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Association between Mir-499, Mir-27a, and Mir-146a polymorphisms and their susceptibility to recurrent spontaneous abortion; *in silico* analysis

Mir-499, Mir-27a ve Mir-146a polimorfizmlerinin tekrarlayan spontan düşüklere yatkınlıkla ilişkisi; in silico analizi

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Abstract

Objective: Recurrent spontaneous abortion (RSA) is defined as two or more pregnancy losses before 24 gestational weeks, accounting for 1-3% of fertile couples. A vast majority of single-nucleotide polymorphisms (SNPs) in some *microRNA (miRNA)* genes can change the miRNA-mRNA interaction and are associated with the risk of RSA. This study was designed to better elucidate the association between miR-27a, miR-499, and miR-146a polymorphisms and RSA risk.

Materials and Methods: SNP genotyping of miR-27a (rs895819), miR-499 (rs3746444), and miR-146a (rs2910164) was performed using polymerase chain reaction (PCR)-restriction fragment length polymorphism and tetra amplification-refractory mutation system PCR in 98 patients with RSA and 105 healthy subjects.

Results: Our results showed that the miR-499 rs3746444 and miR-27a rs895819 polymorphisms were significantly associated with RSA risk, whereas no significant differences were observed between the rs2910164 polymorphism and RSA susceptibility.

Conclusion: We proposed that the miR-499 rs3746444 and miR-27a rs895819 polymorphisms were correlated with RSA in our population, but the miR-146a rs2910164 variant was not associated with the risk of RSA.

Keywords: MiR-499 rs3746444, miR-27a rs895819, miR-146a rs2910164, RSA risk

Öz

Amaç: Tekrarlayan spontan düşük (RSA), 24 gebelik haftasından önce iki veya daha fazla kez gebelik kaybı olarak tanımlanır ve doğurgan çiftlerin %1-3'ünü etkiler. Bazı mikroRNA (miRNA) genlerindeki tek nükleotid polimorfizmlerinin (SNP'lerin) büyük çoğunluğu miRNA-mRNA etkileşimini değiştirebilir ve RSA'nın ortaya çıkma riskiyle ilişkilidir. Bu çalışma, miR-27a, miR-499 ve miR-146a polimorfizmleri ile RSA riski arasındaki ilişkiyi daha iyi açıklamak için tasarlanmıştır.

Gereç ve Yöntemler: Doksan sekiz RSA'lı hastada ve 105 sağlıklı bireyde polimeraz zincir reaksiyonu (PCR)- restriksiyon fragment uzunluk polimorfizmi ve tetra amplifikasyona dirençli mutasyon sistemi PCR yöntemleri kullanılarak miR-27a (rs895819), miR-499 (rs3746444) ve miR-146a rs2910164'ün SNP genotiplemesi gerçekleştirildi.

PRECIS: The current study has investigated the association of three miRNA variations with the susceptibility of RSA in a fraction of the Iranian population. All polymorphisms except miR-146a C>G polymorphism significantly increased the RSA risk in the dominant inheritance model.

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Bulgular: Sonuçlarımız miR-499 rs3746444 ve miR-27a rs895819 polimorfizmlerinin RSA riskiyle anlamlı şekilde ilişkili olduğunu, rs2910164 polimorfizmi ile RSA duyarlılığı arasında anlamlı bir fark gözlenmediğini gösterdi.

Sonuç: MiR-499 rs3746444 ve miR-27a rs895819 polimorfizmlerinin popülasyonumuzda RSA ile ilişkili olduğunu, ancak miR-146a rs2910164 varyantının RSA'nın ortaya çıkma riski ile ilişkili olmadığını düşünmekteyiz.

Anahtar Kelimeler: MiR-499 rs3746444, miR-27a rs895819, miR-146a rs2910164, RSA riski

Introduction

Recurrent spontaneous abortion (RSA) is a common pregnancy complication that occurs in 1-3% of fertile couples. The disease is described as two or more times early pregnancy loss before 24 gestational weeks and accounts for about 10-15% of clinically recognized pregnancies. Although several etiologic factors, including infectious, uterine abnormalities, hormonal disorders, chromosomal abnormalities, and gene polymorphisms, have been reported as pathophysiological mechanisms of RSA, the etiology of 50% of pregnant women with RSA still cannot be explained[1-3]. Therefore, future research is required to elucidate the pathogenesis of RSA.

MicroRNAs (miRNAs) are small endogenous RNAs that modulate the translation and stability of mRNAs through the recruitment of regulatory proteins[4]. miRNAs have important regulatory roles in various biological processes, such as cell growth, apoptosis, and differentiation[5]. Recent studies have reported that single-nucleotide polymorphisms (SNPs) within miRNA sequences may alter miRNA processing and target selection associated with the risk of RSA. In a study by Santamaria and Taylor[6] examined the possible relationship of miRNA polymorphisms, including miR-146aC>G, miR-149T>C, miR-196a2T>C, and miR-499A>G, in patients with RSA was examined. Their result showed that all these polymorphisms were significantly associated with idiopathic RSA. Concomitant mutations have a synergistic effect[6].

Moreover, two SNPs (rs41275794, rs12976445) residing within the pri-miR-125a sequence were associated with increased RSA risk via decreased miR-125a expression[7]. Therefore, this study aimed to assess the significant differences in miR-499, miR-27a, and miR-146a polymorphisms and RSA susceptibility in the southeast Iranian population.

Materials and Methods

Demographic Characteristics

This case-control study enrolled 98 women with a history of two or more early pregnancy losses and 105 control subjects who had no history of abortion and at least one normal birth. In addition, participants with known causes, including anatomical, autoimmune, endocrine, and chromosomal abnormalities, were excluded from this study. The local Ethics Committee of Zahedan University of Medical Sciences (decision no: IR.ZAUMS.REC.1396.218, date: 20.01.2024) approved the study and provided informed consent. Total blood samples were obtained from all participants. The salting-out method was used to extract genomic DNA as described previously[8] and stored at 20°C until use.

Genotyping

Polymerase chainreaction-restriction fragment length polymorphism (PCR-RFLP) method was used to genotype miR-27a (rs895819) and miR-499 (rs3746444) polymorphisms. Genotyping of the miR-146a rs2910164 polymorphism was determined using the Tetra-amplification-refractory mutation system method. Table 1 lists the primers used in this study. In the PCR-RFLP method, each PCR reaction was performed in a final volume of 20 µL including one microliter of extracted DNA, 10 pmol of each primer, 10 L of master mix, and 7 L of DNase-Free Distilled water. The PCR conditions for miR-27a rs895819 consisted of pre-denaturation at 95°C for 6 min, followed by 35 cycles of 95°C for 30 s, 65°C for 35 s, and 72°C for 35 s, and a final extension step of 72°C for 10 min. For miR-499 rs3746444, the PCR conditions were initial denaturation at 95°C for 5 min; 30 cycles of denaturation (95°C, 30 seconds), annealing (63°C 30 s), and extension (72°c, 30 s). After the final extension step, an appropriate restriction enzyme was

Table 1. The primers used to detect the miR-27a, miR-499 and miR-146a polymorphisms

Polymorphism	PCR Primers (5' →3')	Restriction enzyme	Fragment, bp
MiR-27a (rs895819)	F: GAACTTAGCCACTGTGAACACGACTTCG R: GGGTTCCTGGGGATGGGATTTG	BstUI	T allele: 201 C allele: 173+28
MiR-499 (rs3746444)	F: CAAAGTCTTCACTTCCCTGCCA R: GATGTTTAACTCCTCTCCACGTGATC	BclI	C allele: 146 T alleles: 122.24
MiR-146a (rs2910164)	FO: GGCCTGGTCTCCTCCAGATGTTTAT RO: ATACCTTCAGAGCCTGAGACTCTGCC FI (C allele): ATGGGTTGTGTCAGTGTCAGACGTC, RI (G allele): GATATCCCAGCTGAAGAACTGAATTTGAC	-	Control: 364 G allele: 249 C allele: 169

PCR: Polymerase chain reaction

used to digest the PCR products of the miR-27a and miR-499 polymorphisms (according to Table 1). For genotyping miR-146a rs2910164, 10 µL of the master mix was combined with 5 µL of double-distilled water and 1 µL of each primer in each reaction mixture. The amplification parameters for the SNP were similar to the PCR conditions of miR-499 rs3746444, except for the annealing step (61°C, 25 s). Electrophoresis using 2% agarose gel containing 0.5 µg/mL ethidium bromide was performed to visualize the PCR products under ultraviolet light (Figures 1-3). Genotyping quality was confirmed by re-checking approximately 20% of all samples, resulting in a concordance of 100%.

Using SPSS software (version 20, USA), an independent sample t-test and $\chi 2$ test were used to analyze the comparison of differences between the two groups. The odds ratios (ORs) with 95% confidence intervals (CIs) were employed to calculate any possible association between these polymorphisms and RSA risk. The significance probability was <0.05.

Statistical Analysis

In this study, we used "miRDB"[9] and miRWalk[10] software to demonstrate the interaction of miR-27a, miR-499a, and miR-146a with putative target genes. In miRDB, an online database was developed to predict miRNA target sites and generate functional data from large-scale RNA sequencing experiments. The four common features assessed by the program were free energy, seed match, conservation, and site accessibility[9]. In the output of this server, we only included the top 10 results in terms of the target score. Then, we examined the relationship between RSA and possible target genes. Then, the genes were presented in bold. In this analysis, we separately applied both strands (designated 5p- or 3p-) of the miRNA stem-loop structure for gene target prediction. The miRWalk database lists predicted and validated miRNA binding sites from human, dog, cow, and rat genes[10]. First, we used the miRWalk database to download the introduced targets for each miRNA; then we used Cytoscape software to draw the interaction network related to these targets. Cytoscape is also an open-source software for the visualization and analysis of bio-molecular interaction networks, with several plug-ins, including the investigation of biological pathways for further analysis[11]. Finally, using the String App[12] tool, the enrichment of miRNA targets was performed only for confirmed interactions, and in this way, the indirect effects of miRNAs on various processes were investigated (Figure 4).

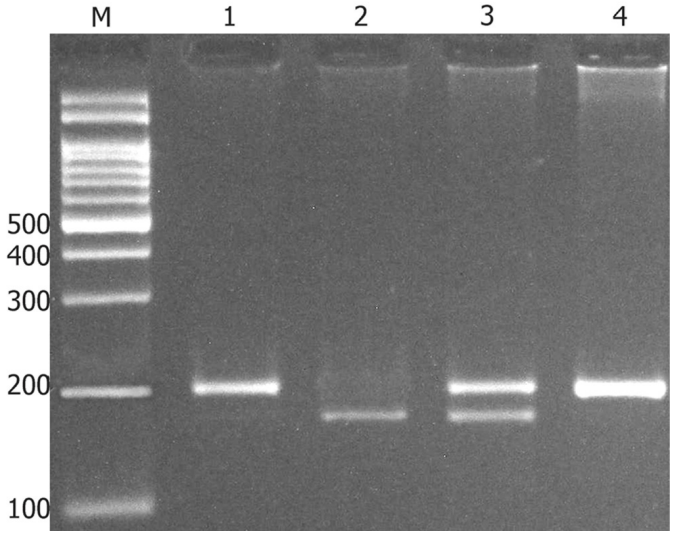

Figure 1. Photograph of the PCR-RFLP method for the detection of miR-27a rs895819 polymorphism

M: DNA marker; Lanes 1 and 4: TT; Lane 2: CC; Lane 3: TC, PCR: Polymerase chain reaction, RFLP: Restriction fragment length polymorphism

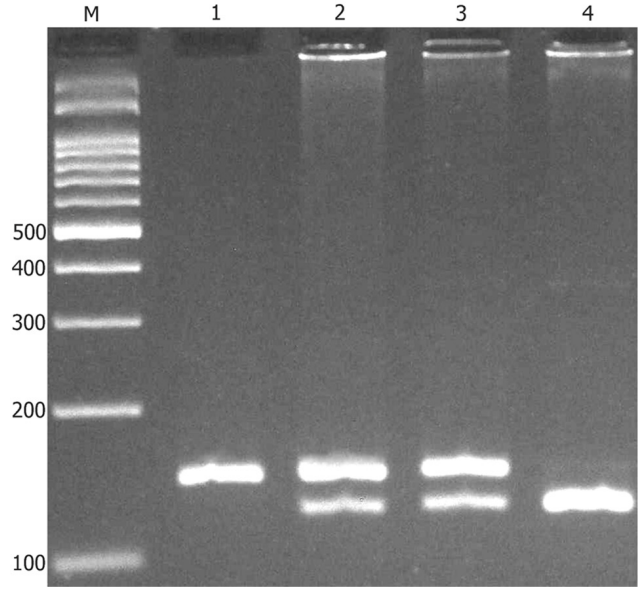

Figure 2. Photograph of the PCR-RFLP method for the detection of the miR-499 rs3746444 polymorphism.

M: DNA marker; Lane 1: CC; Lanes 2 and 3: CT; and Lane 4: TT, PCR: Polymerase chain reaction, RFLP: Restriction fragment length polymorphism

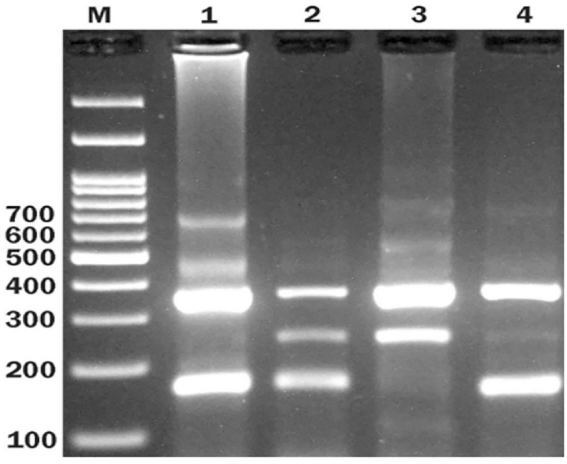

Figure 3. Photograph of the T-ARMS Method for the detection of miR-146a rs2910164

T-ARMS: Tetra amplification-refractory mutation system

Results

In the case-control study, 98 patients with >2 miscarriages, age 28.79±5.00 years, and 105 non-consanguineous healthy women (29.50±4.85 years) were recruited. There was no significant difference in age between the case and control subjects (p=0.306). Table 2 shows the frequency of genotypes and alleles of the miR-27a (rs895819), miR-499 (rs3746444), and miR-146a (rs2910164) polymorphisms in the groups. The miR-27a rs895819 polymorphism increased the susceptibility to RSA in co-dominant (OR=1.98, 95% CI=1.05-3.76, p=0.035 TC vs. TT) models. The miR-499 rs3746444 polymorphism increased the predisposition to RSA in the codominant (OR=2.49, 95% CI=1.36-4.56, p=0.005 TC vs. TT; OR=2.92, 95% CI=1.16-7.34, p=0.036 CC vs. TT) and dominant (OR=2.58, 95% CI=1.47-4.55, p=0.001 TC+CC vs. TT) models. The C allele of miR-499 rs3746444 polymorphism was significantly higher in the patients (OR=2.02, 95% CI=1.32-3.09, p=0.002) than in control subjects. There were no significant differences between cases and controls regarding the miR-146a variants in any inheritance model (Table 2). The genotypes of the miR-27a, miR-499, and miR-146a variants in controls and cases were classified under Hardy-Weinberg equilibrium. (X^2=0.055, 0.136, and 0.337 and X^2=0.078, 0.958, and 0.947 respectively).

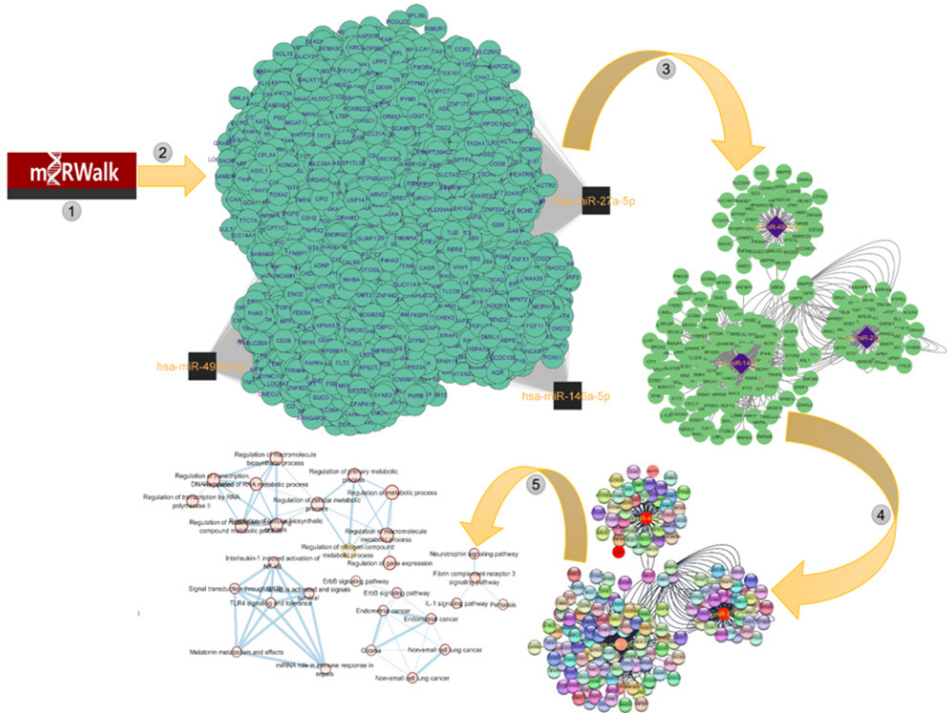

Figure 4. Approach was performed using miRWalk and Cytoscape software. Step 1: search and download process for hsa-miR-27a, hsa-miR-499, and hsa-miR-146a. Step 2: Draw the miRNA target interaction network using the Cytoscape tool. Step 3: Select validated targets and remove other targets from network. Steps 4 and 5: network drawing and enrichment by StringApp

Table 2. Association between *miR-27a, miR-499, and miR-146a* gene polymorphisms and the risk of recurrent spontaneous abortion

Polymorphism	Case number (%)	Control n (%)	OR (95% CI)	p-value
MiR-27a rs895819				
Codominant				
TT	26 (26.5)	38 (36.2)	1	-
TC	57 (58.2)	42 (40.0)	1.98 (1.05-3.76)	**0.050**
CC	15 (15.3)	25 (23.8)	0.88 (0.39-1.97)	0.911
Dominant				
TT	26 (26.5)	38 (36.2)	1	-
TC+CC	72 (73.5)	77 (63.8)	1.37 (0.76-2.47)	0.377
Recessive				
TT+TC	83 (84.7)	80 (76.2)	1	-

Table 2. Continued				
Polymorphism	Case number (%)	Control n (%)	OR (95% CI)	p-value
CC	15 (15.3)	25 (23.8)	0.58 (0.28-1.18)	0.178
Allele				
T	109 (55.6)	118 (56.2)	1	-
C	87 (44.4)	92 (43.8)	1.02 (0.69-1.52)	0.986
MiR-499 rs3746444				
Codominant				
TT	36 (36.7)	63 (60.0)	1	-
TC	47 (48.0)	33 (31.4)	2.49 (1.36-4.56)	**0.005**
CC	15 (15.3)	9 (8.6)	2.92 (1.16-7.34)	**0.036**
Dominant				
TT	36 (36.7)	63 (60.0)	1	-
TC+CC	62 (63.3)	42 (40.0)	2.58 (1.47-4.55)	0.001
Recessive				
TT+TC	83 (84.7)	96 (91.4)	1	-
CC	15 (15.3)	9 (8.6)	1.93 (0.80-4.63)	**0.205**
Allele				
T	119 (60.7)	159 (75.7)	1	-
C	77 (39.3)	51 (24.3)	2.02 (1.32-3.09)	**0.002**
MiR-146a rs2910164				
Codominant				
GG	56 (57.1)	62 (59.1)	1	-
GC	36 (36.7)	35 (33.3)	1.14 (0.63-2.05)	0.778
CC	6 (6.2)	8 (7.6)	0.83 (0.27-2.54)	0.966
Dominant				
GG	56 (57.1)	62 (59.1)	1	-
GC+CC	42 (42.9)	43 (40.9)	1.08 (0.619-1.89)	0.895
Recessive				
GG+GC	92 (93.8)	97 (92.4)	1	-
CC	6 (6.2)	8 (7.6)	0.79 (0.26-2.37)	0.886
Allele				
G	148 (75.5)	159 (75.7)	1	-
C	48 (24.5)	51 (24.3)	1.01 (0.64-1.59)	1.000

OR: Odds ratio, CI: Confidence interval

Bioinformatics Findings

According to Table 3, our analysis illustrated that the *IL2* gene is affected by hsa-miR-27a-5p with a target score of 94. The *SOX6 (SRY-box containing gene 6)* gene with a target score of 100 and the *LEPR* gene with a target score of 96 were affected by hsa-miR-499a-5p and hsa-miR-499a-3p, respectively. The *TRAF6* gene with a target score of 100 and the *TXNIP* gene with a target score of 96 were affected by hsa-miR-499a-5p and hsa-miR-499a-3p, respectively.

Furthermore, functional enrichment analysis using StringApp with a false discovery rate (FDR) threshold of 5% showed that in the KEGG Pathways category, most of the studied target genes of miRNAs are involved in the pathways of cancer, bacterial and viral infections, and hepatitis C.

Discussion

Increasing evidence has confirmed that miRNAs play a vital role in the pathophysiology of RSA and may be potential diagnostic

or prognostic markers for this disease[13]. Moreover, SNPs present both in *miRNA* genes or within miRNA-mRNA binding sites may contribute to susceptibility to RSA by affecting the expression and function of the miRNA target[14]. In this study, we investigated the association between three miRNA polymorphisms (miR-27a, miR-499, and miR-146a) and the risk of RSA in Iranian women.

Our results showed that the mir-499 rs3746444 polymorphisms were statistically associated with an increased risk of RSA in the co-dominant inheritance model. Additionally, we found that the C allele of miR-499 rs3746444 C/T enhanced the risk of RSA compared with the healthy group. The association between miR-499 rs3746444 and RSA susceptibility has been well studied in some populations. The following are some important points of them. In a study conducted on north Indian women affected by RSA, there was a possible correlation between polymorphism and SRA risk[15], which was consistent with our findings. In another study, Fazli and Ghorbian[16] suggested that the miR-499a polymorphism was significantly correlated with susceptibility to idiopathic RSA in the torque ethnic group. Conversely, the bioinformatics analysis predicted that SOX6 and LEPR are affected by miR-499a-5p and miR-499a-3p. Recently published articles have demonstrated that the ***SOX6*** gene (as a direct target of miR-499) can effectively modulate differentiation and cell proliferation during embryonic development via the repression of FGF-3 transcription. It was hypothesized that the deregulation and dysfunction of miR-499 caused by genetic material alteration are likely to influence female reproductive and fertility[17].

Furthermore, the present study assessed the association between rs895819 alleles and genotypes and RSA susceptibility. Our results showed that mir-27a rs895819 polymorphism positively affects the risk of RSA. In 2016, Wang et al.[18] suggested that SNP rs895819 C>T significantly increased the risk of RSA (which is in agreement with our finding), while studies by Rah et al.[19] and Srivastava et al.[14] showed no relationship between the risk of RSA and the risk of RSA. The variant located in the terminal loop of pre-miR-27a is associated with the risk of non-alcoholic fatty liver disease[20], colorectal cancer[21], type 2 diabetes mellitus[22], and primary ovarian insuffciency[23]. Our bioinformatics data suggest that most miRNA target genes are involved in cancer, bacterial and viral infections, and hepatitis C. Previous studies reported that miR-27a regulates the antimicrobial activities of macrophages by targeting the ***IL-10*** gene (inflammatory response gene)[24], which is consistent with our bioinformatics data. In addition, high levels of miR-27a expression were observed in the villus tissue of patients with RSA, and its upregulation may suppress the cycle progression of trophoblasts and induce apoptosis by targeting the regulation of the expression of cyclin D1 and IGF1[23].

Finally, preliminary data suggested significant differences between miR-146a C>G polymorphism and RSA susceptibility in the northeast Iranian population[25]. However, this study did not identify any significant association with RSA risk, which is consistent with the studies by Jeon et al.[26], Parveen and Agrawal[15], and Babakhanzadeh et al.[27]. Previous studies have shown that miR-146a could promote apoptosis in oocytes during folliculogenesis by binding to the 3'-UTR of the ***FAS*** gene, which may then lead to spontaneous abortion[28].

Study Limitations

First, we analyzed only three miRNAs, whereas there are many miRNAs related to RSA. Second, this study registered 98 patients and 105 healthy individuals from a fraction of the southeast Iranian population. However, a large population-based investigation can be more informative. Third, we did not evaluate the correlation between these variants and relative miRNA expression.

Table 3. Predicted target genes for miR-27a, miR-499a, and miR-146a in miRDB

	HSA-miR-27a 5p		HSA-miR-27a-3p		HSA-miR-499a-5p		HSA-miR-499a-3p		HSA-miR-146a-5p		HSA-miR-146a-3p	
Target rank	Gene symbol	Target score	Gene symbol	Target score	Gene symbol	Target score	Gene symbol	Target score	Gene symbol	Target score	Gene symbol	Target score
1	*RFK*	97	*AFF4*	100	***SOX6***	**100**	*TCF7L2*	99	***TRAF6***	**100**	*CPLX2*	99
2	*LTBP1*	97	*GXYLT1*	100	*VAV3*	99	*FOXN2*	99	*IRAK1*	100	*FOXC1*	99
3	*INO80D*	95	*ARFGEF1*	100	*SLC30A4*	99	*NRIP1*	99	*SEC23IP*	99	*STXBP6*	98
4	*BTF3*	95	*GCC2*	100	*EML4*	99	*MEOX2*	98	*NOVA1*	98	*ZFX*	98
5	*IFI30*	94	*DCUN1D4*	100	*EPM2AIP1*	98	*ZC3H6*	96	*PPP1R11*	97	*RIOK3*	97
6	*HECW2*	94	*PLK2*	100	*REEP1*	98	*ZIC2*	96	*UPP2*	97	*FAM126B*	96
7	***IL2***	**94**	*TNPO1*	100	*TMEM100*	98	***LEPR***	**96**	*WWC2*	97	***TXNIP***	**96**
8	*ADCY1*	92	*TRPV3*	100	*UBE2V2*	98	*ADAMTS9*	96	*BCORL1*	96	*C17orf75*	96
9	*EIF5*	91	*GAB1*	100	*PHLDA2*	97	*RAP2B*	95	*ZNF649*	95	*SNAP23*	95
10	*NPM1*	91	*GRIA4*	99	*IKZF2*	97	*UBE2E3*	95	*SORT1*	95	*SORT1*	95

Conclusion

In conclusion, the present study investigated the association between three miRNA variations and susceptibility to RSA in a fraction of the Iranian population. All polymorphisms except miR-146a C>G significantly increased the risk of RSA in the dominant inheritance model.

Ethics

Ethics Committee Approval: The Zahedan University of Medical Sciences Research Ethics Committee approved this study (decision no: IR.ZAUMS.REC.1396.218, date: 20.01.2024).
Informed Consent: All participants provided informed consent before entering the study.

Authorship Contributions

Concept: G.B., A.G., Design: M.M., Data Collection or Processing: M.M., Analysis or Interpretation: M.M., H.S.G., Literature Search: M.T., Writing: M.M.
Conflict of Interest: No conflict of interest was declared by the authors.
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