Association of pre-miRNA-146a rs2910164 and pre-miRNA-499 rs3746444 polymorphisms and susceptibility to rheumatoid arthritis

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Abstract. Single nucleotide polymorphisms in pre-microRNA (miRNA) may alter miRNA expression levels or processing and contribute to susceptibility in a wide range of diseases. The present study aimed to evaluate the possible association between rs2910164 and rs3746444 of the pre-miRNA (hsa-mir-146a and hsa-mir-499) polymorphisms and susceptibility to rheumatoid arthritis (RA) in an Iranian population. This case-control study was performed on 104 patients with RA and 110 healthy individuals. Tetra amplification refractory mutation system-polymerase chain reaction was used to genotype the hsa-mir-499 rs3746444 and hsa-mir-146a rs2910164 polymorphisms. The hsa-mir-499 rs3746444 polymorphism was a risk factor for predisposition to RA in codominant [TT vs. TC: odds ratio (OR), 2.11; 95% confidence interval (CI), 1.08-4.11; P=0.029; TT vs. CC: OR, 3.88; 95% CI, 1.68-8.98; P=0.002], dominant (TT vs. TC-CC: OR, 2.64; 95% CI, 1.48-4.72; P=0.001) and recessive (TC-CC vs. CC: OR, 3.05; 95% CI, 1.36-6.83; P=0.007) tested inheritance models. In addition, the rs3746444 C allele was a risk factor for RA (OR, 2.49; 95% CI, 1.63-3.81; P<0.0001). No significant difference was found between the groups concerning the rs2910164 polymorphism (χ^2=0.348, P=0.841). Our findings demonstrated that the hsa-mir-499 rs3746444, but not mir-146a rs2910164, polymorphism is associated with an increased RA risk in a sample of the Iranian population. Larger studies with different ethnicities are required to validate our findings.

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease which affects 0.5-1% of the general population worldwide (1). A chronic and deforming arthritis, RA is characterized by accelerated inflammatory joint destruction of articular cartilage and bone and synovial hyperplasia, which ultimately leads to severe disabilities and a poor quality of life (2,3). The etiology of RA is unknown, but genetic factors are thought to be important in the pathogenesis and progress of RA (1,4). One class of genetic variants that have recently been the center of attention are DNA polymorphisms that affect microRNA (miRNA) binding (5). miRNAs are approximately 22-nucleotide (nt) non-coding RNAs that are involved in the post-transcriptional regulation of gene expression by affecting the stability and translation of mRNAs (6). Compelling evidence indicates that miRNAs act as key regulators of various processes, including early development, cell proliferation, differentiation, stress resistance, cell fate determination, apoptosis, signal transduction and organ development (7-10). miRNAs are present in dried biological fluids, including semen, saliva, vaginal secretions and menstrual blood and are expected to be diagnostic and prognostic biomarkers of various diseases, including cancer and autoimmune diseases such as RA (11,12).

Abnormal expression of several miRNAs has been detected in RA in various cell types and these miRNAs regulate specific pathways, thus leading to the inflammatory milieu occurring in RA (2). Hsa-mir-499 is involved in autoimmune and inflammatory disease. The targets of hsa-mir-499 include IL-17Rβ, IL-23α, IL-2R, IL-6, IL-2 and IL-18R. IL-6 activates the production of CRP (C-reactive protein) and fibrinogen through the liver and IL-17Rβ, IL-23α, IL-2R, IL-6, IL-2 and IL-18R contribute to the progress and pathogenesis of RA (8). Findings of recent studies have shown that the rs3746444 polymorphism in the pre-miR-499 is correlated with several diseases, including breast cancer (13), cervical squamous cell carcinoma (CSCC) (14), hepatocellular carcinoma (15), RA (8), coronary artery disease (CAD) (16), chronic obstructive pulmonary disease (COPD) (17) and tuberculosis (17).

Table I. Primer sequences for the detection of hsa-mir-146a and hsa-miR-499 gene polymorphisms.

Gene	Primer	Sequence (5'-3')	Amplicon size (bp)
hsa-mir-146a; rs2910164 G>C	FO	GGCCTGGTCTCCTCCAGATGTTTAT	364
	RO	ATACCTTCAGAGCCTGAGACTCTGCC	364
	FI (C allele)	ATGGGTTGTGTCAGTGTCAGACGTC	169
	RI (G allele)	GATATCCCAGCTGAAGAACTGAATTTGAC	249
hsa-miR-499; rs3746444 T>C	FO	GAGTGACCAGGCCCCTTGTCTCTATTAG	422
	RO	TTGCTCTTTCACTCTCATTCTGGTGATG	422
	FI (C allele)	ATGTTTAACTCCTCTCCACGTGACCG	206
	RI (T allele)	GGGAAGCAGCACAGACTTGCTGTTAT	268

FO, forward outer; RO, reverse outer; FI, forward inner; RI, reverse inner.

More recently the miRNA hsa-mir-146a has also received considerable attention, as it was reported to be overexpressed in synovial fibroblasts, synovial tissue, synovial fluid monocytes, peripheral blood mononuclear cells (PBMCs) and serum plasma of RA patients (18). This polymorphism was principally studied for its association with several diseases, including psoriatic arthritis (19), multiple sclerosis (MS) (20), tuberculosis (17), ulcerative colitis, RA (8,21), SLE (16) and various types of cancer (22-29).

Overall, these two single nucleotide polymorphisms (SNPs; rs3746444 and rs2910164) are located at the pre-miRNA regions of hsa-mir-499 and hsa-mir-146a (21), respectively. Given the evidence that SNPs located in the mature miRNA regions affect binding to target mRNAs and the pre-miRNA maturation process (30), the aim of the present study was to evaluate the impact of the rs3746444 and rs2910164 polymorphisms on risk of RA in a sample of the Iranian population.

Materials and methods

Patients. We evaluated the possible association between polymorphisms of hsa-mir-146a and hsa-mir-499 genes (rs2910164 G/C and rs3746444 T/C, respectively) and RA susceptibility in 104 patients meeting the American College of Rheumatology criteria for RA (31). The patients were selected from the Rheumatology Clinic at Zahedan University of Medical Sciences (4,32,33). The control group involved 110 healthy individuals with no relationship to RA patients. The Ethics Committee of Zahedan University of Medical Sciences approved the project and informed consent was obtained from all patients and healthy individuals. Genomic DNA was extracted from peripheral blood samples as previously described (33).

Tetra amplification refractory mutation system-polymerase chain reaction (T-ARMS-PCR). T-ARMS-PCR is a simple and rapid method with a high level of accuracy for the detection of SNPs (34-36). This system was used for genotyping of the rs2910164 G/C and rs3746444 T/C polymorphisms. Genotyping of rs2910164 and rs3746444 was performed using two outer primers (FO and RO) and two inner allele-specific primers (FI and RI) for each SNP, as listed in Table I. PCR was performed using commercially available PCR premix (AccuPower PCR PreMix; Bioneer, Daejeon, South Korea) according to the manufacturer's instructions. The PCR cycling conditions were 95˚C for 5 min followed by 30 cycles of 30 sec at 95˚C, 25 sec at 61˚C for rs2910164, 27 sec at 60˚C for rs3746444 and 25 sec at 72˚C, with a final extension of 72˚C for 10 min. The PCR products were verified on 2% agarose gels containing 0.5 μg/ml ethidium bromide and observed under UV light. The product sizes for rs2910164 were 169 bp for the C allele and 249 bp for the G allele, while the product size for the two outer primers (control band) was 364 bp (Fig. 1). The amplicon sizes for the rs3746444 were 422 bp for the control band, 206 bp for the C allele and 268 bp for the T allele (Fig 2). To ensure genotyping quality, we regenotyped 20% of the random samples and found no genotyping errors.

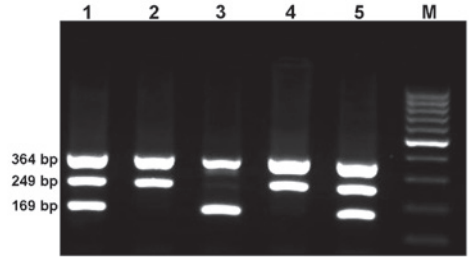

Figure 1. Representative PCR products of tetra amplification refractory mutation system-polymerase chain reaction (T-ARMS-PCR) resolved by agarose gel electrophoresis to detect the pre-miRNA-146a rs2910164 G/C polymorphism. M, DNA marker; lanes 1 and 5, rs2910164 GC; lanes 2 and 4, GG; lane 3, CC.

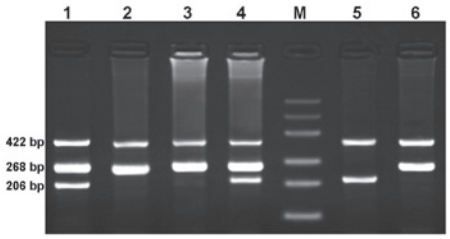

Figure 2. Electrophoresis pattern of tetra amplification refractory mutation system-polymerase chain reaction (T-ARMS-PCR) for the detection of pre-miRNA-499 rs3746444 T/C polymorphism. M: DNA marker; lanes 1 and 4, rs3746444 TC; lanes 2, 3 and 6, TT; lane 5, CC.

Table II. Frequency distribution of hsa-mir-499 genotypes in RA patients and normal subjects.

Model	rs3746444 T>C	RA n (%)	Control n (%)	[a]OR (95% CI)	P-value
Codominant	TT	46 (44.2)	74 (67.3)	Ref.	-
	TC	32 (30.8)	25 (22.7)	2.11 (1.08-4.11)	0.029
	CC	26 (25.0)	11 (10.0)	3.88 (1.68-8.98)	0.002
Dominant	TT	46 (44.2)	74 (67.3)	Ref.	-
	TC+CC	58 (58.8)	36 (32.7)	2.64 (1.48-4.72)	0.001
Recessive	TT+TC	78 (75.0)	99 (90.0)	Ref.	-
	CC	26 (25.0)	11 (10.0)	3.05 (1.36-6.83)	0.007
Alleles	T	124 (59.6)	173 (78.6)	Ref.	-
	C	84 (40.4)	47 (21.4)	2.49 (1.63-3.81)	<0.0001

[a]Adjusted for gender and age. RA, rheumatoid arthritis; OR, odds ratio; CI, confidence interval.

Table III. Frequency distribution of hsa-mir-499 genotypes in RA patients and normal subjects.

Model	rs3746444 T>C	RA n (%)	Control n (%)	[a]OR (95%CI)	P-value
Codominant	GG	57 (54.8)	64 (59.2)	Ref.	-
	GC	39 (37.5)	37 (33.6)	1.06 (0.58-1.94)	0.840
	CC	8 (7.7)	9 (8.2)	0.89 (0.31-2.58)	0.835
Dominant	GG	57 (54.8)	64 (58.2)	Ref.	-
	GC+CC	47 (45.2)	46 (41.8)	1.03 (0.58-1.81)	0.918
Recessive	GG+GC	96 (92.3)	101 (91.8)	Ref.	-
	CC	8 (7.7)	9 (8.2)	0.87 (0.31-2.46)	0.796
Alleles	G	139 (71.6)	165 (75.0)	Ref.	-
	C	55 (28.4)	55 (28.4)	55 (28.4)	0.504

[a]Adjusted for gender and age. RA, rheumatoid arthritis; OR, odds ratio; CI, confidence interval.

Statistical analysis. Statistical analysis was calculated using SPSS 18 software. Data were analyzed by independent sample t-test and χ^2 test. The association between genotypes of hsa-mir-146a and hsa-mir-499 genes and RA were assessed by computing the odds ratio (OR) and 95% confidence intervals (95% CIs) from logistic regression analyses. P<0.05 was considered to indicate a statistically significant difference.

Results

The study group consisted of 104 RA patients (91 females and 13 males) with an average age of 44.7±13.3 years, and 110 healthy subjects (70 females and 40 males) with a mean age of 43.5±10.2 years. No significant difference was found between the groups with respect to age (P=0.458), but a significant difference was observed between the groups with respect to gender (P=0.0001).

The frequency distribution of hsa-mir-499 rs3746444 T/C genotypes in RA patients and normal subjects are demonstrated in Table II. A significant difference was found between case and control groups with regard to rs3746444 T/C polymorphism (χ^2=13.32, P=0.001). The hsa-mir-499 rs3746444 T/C polymorphism was a risk factor for predisposition to RA in codominant (TT vs. TC: OR, 2.11; 95% CI, 1.08-4.11; P=0.029; TT vs. CC: OR, 3.88; 95% CI, 1.68-8.98; P=0.002), dominant (TT vs. TC-CC: OR, 2.64; 95% CI, 1.48-4.72; P=0.001) and recessive (TC-CC vs. CC: OR, 3.05; 95% CI, 1.36-6.33; P=0.007) tested inheritance models (Table II). Furthermore, the rs3746444 C allele was identified as a risk factor for susceptibility to RA (OR, 2.49; 95% CI, 1.63-3.81; P<0.0001).

As demonstrated in Table III, the genotypes and allele frequencies of mir-146a rs2910164 were not found to be as significantly different between RA patients and control subjects (χ^2=0.348, P=0.841).

Discussion

In the present study, we analyzed the correlation between genetic polymorphisms in hsa-mir-146a rs2910164 and hsa-mir-499 rs3746444 and susceptibility to RA in a sample of the Iranian population. The hsa-mir-499 rs3746444 polymorphism was revealed to be associated with an overall

increased risk of RA in codominant, dominant and recessive tested inheritance models. The prevalence of rs3746444 TC (30.8%) and CC (25%) variants in RA patients were identified as significantly higher than that in the healthy individuals (22.7 and 10%, respectively) and the C allele (minor allele) of rs3746444 was found to be as more frequent in patients with RA than that of controls (40.4 vs. 21.4%, respectively). However, no association was detected between the hsa-mir-146a rs2910164 polymorphism and the risk of RA in our population. In contrast to our findings, Yang *et al* (21) did not detect a significant association between hsa-mir-499 rs3746444 polymorphisms and RA. However, the authors observed that carriers of the CT genotype in rs3746444 had a higher level of anti-cyclic citrullinated protein (CCP) antibody. In agreement with results of the present study, no significant difference was detected between the groups with respect to the hsa-mir-146a rs2910164 polymorphism.

Polymorphisms in miRNA genes may alter a wide spectrum of biological processes by affecting the processing and/or target selection of miRNAs (30). A growing number of studies have revealed that polymorphisms in miRNA target sites affect the pathogenesis of several human diseases, including inflammatory bowel (11) and Crohn's disease (37), ulcerative colitis (11), psoriasis (38), COPD (39), SLE (40), MS (20) and RA (41-44). The most severe consequence of RA is joint damage, which leads to disability, deformity, morbidity and mortality (1,45). The principal benefit of the detection of hsa-mir-146a and hsa-mir-499 in circulating PBMCs is for utilization as biomarkers to monitor the course of the disease, without the difficulty of invasive surgical procedures to obtain joint tissues and cells for miRNA analysis (2).

The polymorphism in the hsa-mir-499 rs3746444 has been reported to have a marked correlation with a variety of diseases including breast cancer (13), CSCC (14), hepatocellular carcinoma (15), RA (8), CAD (16), COPD (17) and tuberculosis (17). In their study, Yang *et al* have demonstrated that carriers of the heterozygote genotype (CT) of rs3746444 in RA exhibit higher levels of CRP and erythrocyte sedimentation rate compared to the homozygote carriers (CC and TT), indicative of an important role for rs3746444 in the progress and inflammatory reaction of RA (8). Additional investigation by the same group detected no positive correlation between the SNPs (rs3746444 and rs2910164) and RA. Those authors found that carriers of the genotype CT in rs3746444 had a higher level of anti-CCP antibody in RA and that the SNP rs3746444 may be a candidate biomarker for predicting joint damage in RA patients (21). However, no significant association was observed between rs3746444 and risk of several diseases, including SLE (16), schizophrenia (46), asthma (47) and colorectal (32), gallbladder (48), breast (23) and lung cancer (49).

No association was found between rs2910164 and predisposition to RA in our population. This common hsa-mir-146a polymorphism, rs2910164, involves a G>C nucleotide replacement that causes change from a G:U pair to a C:U mismatch in the stem structure of the hsa-mir-146a precursor, which affects the specificity of mature hsa-mir-146a in binding to its targets, resulting in an elevated expression of hsa-mir-146a (5). The hsa-mir-146a was found to be upregulated in psoriasis (50) and circulating PBMCs of RA patients (18), but is downregulated in SLE (50). Hsa-mir-146a binds several targets, including IRAK2, FADD, IRF-5, Stat-1, PTC1 and FAF1, highlighting the important role of this miRNA in inflammation and apoptosis processes (5).

In conclusion, the present study has shown a marked correlation between the hsa-mir-499 rs3746444 polymorphism and susceptibility to RA in a sample of the Iranian population. However, no association was revealed between the hsa-mir-146a rs2910164 variant and RA susceptibility. Consistent with growing evidence, the present study has demonstrated that miRNA polymorphisms may be suitable for use as diagnostic biomarkers for RA in future.

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