Research Article

Association of Polymorphisms in miR146a, an Inflammation-Associated MicroRNA, with the Risk of Idiopathic Recurrent Spontaneous Miscarriage: A Case-Control Study

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Received 17 February 2022; Revised 9 April 2022; Accepted 13 April 2022; Published 30 April 2022

Academic Editor: Sachchida Nand Rai

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It has been established that microRNAs (miRNAs) are involved in the regulation of immune responses and serve as biomarkers of inflammatory diseases as well as recurrent spontaneous miscarriage (RSM). Herein, we aimed to study the relationship between three functional miR146a gene polymorphisms with idiopathic RSM (IRSM) susceptibility. We recruited 161 patients with IRSM and 177 healthy women with at least one live birth and without a history of abortion. Genotyping was performed using RFLP-PCR and ARMS-PCR methods. We found that the rs6864584 T/C decreased the risk of IRSM under dominant TT+TC vs. CC (OR = 0.029) and allelic C vs. T (OR = 0.028) contrast models. Regarding rs2961920 A/C and rs57095329 A/G polymorphisms, the enhanced risk of IRSM was observed under different genetic contrasted models, including the codominant CC vs. AA (OR = 2.81 for rs2961920) and codominant GG vs. AA (OR = 2.36 for rs57095329). After applying a Bonferroni correction, haplotype analysis revealed a 51% decreased risk of IRSM regarding the ACA genotype combination. This is the first study reporting that miR146a rs57095329 A/G, rs2961920 A/C, and rs6864584 T/C polymorphisms are associated with the risk of IRSM in a southern Iranian population. Performing replicated case-control studies on other ethnicities is warranted to outline the precise effects of the studied variants on the risk of gestational trophoblastic disorders.

1. Introduction

Recurrent spontaneous miscarriage (RSM) is defined as the occurrence of 3 consecutive losses < 20 weeks gestation [1]. This condition has a fascinating yet poorly understood etiology. It affects about 5% of couples; due to its complex and often undefined etiology, RSM has been a tremendous challenge in reproductive medicine [1, 2]. Exposure to environmental factors, genetics and hormonal abnormalities, immune reactions, embryo-fetal infections, uterine pathologies, antiphospholipid syndrome, sperm oxidative stress, drug abuse, and psychological trauma have been implicated as etiologic factors of RSM [3–5]. However, 40-60% of the RSM cases have no specific pathology and thus are classified as unexplained or idiopathic RSM (IRSM) [6]. Lately, several single nucleotide polymorphisms (SNPs) have been associated with IRSM occurrence [7–9]. Of those, SNPs that resided in genes encoding microRNAs (miRNAs or miRs)
have been brought to the attention [10]. Essentially, a significant proportion of IRSM might be due to SNPs in coding- or noncoding genome [11].

Thus far, growing evidence has demonstrated that deregulated miRNA expression is associated with female reproductive diseases, ranging from repeated implantation failure to IRSM [12, 13]. MicroRNAs (miRNAs), as single-stranded RNA molecules of approximately 20-22 nucleotides, act as posttranscriptional regulators of gene expression by translational repression or messenger RNA degradation [14]. Today, it has been established that a single miRNA regulates several targets and therefore acts as a master control of gene expression. Even though miRNAs comprised less than 3% of the human genome, bioinformatics tools have shown that nearly 30% of human genes are targeted by these non-coding RNAs [14], indicating the important function of miRNAs in both pathological and physiological processes. Many miRNAs were already associated with RSM [15]. Among those, miR165a-3p, miR408-5p, miR143-3p, miR4235, miR5386, miR5813, miR5525, miR4407, miR5567, etc., were differentially expressed between normal and abnormal embryos [16]. Besides, while miR-517c, miR-519a-1, miR-522, miR-520h, miR-184, miR-187, and miR-125b-2 were upregulated in RSM, miR-520f, miR-3175, and miR-4672 were downregulated in RSM [17].

As an inflammation-associated miRNA, miR146a is a candidate miRNA found dysregulated in different diseases, including tumors, coronary artery disease, periodontal disease, epilepsy, and, as previously shown, preeclampsia [18–22]. Moreover, miR146a mediates inflammatory pathways in preterm birth patients [23]. It has been shown that miR146a mediates inflammatory changes and tissue fibrosis [24]. Su et al. reported that administration of scavenger receptor/Toll-like receptor 9 agonist (C-miR146a) suppressed the expression of nuclear factor-kappa light chain receptor/Toll-like receptor 9 agonist (C-miR146a) suppresses the expression of nuclear factor-kappa light chain enhancer of activated B cell (NF-kB) target genes [25]. Upregulation of miR146a has been associated with inhibiting inflammatory responses in lipopolysaccharide- (LPS-) induced acute lung injury [26].

Mutations alter the total expression and prematurity processing of miR-146a and affect susceptibility to multiple diseases [27]. Functional miR146a variants, rs57095329 A>G [minor allele frequency (MAF): 0.021-0.5], rs2961920A/C (MAF: 0.168-0.453), and rs6864584 T/C (MAF: 0.001-0.392) reside in the promoter of the miR146a precursor gene. It has already been established that rs2910164 G>C and rs57095329 A>G polymorphisms can potentially alter miR146a function and/or expression [28].

For all we know, no study has integrated these findings into the relationship between these SNPs and IRSM. Hence, in this preliminary study, we aimed to examine the relationship between three miR-146a SNPs (rs2961920A/C, rs57095329 A/G, and rs6864584 T/C) with IRSM incidence in a south-east Iranian population.

2. Subjects and Methods

2.1. Participants. The study group consisted of 161 women with idiopathic IRSM, who presented with a history of at least two consecutive pregnancy losses at less than 20 weeks of gestation and 177 healthy women as controls. The enrollment procedure has been described in our previous work [29]. The local Ethics Committee of Zahedan University of Medical Sciences approved the study (IR.ZAUMS.REC.1399.093). Informed consent was taken from all participants.

2.2. DNA Extraction and Genotyping. The salting-out method was employed to extract DNA from the whole venous blood of participants. A NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) was used to verify the quantity and purity of the extracted DNAs. Electrophoresis on agarose gel was carried out to evaluate the quality of extracted DNA.

Genotyping of rs6864584 polymorphisms was performed via both polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The used primer sequences were already reported in Sargazi et al.’s study [30]. For detecting all three SNPs, PCR was performed in a total volume of 15 μL, containing 1 μL (100 ng/μL) genomic DNA, 1 μL of each primer (6 pmol), 10 μL Taq DNA Polymerase 2X Master Mix Red (Ambion, Denmark), and 2 μL of distilled water. The PCR conditions were as follows: denaturation at 95°C for 5 min, 35 cycles at 95°C for 35 s, annealing temperature (according to Table 1 for each SNP) for 30 s and 72°C for 45 s, and a final extension at 72°C for 5 min. PCR products were electrophoresed on 1.5% agarose gel and stained in 0.25 μg/mL ethidium bromide, and bands were visualized (Figure 1). The C allele of rs6864584 was digested by the ApaI and produced a 152- and 148 bp band, and the undigested T allele has a 300 bp fragment. Regarding rs57095329 and rs2961920, we used allele-specific amplification-refractory mutation system-polymerase chain reaction (ARMS-PCR) [31] in two well-defined conditions. For this purpose, the forward primer for one allele and the common primer were placed in one well. At the same time, the other forward primer specific for the other allele and the common primer were added to another well, and PCR reaction was performed following the condition above. In the presence of either the homozygous genotypes, the amplified band will be visualized in separate wells. In contrast, the bands can be visualized in both wells in the presence of a heterozygous genotype. The amplicon sizes for genotyping rs57095329 and rs2961920 were 153 and 221 bp, for both A and G or A and C alleles, respectively (Table 1). The bands were then displayed via agarose gel electrophoresis (Figure 1). About 30% of the samples were randomly regenotyped for quality control, and the reproducibility of the results was 100%.

2.3. Statistical Analysis. The SPSS 23.0 software (IBM Corporation, Armonk, NY) was used for data analysis. Deviations from Hardy Weinberg equilibrium (HWE) were examined using a chi-square test. Fisher’s exact test was used to compare differences between data sets. The association between the alleles and genotypes of miR146a polymorphisms and IRSM risk was examined by odds ratios (ORs) and 95% confidence intervals (CIs), using unconditional logistic regression analysis. The SHEsis software platform
Table 1: The primers used for genotyping of miR146a gene polymorphisms.

| SNP     | Primers       | Sequence (5’ to 3’)          | Annealing temperature | Length of PCR product (bp) | Restriction enzyme |
|---------|---------------|------------------------------|-----------------------|--------------------------|-------------------|
| rs2961920 C/A | F (C-allele) | GTGTATAAGGAAGCCTCACGC        | 58°C                  | C:221                    | -                 |
|         | F (A-allele)  | GTGTATAAGGAAGCCTCACGA        |                       | A:221                    |                   |
|         | R (common)    | TGAACACCGGACATCTGAC          |                       |                          |                   |
| rs6864584 C/T | F             | CGGAGAGTACAGACAGGAAGC        | 58.5°C                | T: 300                   | ApaI              |
|         | R             | TAGATCCCTCCTCGGACACGC        |                       | C: 148 + 152             |                   |
| rs57095329 A/G | F (A-allele) | CGGGACTCGGAGAGTACGGA         | 61°C                  | A:153                    | -                 |
|         | F (G-allele)  | CGGGACTCGGAGAGTACGGG         |                       | G:153                    |                   |
|         | R (common)    | TTGGAGCACGTGTCAGGACG         |                       |                          |                   |

F: forward; R: reverse; PCR: polymerase chain reaction; SNP: single-nucleotide polymorphism.

Figure 1: Gel photograph of PCR amplification products of the miR146a (a) rs2961920 A/C, (b) rs6864584 C/T, and (c) rs57095329 A/G polymorphisms.
was used to determine the amount of linkage disequilibrium (LD) between the studied SNPs [32]. Statistical significance considered as a $p$ value $< 0.05$.

### 3. Results

The subject’s ages differed from 15 to 55 years, with the mean age of 31.6 ± 6.8 (in controls) and 32.5 ± 6.6 (in IRSM patients). The mean (SD) BMI of controls was 26.2 ± 4.4 versus 26.3 ± 4.7 in cases. We found no marked difference between cases and controls regarding age ($p = 0.23$) and body mass index (BMI, $p = 0.77$). For all the studied SNPs, we did not observe any departure from HWE in healthy women ($p$ value for HWE $> 0.05$ as shown in Table 2), indicating the proper selection of the controls.

Genotypic and allelic distribution of miR146a SNPs is presented in Table 2. We found that the AC genotype of rs2961920, the TC genotype of rs6864584, and the AG genotype of rs57095329 were more frequent in controls and selected as the reference genotypes. Both rs57095329 A/G and rs2961920 A/C enhanced the risk of IRSM in our population, whereas alleles and genotypes of rs6864584 C/T showed a protective role against the disease. The C allele of rs2961920 A/C increased the risk of IRSM under codominant CC vs. AA (OR $= 2.81$, CI $= (1.34-5.88)$, and $p = 0.005$), recessive CC vs. AC+AA (OR $= 2.77$, CI $= (1.39-5.54)$, and $p = 0.003$), and allelic C vs. A (OR $= 1.41$, CI $= (1.03-1.93)$, and $p = 0.032$) genetic patterns. In terms of rs57095329 A/G, an increased risk of developing IRSM was found under the codominant GG vs. AA (OR $= 2.36$), codominant AG vs. AA (OR $= 1.87$), and dominant AA+AG vs. GG (1.94) modes of inheritance. The G allele of this SNP enhanced the risk of IRSM by 1.52 folds ($OR (95\% CI)=1.52 \times 1.11 = 1.68 \times 0.95$, $p = 0.028$). A diminished risk of IRSM was also noticed under the dominant TT+TC vs. CC (OR $= 0.68$) genetic pattern of this variation.

| SNP          | Type | IRSM (%) | Control (%) | Genetic model | OR (95% CI) | $p$ value |
|--------------|------|----------|-------------|---------------|-------------|-----------|
| rs2961920 A/C | HWE  | 0.865    | 0.073       | Recessive CC vs. AC+AA | 2.77 (1.39-5.54) | 0.003     |
|              | A    | 190 (59.0) | 237 (66.9) | Overdominant AC vs. AA+CC | 0.80 (0.52-1.23) | 0.317     |
|              | C    | 132 (41.0) | 117 (33.1) | Allelic C | 1 [reference] | 1.41 (1.03-1.93) | 0.032     |
| rs6864584 C/T | HWE  | 0.768    | 0.358       | Recessive CC vs. TT+CC | 0.69 (0.44-1.07) | 0.118     |
|              | T    | 243 (75.5) | 239 (67.5) | Allelic T | 1 [reference] | 0.68 (0.48-0.95) | 0.028     |
|              | C    | 79 (24.5)  | 115 (32.5) | Allelic C | 1.41 (1.03-1.93) | 0.032     |
| rs57095329 A/G | HWE  | 0.008    | 0.171       | Recessive GG vs. AG+AA | 1.62 (0.80-3.26) | 0.237     |
|              | A    | 185 (57.5) | 238 (67.2) | Overdominant AG+GG vs. AA+CC | 1.52 (0.99-2.34) | 0.070     |
|              | G    | 137 (42.5) | 116 (32.8) | Allelic G | 1 [reference] | 1.52 (1.12-2.08) | 0.011     |

SNP: single-nucleotide polymorphism; CI: confidence interval; OR: odds ratio; IRSM: idiopathic recurrent spontaneous miscarriage; HWE: Hardy-Weinberg equilibrium. $p < 0.05$ was considered statistically significant.
Bonferroni correction.

miR146a has a pivotal role in modulating inflammatory responses, as it negatively modulates the inflammatory cytokines, including interleukin 6 (IL-6) and interleukin 8 (IL-8) [36], T-helper 17 (Th17) [34], T-helper 1 (Th1) [35], and NF-kB pathway [36]. Genetic variations in the miR146a gene have been correlated with the risk of different diseases. We had already shown that different haplotypes and genotypes of miR146a polymorphisms could influence the odds of developing chronic kidney disease, another inflammatory condition [30]. However, no study has reported the link between these functional miR146a polymorphisms and IRSM in the Iranian population. The current study’s findings demonstrated that alleles and genotypes of miR146a SNPs were associated with the risk of IRSM. Besides, we found that a haplotype of miR146a polymorphisms decreased the disease risk by 51%. Our results suggest that miR146a might be a biomarker for IRSM susceptibility.

A plethora of studies has established the link between miR146a variations and susceptibility to sepsis [37], lupus erythematosus [28], type 1 diabetes [38], type 2 diabetes [39], coronary heart disease [40], ischemic stroke [41], pre-eclampsia [21, 42], and different types of cancer including hepatocellular carcinoma [43], prostate cancer [44], breast cancer [45], and cervical cancer [46]. Still, a minority of studies have failed to report a correlation between SNPs in this gene and susceptibility to ischemic stroke [40] and colorectal cancer [47].

As the first report on the Iranian population, Alipour and coworkers demonstrated an association between an SNP located in miR146a and IRSM risk in 2019. In this connection, they screened the rs2910164 C/G polymorphism on 120 Iranian women diagnosed with IRSM and 90 controls. They discovered significant associations between this SNP and RSM incidence in CC vs. GG (OR = 0.481, p = 0.015) and CG vs. GG (OR = 2.125, p = 0.014) genetic models. Besides, individuals who had either heterozygous or homozygous specified polymorphism (carriers of CG+GG genotype) had a 2.077-fold increased RSM risk [48]. The findings of this report have established the role of miR146a, as a circulating RNA, and SNPs located in miR146a in the pathophysiology of IRSM. Two years later, Babakhazadeh et al. failed to report an association between CC vs. CG +GG (p = 0.854) and CC+CG vs. GG (p = 0.282) genotypes of miR146a rs2910164 polymorphism and risk of IRSM in women from Yazd Province, located in the center of Iran [49]. Other SNPs in the miR146a gene, rs6864584 T/C, rs2961920 A/C, and rs57095329 A/G, were less studied. Still, the findings of some studies support the contribution of these variants to susceptibility to different diseases. Zha et al. found a significant correlation between allele and genotypes of miR146a rs57095329 A/G polymorphism and the risk of developing coronary artery lesions in Kawasaki patients. Meanwhile, the miR146a rs6864584 T/C was not correlated with the risk of developing Kawasaki disease [50]. In another case-control study, Devanandan et al. proposed that the A_rs57095329G_rs2910164 enhances the risk of developing acute lymphoblastic leukemia by fold, whereas the G_rs57095329G_rs2910164 represented a protective haplotype for the disease [51].

To this date, no study has reported the possible correlation between these two miR146a SNPs with the risk of gestational trophoblastic diseases. There are very limited reports on the correlation between miR146a rs2961920 A/C and the risk of a multifactorial disease. In this context, Guo

4. Discussion

miR146a has a pivotal role in modulating inflammatory responses, as it negatively modulates the inflammatory cytokines, including interleukin 6 (IL-6) and interleukin 6 (IL-8) [33], T-helper 17 (Th17) [34], T-helper 1 (Th1) [35], and NF-kB pathway [36]. Genetic variations in the miR146a gene have been correlated with the risk of different diseases. As illustrated in Figure 3, miR146a rs57095329 A/G and rs2961920 A/C polymorphisms are not located in the conserved regions across multiple mammalian species.

We used the WebLogo server to determine the conservation of DNA sequences containing the studied polymorphism sites. As illustrated in Figure 3, miR146a rs57095329 A/G and rs6864584 C/T polymorphisms are not located in the conserved regions across multiple mammalian species.
and associates genotyped 502 healthy controls and 529 patients diagnosed with glioma using the Agena MassArray Gold technique. Their findings revealed that rs2961920, as a promotoric variant for miR146a and an intronic variant for MIR3142HG, enhanced the glioma susceptibility in Chinese subjects (OR = 1.53, p = 0.019) [52]. In another study, Oetting et al. showed that rs2961920, a proxy for rs2910164, can independently predict acute rejection in kidney transplant recipients [53]. In agreement with these reports, we found the enhanced risk of IRSM in Iranian subjects carrying the CC genotype of miR146a rs2961920 A/C under codominant CC vs. AA, recessive CC vs. AC+AA, and allelic C vs. A mode of inheritance.

As a circulating miRNA, miR146a controls megakaryocyte differentiation, adaptive immunity, and cancer [54]. On the other hand, completion of pregnancy highly depends on maternal immune tolerance to the fetal-placental unit, where imbalances between some signaling molecules (specifically Th1- and Th2-type cytokines) increase the incidence of miscarriage [55].

The decidual tissue contains different cytokine-producing cells [56]. Zhao et al. showed that miR146a-5p is downregulated in the decidua of RSM patients compared with healthy controls [55]. Moreover, it has been established that miR146a improves the decidual cytokine microenvironment through modulating the Toll-like receptor (TLR) pathway and other possible mechanisms of RSM pathogenesis [57]. Furthermore, Chen et al. proposed that miR146a down-regulates TLR4, tumor necrosis factor receptor-associated factor-6 (TRAF6), and IL-1 receptor-associated kinase 1 (IRAK1) and phosphorylates NF-κB. Consequently, suppressing TLR4/TRAF6/JNK and TLR4/NF-κB pathways inhibits proinflammatory cytokine production [58]. This is important since the upregulation of NF-κB at the protein or gene level is involved in RSM pathogenesis [59, 60]. Gysler and colleagues reported that transfection of trophoblastic cells with miR146a-5p mimic caused increased IL8 secretion. However, in contrast, patients with adverse pregnancy outcomes express higher levels of miR146a-3p than healthy pregnant women experiencing no pregnancy complications [61]. Together, TLR4- and NF-κB-dependent miR-146a expression might contribute to the protective role of miR-146a polymorphisms against IRSM.

RSM is a very heterogeneous disease, and various etiologies are implicated in RSM, including factors known to be causative and those implicated as possible causative agents [i.e., genetic causes (aneuploidy, somatic mutations, and SNPs) and immunologic causes]. Autoimmune and alloimmune causes, anatomic causes, uterine Müllerian anomalies, infectious causes, environmental causes, smoking, endocrine factors, diabetes mellitus, and hematologic disorders are the common etiologic factors for recurrent abortion [62–66]. Dysregulation of many genes, including miR146a, is important in preserving pregnancy; therefore, SNPs within these genes might predict RSM susceptibility. Although the etiology of RSM is still unclear, discovering candidate SNPs can help understand the pathophysiology of this complex disease. Still, other mechanisms are involved in the pathogenesis of IRSM that remains to be fully elucidated.

Table 4: Haplotype analysis of miR146a polymorphisms in women with IRSM and healthy women.

| rs2961920 A/C | rs6864584 C/T | rs57095329 A/G | IRSM | Control | OR (95% CI) | p value* |
|--------------|--------------|----------------|------|---------|-------------|---------|
| A            | T            | T              | 90 (27.9) | 110 (31.2) | 1 [reference] |        |
| A            | C            | A              | 24 (7.5)  | 60 (17.1)  | 0.49 (0.28–0.85) | 0.010  |
| A            | C            | G              | 21 (6.5)  | 19 (5.3)   | 1.35 (0.68–2.67) | 0.385  |
| A            | T            | G              | 55 (17.1) | 47 (13.4)  | 1.43 (0.89–2.31) | 0.142  |
| C            | C            | A              | 13 (3.9)  | 11 (3.0)   | 1.44 (0.62–3.38) | 0.395  |
| C            | C            | G              | 21 (6.6)  | 25 (7.1)   | 1.03 (0.54–1.95) | 0.936  |
| C            | T            | A              | 58 (18.1) | 57 (16.0)  | 1.24 (0.78–1.97) | 0.352  |
| C            | T            | G              | 40 (12.4) | 25 (7.0)   | 1.96 (1.10–3.47) | 0.020  |

OR: odds ratio; CI: confidence interval; IRSM: idiopathic recurrent spontaneous miscarriage. *p < 0.017 was considered statistically significant after the Bonferroni correction.
DNA variations might serve as a valuable prognostic factor to predict the risk of developing such gestational diseases and can be exploited in “Personalized Medicine” applications [67, 68].

Despite all the efforts made, there were some limitations to the current study. The main limitation was using a relatively small sample size. Although we could not perform sequencing to validate our results, random regenotyping of samples showed a very high genotyping accuracy. Moreover, an assessment of different hormones in the serum samples of IRSM patients and healthy controls is needed to correlate miR-146a with serum hormones and give a better idea of the clinical significance of this circulating miRNA. Finally, mRNA levels of miR146a were not evaluated to determine the link between genotypes of miR146a polymorphisms and miR146a expression, which requires further study.

5. Conclusion

This study first reported that miR146a rs57095329 A/G, rs2961920 A/C, and rs6864584 T/C polymorphisms are correlated with the risk of IRSM in a southern Iranian population. These results provide an opportunity to approach the problems of prognosis and diagnosis of RSM. Still, performing replicated case-control studies on other ethnicities is warranted to outline the precise effects of the studied variants on the risk of gestational disorders.

Data Availability

Derived data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

The local Ethics committee of Zahedan University of Medical Sciences approved the study (Ethical code: IR.ZAUMS.REC.1399.093) (the webpage of ethical approval code is available at https://ethics.research.ac.ir/EthicsProposalViewEn.php?id=138328).

Consent

Informed consent was taken from all participants.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors’ Contributions

SS and SS contributed to methodology and writing the draft, BM, MM, SS, and SM contributed to genotyping, MHN carried out data analysis, MG contributed to clinical patient assessment, and SS contributed to supervision and editing.
Disease Markers

Acknowledgments

Zahedan University of Medical Sciences (No. 9729) provided financial support for the present research. The authors would like to thank the technical operators of Moloud Infertility Center (Zahedan, Iran).

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