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GENETICS

Association between miR-146a C > G, miR-149 T > C, miR-196a2 T > C, and miR-499 A > G polymorphisms and susceptibility to idiopathic recurrent pregnancy loss

Meysam Alipour[1] · Maryam Abtin[2] · Asghar Hosseinzadeh[3] · Masoud Maleki[1]

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Abstract

Background A growing body of evidence suggests that microRNAs play fundamental regulatory roles in embryo implantation and maintenance of pregnancy. The aim of this study was to investigate the possible association between miR-146a C > G, miR-149 T > C, miR-196a2 T > C, and miR-499 A > G polymorphisms and genetic susceptibility to recurrent pregnancy loss (RPL).

Material and methods One hundred and twenty women with a history of two or more unexplained consecutive miscarriages and 90 ethnically matched healthy women with a history of at least two successful pregnancy outcomes and without a history of miscarriage were enrolled in a case-control study. Genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

Results Our findings showed that the prevalence of miR-149 T > C polymorphism in RPL patients was significantly higher than those in healthy controls ($p < 0.05$). We also found that the presence of miR-149 C and miR-499 G alleles was significantly associated with susceptibility to RPL ($p < 0.05$). The miR-146a CC/miR-499 GG, miR-149 TC/miR-499 AG, and miR-196a2 TT/miR-499 GG combined genotypes were associated with the high risk of RPL ($p < 0.05$).

Conclusion This study suggests that miR-149 T > C polymorphism and the presence of miR-149 C, and miR-499 G alleles are a genetic determinant for the risk of idiopathic RPL.

Keywords miR-146a C > G · miR-149 T > C · miR-196a2 T > C · miR-499 A > G · Polymorphism · Recurrent pregnancy loss

Introduction

Recurrent pregnancy loss (RPL) is defined as the spontaneous loss of two or more idiopathic consecutive clinical pregnancies prior to 22 completed weeks of gestation according to American Society of Reproductive Medicine (ASRM), European Society of Human Reproduction and Embryology (ESHRE), and several medical societies guidelines [1, 2]. RPL is a distressful experience which affects approximately 1–5% of couples trying to conceive [3]. There are well-documented causes in the literature which can be related with RPL, including chromosomal abnormalities, uterine anatomical anomalies, endocrine dysfunction, maternal inherited thrombophilia, infections, immunological, and environmental factors [4–7]. Although an increasing amount of research has been previously carried out in this complicated area to elucidate the genetic basis and molecular pathophysiology of RPL, there are still many questions that remain unanswered [6–9].

In the recent years, microRNAs (miRNAs), a class of endogenous ~ 22 nucleotide noncoding small RNAs, are considered as an interesting research topic in the field of reproductive medicine and science [10–13].

Following transcription, miRNAs are precisely excised from hairpin RNA precursors (pri-miRNAs) through a coordinated two-step sequential processing events mediated by microprocessor machinery [14]. MiRNAs post-transcriptionally regulate the expression of protein-encoding genes through translational repression and/or mRNA degradation usually by pairing to sites in the 3′ untranslated region (3′UTR) of their complementary

✉ Masoud Maleki
Maleki.masoud@iaut.ac.ir

[1] Department of Biology, Tabriz Branch, Islamic Azad University, Tabriz, Iran

[2] Department of Medical Genetics, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

[3] Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran

target mRNAs [15–17]. More than 60% of all human protein-coding transcripts are computationally predicted to be under control of miRNAs [18]. This means that virtually, a wide array of accurately regulated biological processes such as cell proliferation, cell differentiation, cell cycle progression, and programmed cell death is subject to miRNA-dependent regulation [19]. Moreover, evidence suggests that miRNAs play fundamental roles in the embryo implantation, embryo-maternal communication, pregnancy establishment, and embryo development [20–22]. Therefore, there is no doubt that deregulation of miRNA expression and functions contributes to the pathophysiology of the female reproductive disorders, especially RPL. Altered miRNA expression and function could be caused by a variety of mechanisms, including promoter methylation of miRNA genes, improper histone modification, DNA copy number aberrations, alteration in the miRNAs processing pathway, and genetic mutations existing in miRNA genes [23]. A growing number of studies have shown that single nucleotide polymorphisms (SNPs) in human miRNAs genes affecting the expression and function of them are closely associated with various human diseases [24–28].

Recently, the association of miR-146a C > G (rs2910164), miR-149 T > C (rs2292832), miR-196a2 T > C (rs11614913), and miR-499 A > G (rs3746444) polymorphisms with RPL has also been investigated [29–34]. However, the association of these polymorphisms with RPL has not been fully understood yet. Therefore, the present study was designed to address the question of whether miR-146a C > G, miR-149 T > C, miR-196a2 T > C, and miR-499 A > G polymorphisms can be a genetic predisposing factors in the pathogenesis of RPL.

Material and methods

Subjects

This case-control study consisted of 120 women (mean age 31.79 ± 5.32 years, ranged between 22 and 43 years) who had a documented history of two or more idiopathic consecutive pregnancy losses in the first trimester and were recruited as case group at the ART and Stem Cell Research Center (ACECR), Tabriz, Iran, between May 2014 and February 2017. Patients were excluded from the study if they had uterine anomalies, chromosomal abnormalities, sex-hormones imbalance, thyroid dysfunction, genital infections, and autoimmune or thrombotic causes. The mean number of miscarriages in the case group was 2.41 ± 0.931 (range 2–5). Ninety ethnically matched healthy women (mean age 29.46 ± 4.91 years, ranged between 21 and 39 years) who had regular menstrual cycles, a history of at least two successful pregnancy outcomes, and without a history of miscarriage were enrolled as control group. The mean number of successful pregnancies in the control group was 2.47 ± 0.678 (range 2–5). A structured questionnaire form was used to obtain demographic and gynecologic data from all subjects by gynecologist. After approval of the Institutional Review Board/Ethics Committee, written informed consents were filled by all participants in accordance with principles of the declaration of Helsinki.

DNA extraction and genotyping

Venous blood samples were taken from all participants by venipuncture in EDTA containing tubes, and genomic DNA was extracted from whole blood leukocytes using the salting-out method as described by Miller et al. [35]. The quality and quantity of extracted genomic DNA were evaluated by a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, USA). We used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique for genotyping the miR-146a C > G, miR-149 T > C, miR-196a2 T > C, and miR-499 A > G polymorphisms. The desired DNA fragments were amplified using the specific primers that are given in Table 1. PCR amplification was conducted in a final volume of 25 μL containing 12.5 μL Taq DNA polymerase 2× Master Mix Red (Ampliqon, Odense M, Denmark), 10 pmol of each primers, and 100 ng genomic DNA. Thermocycling conditions were as follows: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 45 s, annealing at temperature that are given in Table 1 for 45 s and extension at 72 °C for 60 s, and final extension at 72 °C for 5 min. The PCR products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide and then visualized under ultraviolet transilluminator. To detect the miR-146a C > G, miR-149 T > C, miR-196a2 T > C, and miR-499 A > G polymorphisms, PCR products were digested by restriction endonucleases *SacI*, *PvuII*, *MspI*, and *BclI*, respectively (New England Biolabs, Beverly, MA) (Table 2). Finally, determination of genotype for miR-146a C > G, miR-149 T > C, miR-196a2 T > C, and miR-499 A > G polymorphisms was characterized based on length of digested fragments (Table 1), after separation of digested fragments by electrophoresis on 3% ethidium bromide-stained 3% agarose gels.

Statistical analysis

Statistical analysis was carried out using SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA), and all continuous variables were expressed as mean ± standard deviation (SD). Differences in SNP (miR-146a C > G, miR-149 T > C, miR-196a2 T > C, and miR-499 A > G) genotype and allele distributions between RPL patients and healthy controls were compared using Pearson's chi-square exact test. The strength of association between miR-146a C > G, miR-149 T > C, miR-196a2 T > C, and miR-499 A > G polymorphisms and RPL was evaluated by odds ratios (ORs) and 95% confidence intervals (CIs). To calculate the expected genotype distribution, we used Hardy-Weinberg Equilibrium (HWE) equation. The observed numbers of each

Table 1 Primers and restriction enzymes used to detect miR-146a C > G, miR-149 T > C, miR-196a2 T > C, and miR-499 A > G polymorphisms

Polymorphism	Sequence 5′ to 3′	Annealing temperature (°C)	PCR product (bp)	Restriction enzyme	Wild type	Heterozygote	Mutant
miR-146a C > G	F 5′-CATGGGTTGTGTC AGTGTCAGAGC-3′ R 5′-TGCCTTCTGTCTC CAGTCTTCCA-3′	62	147	*SacI*	147	147/122/25	122/25
miR-149 T > C	F 5′-TGTCTTCACTCCC GTGCTTGTCC-3′ R 5′-TGAGGCCCGAAA CACCCGTA-3′	65	254	*PvuII*	254	254/194/60	194/60
miR-196a2 T > C	F 5′-CCCCTTCCCTTCT CCTCCAGATA-3′ R 5′-CGAAAACCGACT GATGTAACTCCG-3′	63	149	*MspI*	149	149/125/24	125/24
miR-499 A > G	F 5′-CAAAGTCTTCACT TCCCTGCC-3′ R 5′-ATGT TTAACTCCTC TCCACGTGATC-3′	65	145	*BclI*	145	145/120/25	120/25

Table 2 The genotype distribution of miR-146a C > G, miR-149 T > C, miR-196a2 T > C, and miR-499 A > G polymorphisms in the case and control subjects

Genotypes	Cases (n = 120) (%)	Controls (n = 90) (%)	p value[a]	OR (95% CI)
miR-146a C > G			0.031	–
CC	67.50	50.00	0.015	0.481 (0.274–0.845)
CG	29.20	46.70	0.014	2.125 (1.200–3.763)
GG	3.30	3.30	1.000	1.000 (0.218–4.584)
Carrier (CG + GG)[b]	32.50	50.00	0.015	2.077 (1.183–3.646)
Frequency of G allele	17.91	26.65	0.042	0.600 (0.376–0.957)
HWE p value[c]	0.008	2.017		
miR-149 T > C			0.034	–
TT	58.30	75.60	0.012	2.208 (1.209–4.032)
TC	40.00	23.30	0.012	0.457 (0.248–0.840)
CC	1.70	1.10	1.000	0.663 (0.059–7.427)
Carrier (TC + CC)[b]	41.70	24.40	0.012	0.453 (0.248–0.827)
Frequency of C allele	21.66	12.77	0.020	1.888 (1.106–3.222)
HWE p value[c]	3.819	0.197		
miR-196a2 T > C			0.627	–
TT	74.20	78.90	0.513	1.302 (0.679–2.495)
TC	23.30	20.00	0.615	0.821 (0.421–1.601)
CC	2.50	1.10	0.637	0.438 (0.045–4.284)
Carrier (TC + CC)[b]	25.80	21.10	0.513	0.768 (0.401–1.473)
Frequency of C allele	14.15	11.11	0.380	1.320 (0.732–2.381)
HWE p value[c]	0.198	0.014		
miR-499A > G			0.083	–
AA	12.50	17.80	0.328	1.514 (0.705–3.251)
AG	47.50	56.70	0.211	1.145 (0.834–2.504)
GG	40.00	25.50	0.039	0.515 (0.283–0.936)
Carrier (AG + GG)[b]	87.50	82.20	0.328	0.661 (0.308–1.419)
Frequency of G allele	64.16	53.90	0.045	1.505 (1.015–2.231)
HWE p value[c]	0.092	1.769		

HWE Hardy-Weinberg Equilibrium, *OR* odds ratio, *CI* confidence interval

[a] Evaluated by Pearson's chi-squared test

[b] Carriers; individuals who had either heterozygous and homozygous specified polymorphism

[c] Calculated by Hardy-Weinberg Equilibrium

genotype were compared with those expected for a population in HWE by chi-square test available on (https://wpcalc.com/en/equilibrium-hardy-weinberg/). p values less than 0.05 were statistically considered as significant.

Results

The genotype distribution of miR-146a C > G, miR-149 T > C, miR-196a2 T > C, and miR-499 A > G polymorphisms in patients suffering from RPL and healthy controls is displayed in Table 2. Our findings revealed that the miR-149 T > C polymorphism was significantly higher in cases compared with healthy controls ($p < 0.05$) (Table 2). Surprisingly, the prevalence of miR-146a C > G polymorphism was more frequent in healthy controls compared with RPL patients (Table 2). We did not find any differences between cases and controls concerning miR-196a2 T > C and miR-499 A > G polymorphisms ($p > 0.05$) (Table 2).

When the heterozygosity frequency of all studied polymorphisms was compared, the difference between two groups was not statistically significant for miR-196a2 T > C and miR-499 A > G polymorphisms ($p > 0.05$) (Table 2). For miR-149 T > C polymorphism, we observed that TC genotype was more frequent in the case group than in control group ($p < 0.05$) (Table 2). Concerning miR-146aC > G polymorphism, the frequency of individuals with the heterozygous genotype was 46.70%, whereas 29.20% of RPL patients had such genotype. This difference is statistically significant ($p < 0.05$) (Table 2). No statistically significant difference was found for miR-146a C > G, miR-149 T > C, and miR-196a2 T > C polymorphisms, when the homozygosity frequency was compared between two groups ($p > 0.05$) (Table 2). Our results showed that the homozygous genotype for miR-499 A > G polymorphism was significantly higher in patients experiencing RPL compared with healthy controls ($p < 0.05$) (Table 2).

The prevalence of miR-146a G, miR-149 C, miR-196a2 C, and miR-499 G alleles in case and control subjects was also compared (Table 2). Our results revealed that the frequency of miR-149 C, and miR-499 G alleles was significantly higher in RPL patients in comparison with healthy controls ($p < 0.05$) (Table 2). For miR-196a2 C allele, the prevalence was different between patients and controls, but the difference was not statistically significant ($p > 0.05$) (Table 2). Also, the frequency of miR-146a G allele was significantly higher in the healthy women compared with patients ($p < 0.05$) (Table 2).

We also performed combination analyses for miR-146a C > G, miR-149 T > C, miR-196a2 T > C, and miR-499 A > G polymorphisms (Table 3). Our results revealed that the frequencies of miR-146a CC/miR-499 GG, miR-149 TC/miR-499 AG, and miR-196a2 TT/miR-499 GG combined genotypes were significantly higher in RPL patients ($p < 0.05$) (Table 3).

Discussion

Recently, a number of studies have provided convincing evidence of involvement of miRNA in the control of embryo implantation, embryo-maternal communication, and embryo development processes [20–22].

The success of each event is essential for a successful pregnancy. In this context, there has been accumulating evidence that the miR-146a, miR-149, miR-196a2, and miR-499 play crucial roles in such processes in female reproduction [36–48]. Cho et al. reported that the regulation of FOXL2 and CCND2 genes expression at mRNA levels is regulated by miR-146a [36]. The human FOXL2 gene encodes the multifunctional forkhead/HNF3 transcription factor and displays a highly conserved expression in the ovary [37]. High-throughput expression profiling experiments suggested the highest levels of FOXL2 expression in human endometrium during the proliferative phase [38]. Bellessort et al. suggested that conditional Foxl2 inactivation in the uterus results in infertility due to myometrial disorganization and vascular defects [39]. The human CCND2, the gene encoding cyclin D2, regulates G1-to-S phase transition during cell cycle progression and without sufficient cyclin D2, the cell will enter a non-proliferating state [40]. In the ovary, CCND2 is expressed mainly in granulosa cells and is required for granulosa cell proliferation during ovarian folliculogenesis [41]. It has also been reported that miR-146a downregulated Fas gene expression via targeting its 3′-UTR of this gene [42]. Panzan et al. revealed that women with idiopathic infertility and recurrent pregnancy loss have lower expression of FAS, which suggests a decrease in apoptotic signaling mechanisms in the epithelial cells during embryo implantation [43]. Lin et al. suggested that serine/threonine protein kinase AKT1 and transcription factor E2F1 represent direct target genes of miR-149 [44]. They also found that miR-149 is a pro-apoptotic miRNA by repressing the expression of Akt1 and E2F1 [44]. The inhibitory function of miR-196a on progesterone receptor (PGR) mRNA expression through activation of mitogen-activated protein kinase kinase/extracellular signal regulated kinase (MEK/ERK) signaling pathway has been previously reported [45]. MEK/ERK signaling pathway promotes cell proliferation, cell survival, and cell differentiation, and is closely related to embryo implantation during early pregnancy [46]. The human SOX6 gene was identified as a direct target of miR-499, which recruits c-terminal binding protein 2 (CtBP2) to repress transcription of fibroblast growth factor-3 (FGF-3) [47, 48]. FGF-3 is involved in cell proliferation and differentiation during developing embryonic tissues [47]. Therefore, it is not surprising that deregulation of miR-146a, miR-149, miR-196a2, and miR-499 expression and functions caused by gene mutation can affect female

Table 3 Combination analysis of miR-146a C > G, miR-149 T > C, miR-196a2 T > C, and miR-499 A > G polymorphisms in the case and control subjects

Genotypes	Cases (n = 120) (%)	Controls (n = 90) (%)	p value[a]	OR (95% CI)
miR-146a/miR-149				
CC/TT	45 (37.50)	27 (30.00)	0.304	1.400 (0.782–2.508)
CC/TC	34 (28.33)	17 (18.88)	0.143	1.698 (0.877–3.286)
CC/CC	2 (1.66)	1 (1.11)	1.000	1.508 (0.135–16.89)
CG/TT	23 (19.16)	38 (42.22)	0.0001	0.324 (0.175–0.602)
CG/TC	12 (10.00)	4 (4.44)	0.189	2.389 (0.744–7.670)
CG/CC	0 (0.00)	0 (0.00)	ND	ND
GG/TT	2 (1.66)	3 (3.33)	0.653	0.492 (0.080–3.005)
GG/TC	2 (1.66)	0 (0.00)	0.508	0.983 (0.961–1.007)
GG/CC	0 (0.00)	0 (0.00)	ND	ND
miR-146a/miR-196a2				
CC/TT	60 (50.00)	33 (36.66)	0.068	1.727 (0.988–3.019)
CC/TC	19 (15.83)	11 (12.22)	0.552	1.351 (0.608–3.003)
CC/CC	2 (1.66)	1 (1.11)	1.000	1.508 (0.135–16.89)
CG/TT	26 (21.66)	35 (38.88)	0.009	0.435 (0.237–0.797)
CG/TC	8 (6.66)	10 (11.11)	0.321	0.571 (0.216–1.512)
CG/CC	1 (0.83)	0 (0.00)	1.000	0.992 (0.976–1.008)
GG/TT	3 (2.50)	3 (3.33)	1.000	0.774 (0.147–3.773)
GG/TC	1 (0.83)	0 (0.00)	1.000	0.992 (0.976–1.008)
GG/CC	0 (0.00)	0 (0.00)	ND	ND
miR-146a/miR-499				
CC/AA	11 (9.16)	8 (8.88)	1.000	1.034 (0.398–2.689)
CC/AG	42 (35.00)	27 (30.00)	0.462	1.256 (0.699–2.259)
CC/GG	28 (23.33)	10 (11.11)	0.029	2.435 (1.114–5.321)
CG/AA	4 (3.33)	7 (7.77)	0.212	0.409 (0.116–1.44)
CG/AG	13 (10.83)	25 (27.77)	0.002	0.316 (0.151–0.661)
CG/GG	18 (15.00)	13 (14.44)	1.000	1.045 (0.483–2.263)
GG/AA	1 (0.83)	1 (1.11)	1.000	0.748 (0.046–12.12)
GG/AG	1 (0.83)	2 (2.22)	0.586	0.370 (0.033–4.412)
GG/GG	2 (1.66)	0 (0.00)	0.508	0.983 (0.961–1.007)
miR-149/miR-196a2				
TT/TT	53 (44.16)	52 (57.77)	0.070	0.578 (0.333–1.004)
TT/TC	16 (13.33)	15 (16.66)	0.558	0.769 (0.358–1.652)
TT/CC	1 (0.83)	1 (1.11)	1.000	0.748 (0.046–12.12)
TC/TT	34 (28.33)	18 (20.00)	0.197	1.581 (0.824–3.034)
TC/TC	12 (10.00)	3 (3.33)	0.102	3.222 (0.881–11.77)
TC/CC	2 (1.66)	0 (0.00)	0.508	0.983 (0.961–1.007)
CC/TT	2 (1.66)	1 (1.11)	1.000	1.508 (0.135–16.89)
CC/TC	0 (0.00)	0 (0.00)	ND	ND
CC/CC	0 (0.00)	0 (0.00)	ND	ND
miR-149/miR-499				
TT/AA	8 (6.66)	9 (10.00)	0.447	0.643 (0.238–1.738)
TT/AG	31 (25.83)	41 (45.55)	0.003	0.416 (0.223–0.745)
TT/GG	31 (25.83)	18 (20.00)	0.410	1.393 (0.721–2.692)
TC/AA	7 (5.83)	7 (7.77)	0.598	0.735 (0.248–2.174)
TC/AG	25 (20.83)	9 (10.00)	0.038	2.368 (1.046–5.364)
TC/GG	16 (13.33)	5 (5.55)	0.068	2.165 (0.920–7.431)
CC/AA	1 (0.83)	0 (0.00)	1.000	0.992 (0.976–1.008)

Table 3 (continued)

Genotypes	Cases ($n = 120$) (%)	Controls ($n = 90$) (%)	p value[a]	OR (95% CI)
CC/AG	0 (0.00)	1 (1.11)	0.429	1.011 (0.989–1.034)
CC/GG	1 (0.83)	0 (0.00)	1.000	0.992 (0.976–1.008)
miR-196a2/miR-499				
TT/AA	12 (10.00)	13 (14.44)	0.391	0.658 (0.285–1.520)
TT/AG	40 (33.33)	42 (46.66)	0.063	0.571 (0.326–1.002)
TT/GG	37 (30.83)	16 (17.77)	0.037	2.062 (1.060–4.009)
TC/AA	3 (2.50)	3 (3.33)	1.000	0.774 (0.147–3.773)
TC/AG	14 (11.66)	8 (8.88)	0.650	1.354 (0.542–3.381)
TC/GG	11 (9.16)	7 (7.77)	0.807	1.197 (0.445–3.219)
CC/AA	1 (0.83)	0 (0.00)	1.000	0.992 (0.976–1.008)
CC/AG	2 (1.66)	1 (1.11)	1.000	1.508 (0.135–16.89)
CC/GG	0 (0.00)	0 (0.00)	ND	ND

OR odds ratio, *CI* confidence interval, *ND* not determined

[a] Evaluated by Fisher's chi-squared test

reproduction and fertility. Hence, in the present study, we investigated the possible association between miR-146a C > G, miR-149 T > C, miR-196a2 T > C, and miR-499 A > G polymorphisms and the occurrence of RPL in North-West of Iran. In summary, our results revealed that there is a positive association between miR-149 T > C polymorphism and increased susceptibility to RPL ($p < 0.05$) (Table 2). The miR-149 C and miR-499 G alleles were also found to be significantly associated with RPL in our population ($p < 0.05$) (Table 2). Our results are consistent with some previous findings that have been published in this field [30–32]. However, other studies have shown conflicting results and the possible association between miR-146a C > G, miR-149 T > C, miR-196a2 T > C, and miR-499 A > G polymorphisms and RPL is still controversial [29, 33]. For miR-146a C > G polymorphism, we found no statistically significant difference when comparing the prevalence of this polymorphism between RPL cases and healthy controls ($p > 0.05$) (Table 2). Our findings are in agreement with two previous studies reporting no associations between miR-146a C > G polymorphism and RPL [29, 30]. Jeon et al. studied 330 patients with RPL and 200 healthy women for miR-146a C > G polymorphism, and they failed to find an association between this polymorphism and occurrence of RPL in the Korean population [29]. Similarly, Parveen et al. found no significant association between miR-146a C > G polymorphism and RPL [30]. In the case of miR-149 T > C polymorphism, our findings showed that the allele frequency and genotype distribution of this polymorphism were significantly higher in cases in comparison with healthy controls ($p < 0.05$) (Table 2). This data suggest a positive association between miR-146a C > G polymorphism and RPL, and it seems that the presence of the miR-149 C allele increases risk of early fetal loss. This result is inconsistent with previous studies. To the authors knowledge, two studies have been previously investigated the association between the miR-149 T > C polymorphism and risk of unexplained RP [29, 30]. Both groups of investigators found that there is no association between miR-149 T > C polymorphism and RPL [29, 30]. In the case of miR-196a2 T > C polymorphism, we found that the genotypic and allelic frequencies of this polymorphism were the same in both case and control groups ($p > 0.05$) (Table 2).

Our results are in accordance with the findings of several previous studies that demonstrated no association between miR-196a2 T > C polymorphism and RPL [29–31, 33]. By contrast, Amin-Beidokhti et al. reported that miR-196a2 T > C polymorphism is associated with the risk of RPL [34]. For miR-499 A > G polymorphism, miR-499 G allele was significantly associated with RPL in our population ($p < 0.05$) (Table 2). A case-control study conducted that Parveen et al. showed that miR-499 A > G polymorphism might be a susceptibility factor for RPL among North Indian women [30]. Fazli and coworker studied 100 RPL patients and 100 healthy controls for miR-499 A > G polymorphism, and reported that miR-499 A > G polymorphism predispose women to an increased risk for RPL [31]. Similar data were obtained in a study by Rah et al. who investigated the miR-499 A > G polymorphism in 387 RPL patients and 225 healthy controls [32]. By contrast, no statistically significant differences in genotypic or allelic frequencies for miR-499 A > G polymorphism were observed when RPL patients was compared with controls in other studies [29, 33, 34]. Discrepancies of miR-146a C > G, miR-149 T > C, miR-196a2 T > C, and miR-499 A > G genotype/allele frequencies and their association with increased risk of RPL between the current study and published data may be explained in part by the racial differences of

populations, differences in number of studied subjects, and multifactorial etiology of RPL. Therefore, further large-scale population-based studies in various populations are needed to firmly validate these findings.

In conclusion, our study suggests that the miR-149 T > C polymorphism and the presence of miR-149 C and miR-499 G alleles may be associated with increased risk of RPL.

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Compliance with ethical standards

This project was approved by faculty of sciences, Islamic Azad University of Tabriz branch, Tabriz, Iran on 2016-05-12. the questionnaires were taken by research committee of faculty of sciences and then they were reviewed in accordance with research ethical standards to provide ethical code and finally they were archived there confidential, so we don't access to them now.

Conflict of interest The authors declare that they have no conflicts of interest.

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