]. miR-146a is important in cancer metastasis in various types of tumours, including breast cancer [14,15], and have been reported to be implicated in breast cancer initiation, progression and/or metastasis [16].

miR-SNPs (single nucleotide polymorphisms) could affect the transcription of the target genes, change the

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processing of pri-miRNA or pre-miRNA and affect interaction between miRNA and mRNA [17]. Furthermore, miR-SNPs also represent novel molecular biomarkers. Genetic polymorphisms in miRNAs are associated with many tumours, including gastric [18], hepatocellular [19], and breast cancer [20]. Thus, given the critical role played by miRNAs such as miR-27a, miR-196a2 and miR-146a in cancer biology and their dysregulation in breast cancer, we hypothesized that three putatively functional SNPs (rs895819 A > G in *miR-27a*; rs11614913 C > T in *miR-196a2* and rs2910164 G > C in *miR-146a*) ultimately influence an individual's genetic susceptibility to breast cancer.

Materials and methods

Three hundred and fifty-three breast cancer cases were recruited from the Razi hospital of Guilan Medical University, Rasht, between January 2012 and September 2016. They were all newly diagnosed, histologically confirmed breast cancer patients between the ages of 29 and 72 without a prior history of cancer or previous chemo- or radiotherapy, who were alive at the time of the interview. Menopausal status was defied as the date of last menses followed by 12 months without menses. The clinico-pathological variables and prognostic factors including tumour size, histology, lymph node involvement, hormone receptor (including oestrogen receptor [ER] and progesterone receptor [PR]) status and human epidermal growth factor receptor (HER2) status, were taken from medical records. Histological determination, including tumour type and disease stage, was performed according to the WHO criteria and the 7th Edition of the American Joint Committee on Cancer (AJCC), respectively. Three hundred and fifty-three control subjects were randomly selected from healthy women who got a routine health check-up and normal mammography. All cases and control subjects were genetically unrelated and control subjects had no personal or family history of cancer. Women suffering from liver, metabolic, cardiovascular, kidney disease or those reporting other types of malignancy were excluded. All participants signed informed written consent prior to interview, and the protocol for this study was approved by the Ethical and Protocol Review Committee of the Guilan University of Medical Sciences, Rasht (IR.GUMS.REC.1396.25). This research was conducted in agreement with the principles of the Declaration of Helsinki.

Based on previous reports about miRNA polymorphisms and cancer risk [21,22], we chose three potentially functional SNPs (rs895819 A > G in *miR-27a*, rs11614913 C > T in *miR-196a2* and rs2910164 G > C in *miR-146a*) for genotyping. The *miR-27a*, *miR-196a2* and *miR-146a* genes are located at 19p13.12, 12q13.13 and 5q33.3, respectively. The rs895819, rs11614913 and rs2910164 have a Global minor allele frequency (MAF) greater than 10% (0.36, 0.33 and 0.27, respectively).

Genomic DNA was isolated (AccuPrep, Bioneer, Korea) from 200 µl of whole blood. Oligonucleotide primers used for amplification of the target sequences of *miR-27a*, *miR-146a* and *miR-196a2* were designed by means of Oligo primer analysis software (version 7.54, Molecular Biology Insights Inc., Cascade, CO, USA). The primers sequences used for *miR-27a* were F1 (5′-GAACTTAGCCACTGTGAACACCACTTGG-3′) and R1 (5′-TTGCTTCCTGTCACAAATCACATTG-3′) and for *miR-146a* polymorphic site were F2 (5′-CATGGGTTGTGTCAGTGTCAGAGCT-3′) and R2 (5′-TGCCTTCTGTCTCCAGTCTTCCAA-3′). Following an initial denaturation step (5 min at 95 °C), samples were subjected to 30 rounds of PCR at 95 °C for 30s, 60 °C (*miR-27a*) or 62 °C (*miR-146a*) for 40s, and 72 °C for 45 s with a final extension time of 2 min at 72 °C. The PCR amplicons generated for *miR-27a* (182 bp) and *miR-146a* (147 bp) were subjected to restriction digestion. *DraIII* and *SacI* restriction enzymes (New England Biolabs, UK) were used to detect polymorphisms in the *miR-27a* and *miR-146a*, respectively. A gain of *DraIII* restriction site in the polymorphic allele resulted in 155 and 27-bp products for homozygous major type (AA), 182, 155, 27 bp for heterozygous (AG) and 182 for homozygous minor (GG). For the *miR-146a*, the C allele was cut into two fragments of 122 and 25-bp, while the G allele remained uncut (147-bp).

Polymorphism-spanning fragments of *miR-196a2* were analysed by tetra-primers amplification refractory mutation system. The primers were as follow: F3 (5′-CAGTCAGACCCCTTACCCA-3′) and R3 (5′-AAAACCGACTGATGTAACTCTGG-3′) for C allele; F4 (5′- AACTCGGCAACAAGAAACGGT-3′) and R4 (5′-TTGTTCTGCAACCCCACTCA-3′) for T allele. Two amplification reactions were necessary for each one of the subjects analysed one with the C allele primers and another with the T allele primers. PCR cycling conditions were an initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 45 s; and a final extension at 72 °C for 2 min. At least 10% of samples were randomly selected for repeat analysis, yielding 100% concordance.

Table 1. The demographic characteristics of controls and patients with breast cancer.

	Cases	Controls	
Variables	*n* (%)	*n* (%)	*P*-value
Age	51.8 ± 8.2	51.0 ± 10.2	0.52
Age of menarche	12.9 ± 1.2	13.0 ± 0.9	0.08
Age of pregnancy	24.3 ± 4.6	23.8 ± 3.6	0.45
BMI	27.9 ± 17.5	26.7 ± 3.9	0.67
OCP history			
Yes	131 (31.10)	79 (22.40)	<0.001
No	222 (62.90)	274 (77.6)	
Family history of breast cancer			
Positive	11 (3.10)	0 (0)	0.001
Negative	342 (96.90)	353 (100)	
Menopausal status			
Premenopausal	132 (37.40)	136 (38.5)	0.81
Postmenopausal	221 (62.60)	217 (61.50)	
Breast-feeding history			
Positive	270 (76.50)	306 (86.70)	0.001
Negative	83 (23.50)	47 (13.30)	

BMI = Body mass index, OCP = Oral Contraceptive Pill. Data are mean(SD) or n.

Table 2. The association of miRNAs genotypes and breast cancer susceptibility.

Gene (Accession number)	Genotype	Cases *n* (%)	Controls *n* (%)	OR[a] (95%CI, *P*-value)	AOR[b] (95%CI, *P*-value)
miR-27a (rs895819)	AA	167 (47.30)	127 (35.97)	Ref	Ref
	AG	156 (44.19)	155 (43.90)	0.76 (0.55–1.05, $p = 0.10$)	0.64 (0.46–0.91, $p = 0.013$)
	GG	30 (8.49)	71 (20.11)	0.32 (0.20–0.52, $p < 0.001$)	0.24 (0.14–0.42, $p < 0.001$)
miR-196a2 (rs11614913)	CC	142 (40.22)	149 (42.20)	Ref	Ref
	CT	169 (47.87)	158 (44.75)	1.12 (0.81–1.53, $p = 0.47$)	1.10 (0.78–1.55, $p = 0.57$)
	TT	42 (11.89)	46 (13.03)	0.98 (0.59–1.54, $p = 0.86$)	1.10 (0.66–1.83, $p = 0.71$)
miR-146a (rs2910164)	GG	130 (36.82)	190 (53.82)	Ref	Ref
	GC	178 (50.42)	145 (41.07)	1.79 (1.31–2.45, $p < 0.001$)	1.85 (1.32–2.59, $p < 0.001$)
	CC	45 (12.74)	18 (5.09)	3.65 (2.02–6.59, $p < 0.001$)	4.09 (2.19–7.67, $p < 0.001$)

[a]Crude. [b]Adjusted odds ratio for age of menarche, age of pregnancy, menopausal status, family history of breast cancer, breastfeeding history, oral contraceptive pills use and BMI.

Table 3a. Relationship of clinic-pathologic status and miRNAs genotypes in breast cancer patients.

	miR-27a (rs895819)		
	AA	AG	GG
Variables	*n* = 167	*n* = 156	*n* = 30
Tumor size (cm)			
≥5/<5	87/80	131/25	21/9
OR(95%CI)	Ref	4.81(2.85–8.14)	1.99(0.86–4.60)
P-value		<0.0001	0.1
Tumor site			
Ductal/Lobular	136/31	122/34	22/8
OR(95%CI)	Ref	0.81(0.47–1.41)	0.62(0.25–1.53)
P-value		0.46	0.3
Nodal status			
Positive/Negative	130/64	121/35	14/16
OR(95%CI)	Ref	1.70(1.05–2.75)	0.17(0.07–0.40)
P-value		0.03	<0.001
Stage			
III-IV/0-II	87/80	122/34	21/9
OR(95%CI)	Ref	3.29(2.02–5.36)	2.14(0.92–4.95)
P-value		<0.0001	0.07
Estrogen receptor			
Positive/Negative	96/71	102/54	17/13
OR(95%CI)	Ref	1.39(0.89–2.19)	0.96(0.44–2.11)
P-value		0.14	0.93
Progesterone receptor			
Positive/Negative	114/53	113/43	20/10
OR(95%CI)	Ref	1.22(0.75–1.97)	0.92(0.40–2.12)
P-value		0.41	0.86
HER2 status			
Positive/Negative	44/123	38/118	24-Jun
OR(95%CI)	Ref	0.90(0.54–1.48)	0.69(0.26–1.82)
P-value		0.68	0.46

Table 3b. Relationship of clinic-pathologic status and miRNAs genotypes in breast cancer patients.

	miR-196a2 (rs11614913)		
	CC	CT	TT
Variables	*n* = 142	*n* = 169	*n* = 42
Tumor size (cm)			
≥5/<5	115/27	106/63	19/23
OR(95%CI)	Ref	0.39(0.23–0.66)	0.19(0.09–0.40)
P-value		0.0005	<0.0001
Tumor site			
Ductal/Lobular	113/29	135/34	32/10
OR(95%CI)	Ref	1.01(0.58–1.77)	0.82(0.36–1.86)
P-value		0.94	0.63
Nodal status			
Positive/Negative	113/29	113/56	23/19
OR(95%CI)	Ref	0.51(0.30–0.86)	0.31(0.14–0.64)
P-value		0.01	0.001
Stage			
III-IV/0-II	109/33	102/67	19/23
OR(95%CI)	Ref	0.46(0.28–0.75)	0.25(0.12–0.51)
P-value		0.002	0.0002
Estrogen receptor			
Positive/Negative	87/55	100/69	28/14
OR(95%CI)	Ref	0.91(0.58–1.44)	1.26(0.61–2.61)
P-value		0.7	0.52
Progesterone receptor			
Positive/Negative	107/35	108/61	32/10
OR(95%CI)	Ref	0.57(0.35–0.94)	1.04(0.46–2.39)
P-value		0.3	0.91
HER2 status			
Positive/Negative	33/109	43/126	30-Dec
OR(95%CI)	Ref	1.12(0.66–1.89)	1.32(0.60–2.86)
P-value		0.65	0.48

The OpenEpi software (www.OpenEpi.com) based on minor allele frequency of SNPs was used to calculate a required sample size. Our study had 80% power to detect the associations of miRNAs SNPs and breast cancer. We used Hardy Weinberg equilibrium to evaluate deviation between observed and expected frequencies. The differences in allele frequencies between case and control subjects were tested using the likelihood ratio χ^2 tests for 2×2 tables (two alleles, case vs. control subjects). Logistic regression models were used to estimate odds ratios (OR) and 95% confidence intervals (CI) for breast cancer associated with SNPs, adjusting for age of menarche, age of pregnancy, menopausal status, family history of breast cancer, breastfeeding history, oral contraceptive pill history and body mass index (BMI). For data analysis, Statistical Package for the Social Sciences (SPSS) version 20.0 (Chicago, IL, USA) was used. *P*-value < 0.05 was considered statistically significant.

Results

The demographic characteristics of the patients and controls enrolled in this study are given in Table 1. No statistically significant differences in age, age of menarche, age

Table 3c. Relationship of clinic-pathologic status and miRNAs genotypes in breast cancer patients.

	miR-146a (rs2910164)		
	GG	GC	CC
Variables	*n* = 130	*n* = 178	*n* = 45
Tumor size (cm)			
≥5/<5	53/77	152/26	35/10
OR(95%CI)	Ref	8.49(4.93–14.62)	5.08(2.31–11.14)
P-value		<0.001	<0.001
Tumor site			
Ductal/Lobular	103/27	141/37	36/9
OR(95%CI)	Ref	0.99(0.57–1.74)	1.04(0.45–2.43)
P-value		0.99	0.91
Nodal status			
Positive/Negative	66/64	149/29	34/11
OR(95%CI)	Ref	4.98(2.94–8.42)	2.99(1.39–6.42)
P-value		<0.0001	0.004
Stage			
III-IV/0-II	52/78	145/33	33/12
OR(95%CI)	Ref	6.59(3.09–11.03)	4.12(1.95–8.71)
P-value		<0.0001	0.0002
Estrogen receptor			
Positive/Negative	79/51	108/70	28/17
OR(95%CI)	Ref	0.99(0.62–1.58)	1.06(0.52–2.13)
P-value		0.98	0.8
Progesterone receptor			
Positive/Negative	96/34	124/54	27/18
OR(95%CI)	Ref	0.81(0.49–1.34)	0.53(0.26–1.08)
P-value		0.42	0.08
HER2 status			
Positive/Negative	33/97	40/138	15/30
OR(95%CI)	Ref	0.85(0.50–1.44)	1.46(0.70–3.06)
P-value		0.55	0.3

of pregnancy, BMI and menopausal status were identified between breast cancer cases and controls ($p > 0.05$).

The genotype distributions of the three polymorphisms between the cases and controls are displayed in Table 2. The observed genotype frequencies for all polymorphisms were in Hardy-Weinberg equilibrium ($p = 0.06$ for rs895819, $p = 0.68$ for rs11614913 and $p = 0.18$ for rs2910164).

The frequencies of *miR-27a* homozygous major (AA), heterozygous (AG) and homozygous minor (GG) genotypes in healthy controls were 35.97, 43.90 and 20.11%, whereas the same were 47.30, 44.19 and 8.49% in breast cancer patients, respectively. The frequency of alleles between healthy individuals were A: 58%, G: 42% and A: 68%, G: 32% among cases ($\chi^2 = 18.59$, $p < 0.001$). The minor allele G was associated with a decreased risk of breast cancer (OR 0.61 [95% CI 0.49–0.77] $p < 0.001$).

The overall allele frequencies and genotype distribution of the *miR-196a2* polymorphism in breast cancer patients were similar to those in the controls. The frequency of C allele was more common (0.64 in both groups). There was no significant association between *miR-196a2* (rs11614913) and breast cancer risk ($p > 0.05$).

In the case of *miR-146a*, the rate of allele C was 38% (268/706) in breast cancer group, being significantly higher than that in the control group (26% [181/706]) (OR 1.77 [95% CI: 1.41–2.22] $p < 0.001$).

The distribution of clinical features and miRNAs SNPs in breast cancer patients was determined to clarify the role of miRNAs SNPs in the clinic-pathologic status (Table 3a–c). Patients carrying *miR-27a* AG, *miR-146a* GC and *miR-146a* CC genotypes had a significantly increased risk of larger breast tumour (≥5 cm), lymph node involvement and advanced tumour stages ($p < 0.05$). Contradictory to *miR-27a* and *miR-146a* polymorphisms, *miR-196a2* CT or TT genotypes gave protection against lymph node involvement, larger tumour size and advanced stages of breast cancer. In addition, there were no significant correlations between microRNAs polymorphism and ER, PR and Her2 status.

Discussion

In this case-control study with 353 breast cancer patients and 353 cancer-free controls, we investigated *miR-27a* (rs895819), *miR-196a2* (rs11614913) and *miR-146a* (rs2910164) polymorphisms and breast cancer. The most important result of our study was the significant association of *miR-146a* CC genotype with increased risk of BC, indicating the possible association of *miR-146a* (rs2910164) polymorphism with breast cancer. In addition, the minor allele G of *miR-27a* was associated with a decreased risk of breast cancer. However, the *miR-196a-2* (rs11614913) was not related to an altered risk for breast cancer. Our data also suggests that the *miR-27a* A allele and *miR-146a* C allele had detrimental effects on breast cancer progression and invasion.

MicroRNAs play a role in regulating many biological pathways and deregulation of its expression was shown in many cancers [16,23–25]. Many researches focused on the association of microRNAs polymorphism with susceptibility to breast cancer, but their results were inconsistent and inconclusive. The *miR-27a* rs895819 polymorphism is an unusual miRNA-SNP due to its location in the coding region of the pre-miR-27a hairpin in the stem-loop, which could be cut by Dicer in the process of pre-miRNA maturation. This polymorphic site may change its secondary structure, leading to dysfunction of miRNA-27a or aberrant expression of its targeted gene [26]. A meta-analysis of *miR-27-a* (rs895819) and cancer vulnerability indicated that the variant G allele is consistently associated with reduced risk of breast cancer (OR 0.92, 95% CI = 0.85–0.99) [27]. In addition, an earlier study revealed that the *miR-27a* (rs895819) G/G genotype is significantly associated with reduced breast cancer risk in the families with a moderate history (OR 0.3 [95% CI 0.1 –0.8] $p = 0.01$) [28]. However, in a recent meta-analysis of 10 studies, the G allele and AG genotype were associated with a decreased risk of breast cancer in Caucasian population, whereas a subtly increased risk was observed in a Asian population

[21]. A recent case-control study showed no significant association between the *miR-27a* rs895819 and breast cancer risk [29]. Publicly available data indicate that the rs895819 G allele frequency varies among populations of different ethnicity. The mean G-allele frequency from the '1000 Genomes Project' is 0.36, with a higher value observed in Nigerians (0.55) and a lower value observed in Chinese (0.25).

The G to C SNP of *miR-146a* rs2910164 at position +60 relative to the first nucleotide of the precursor miR-146a causes a structure disturbance in the stem region of the miR-146a and leads to the increased production of mature miR-146a [30]. Recently published meta-analysis indicated that there is no association between miR-146a (rs2910164) polymorphism and breast cancer [31]. However, the genetic polymorphism in *miR-146a* (rs2910164) is associated with young age of familial breast/ovarian cancer diagnosis [32]. In addition, a case-control study of 101 Italian probands with ascertained familiarity for breast/ovarian cancer patients also reported that the C allele of *miR-146a* (rs2910164) was associated with early familial breast/ovarian tumour [33].

An *in vitro* study suggested that *miR-196a2* (rs11614913) genotype may result in altered processing of the pre-miR as well as diminished capacity to regulate target genes [34]. A case-control study in German and Italian women concluded that the SNP (rs11614913) in *miR-196a2* is not associated with breast cancer [22]. Consistent with this, we found no association between rs11614913C > T polymorphism and breast cancer ($p = 0.60$). However, a meta-analysis showed that CC genotype was associated with an increased breast cancer risk (CC versus TT, OR 1.30, 95%CI = 1.01–1.68) [35]. The TT genotype of rs11614913 was associated with a decreased cancer risk, while the G allele of rs3746444 was related with an increased risk of breast cancer in China [27]. Discrepancies in these results may be due to differences in sample size, ethnicity, study design, heterogeneity of the cancer subtype and environmental backgrounds.

Some limitations should be considered when we interpret our results. First, our study had been implemented in Iranian population; whether there is some difference for the same SNP in the characteristics breast cancer among different populations needs to be further tested. Second, more research using larger patient populations is needed to confirm our findings due to a relatively small sample size in analysis. Thirdly, only three potentially functional SNPs of microRNAs were investigated, which did not cover all variants. Finally, we do not have any data about mutational status of *BRCA1* and *BRCA2* in studied population. So, we should involve more factors in our future work.

This work represents an advance in biomedical science because it provides evidence of the effect of *miR-146a* (rs2910164) and *miR-27a* (rs895819) variant on breast cancer.

Summary table

What is known about this subject

- miR-27a is related with poor overall survival of breast cancer patients and may be a valuable marker of tumour progression.
- miR-196a2 is over-expressed in head-neck, oral and cervical cancers.
- A meta-analysis indicated that there is no association between miR-146a (rs2910164) polymorphism and breast cancer susceptibility.

What this paper adds

- This work provides evidence of the effect of *miR-146a* (rs2910164) and *miR-27a* (rs895819) variant on breast cancer susceptibility risk.
- CC genotype of miR-146a and G allele of miR-27a are associated with an increased and decreased risk of breast cancer, respectively.

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Disclosure statement

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