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Recurrent Miscarriage and Micro-RNA Among North Indian Women

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Abstract

Micro-RNAs (miRNAs) regulate diverse cellular processes such as cell differentiation, proliferation and apoptosis. Mutation in miRNAs results in various pathological conditions such as inflammation, viral infections, neurodegeneration, autoimmunity, and so on. We have evaluated the association of miR-146aC > G (rs2910164), miR-149T > C (rs2292832), miR-196a2T > C (rs11614913), and miR-499A > G (rs3746444) among patients with recurrent miscarriage (RM) and controls from North India. All the 200 patients with RM reported to experience at least 3 unexplained miscarriages before 20th week of gestation. Three hundred fertile women with no history of RMs were taken as controls. Both patients and controls were genotyped by the polymerase chain reaction amplification followed by restriction fragment length polymorphism. Variant alleles and genotypes of miR-499 A > G (Single Nucleotide Polymorphism Database [dbSNP] ID rs3746444) were found to be significant risks associated with patients having RM (odds ratio [OR] = 1.98; 95% confidence interval [CI] = 1.40-2.81; *P* value = .0001) and controls (OR = 3.64; 95% CI = 1.33-9.94; *P* value = .0109). A significant susceptible effect was found at allelic level in miR-196aT > C (dbSNP ID rs11614913) and miR-499 A > G (dbSNP ID rs3746444).

Keywords

recurrent miscarriage, single-nucleotide polymorphism, micro-RNA

Introduction

Recurrent miscarriage (RM) is defined as the loss of 3 or more consecutive pregnancies before 20th week of gestation, affecting 1% of couples trying to conceive. There are various factors involved in RMs. These are uterine anomalies, chromosomal abnormalities, endocrine dysfunction, thrombophilia, immune disorders, environmental factors, maternal infections, and so on. Among immune disorders, it is known that inflammation may regulate micro-RNA (miRNA) biogenesis. There are specific transcription factors found in the Toll-like receptor ligands, cytokines, and so on, which may alter the miRNA expression level.[1] It has been reported that various cytokines may be responsible for the deregulation of Dicer expression resulting in an aberrant premature miRNA (pre-miRNA) processing.[2]

It has been reported that there are placenta-derived RNAs that can be detected in maternal plasma.[3,4] The latter offers the possibility for noninvasive profiling of placental gene expression and the detection of diseases associated with placental dysfunction.[5] Fetal miRNAs in maternal plasma can be used to obtain valuable information about the fetus or pregnancy, either for prenatal diagnosis and monitoring or for the detection of pregnancy disorders such as fetal growth restriction and so on.[6] The small noncoding RNA molecules consisting of 21 to 24 nucleotides act as negative regulators of gene expression and belong to the family of miRNA. They are also involved in the posttranscriptional regulation by binding to complementary sequences on target messenger RNAs (mRNAs) and promote mRNA degradation or translational repression.[7] The binding of miRNAs to mRNAs occurs in the cytoplasm in conjunction with the RNA-induced silencing complex and suppresses mRNA translation.[8] Till date, more than 1500 human miRNAs have been identified and it has been estimated that miRNA targets more than 5300 human genes, which may modulate several developmental and physiological processes including pregnancy.

Most of the miRNAs are placed in the intergenic regions or antisense sequences of genes[9] and are usually transcribed by RNA polymerase II to generate primary miRNAs (pri-miRNAs).[10] The Pri-miRNAs get converted into pre-miRNAs by the DGCR8–Drosha complex. Premature miRNAs are further transported to the cytosolic matrix in a process involving the shuttle protein exportin 5 and cleaved into the mature miRNA form by the endoribonuclease Dicer.[11] Polymorphisms

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of pre-miRNAs were first reported in 2005 and since then several miRNA polymorphism-association studies have been reported.[12,13] Some pre-miRNA polymorphisms have negative effect on mature miRNA expression,[14,15] showing that pre-miRNA sequences are important for miRNA processing.

Several studies have revealed that miRNAs play important role in different regulatory pathways such as control of developmental timing, hematopoietic cell differentiation, apoptosis, cell proliferation, and organ development.[16] Micro-RNAs have also been implicated in various human diseases such as cancers, human brain dysfunctions, glioma, cardiovascular disease, primary muscular disorders, diabetes, endometriosis, preeclampsia, infertility, and RMs.[17-22] The association of variants of miRNAs with RM is still not very clear. Therefore, in order to investigate whether or not there is a genetic association of the miRNA with the outcome of pregnancy, we made an attempt to investigate the role of miR-146aC > G (rs2910164), miR-149T > C (rs2292832), miR-196a2T > C (rs11614913), and miR-499A > G (rs3746444) gene polymorphisms in miscarriages. We selected these miRNAs on the basis that targets of miR-146a, -149, -196a2, and -499 are *FAS*,[23] *E2F1*,[24] *HOXB8*,[25] and *SOX6*,[26] respectively. *FAS* induces apoptosis,[27] *E2F1* regulates cell cycle progression,[27] *HOXB8* is involved in the suppression of myeloid differentiation,[28] and *SOX6* is associated with transrepression of fibroblast growth factor 3 (FGF-3), promoting cell growth.[26] The 146aG > C single-nucleotide polymorphism (SNP) exists in the stem region opposite to the mature miR-146a sequence, the 149C > T SNP exists in the terminal loop of the pre-miR-149 sequence, the 196a2C > T SNP is located in the 3p mature miRNA region of miR-196a2, and the 499A > G SNP is in the seed region of miR-499-3p. To the best of our knowledge, this is the first study from this part of the country.

Materials and Methods

All patients with RM were selected from patients referred to the outpatient department of Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS) Lucknow and Queen Mary Hospital of King George Medical College (Lucknow, Uttar Pradesh, India) for the evaluation of RM. In the present study, we selected 200 patients with RMs who had no known cause of RM. Patients with RM had at least 3 spontaneous miscarriages (mean 4, range 3-7) and no history of successful pregnancy. All selected patients were with primary abortion, having no live child. The patient's detailed clinical information was recorded before inclusion in this study.

All the patients were screened for various known causes of miscarriages, including parental chromosomes, day 2 hormone levels of follicle-stimulating hormone (3-11 U/L), luteinizing hormone (3-12 U/L), troponin (0.5-3 nmol/L), antiphospholipid antibodies, including lupus anticoagulant (PLR 0.8-1.05) and anticardiolipin antibodies (immunoglobulin [Ig] G 0-12 GPL units, IgM 0-5 MPL units). Because all the patients were referrals from other hospitals, karyotyping of the product of miscarriage was not possible and only the karyotyping of both partners was carried out. Other factors screened were prothrombotic risk factors, including activated protein C resistance (2.6:4.36 ratio), factor V Leiden, and prothrombin mutations; investigation of luteal phase insufficiency, prolactin dosage, glycemic curve, thyroid hormone levels; and investigation of toxoplasmosis, cytomegalovirus, rubella, HIV, group B Streptococci, *Chlamydia trachomatis*, hepatitis B and C, and bacterial vaginosis. The uterine cavity was investigated for cervical incompetence by hysteroscopy, hysterosalpingography, and serial ultrasound.

Table 1. Case–Control Study of the SNPs of 4 miRNAs.

Gene	SNP ID	Phenotype	N[a]	HWE	MAF
miR-146aG > C	rs2910164	Cases	200	0.188	0.41
		Control	300	0.085	0.37
miR-149T > C	rs2292832	Cases	200	0.484	0.20
		Control	300	0.879	0.17
miR-196aT > C	rs11614913	Cases	200	0.635	0.43
		Control	300	0.075	0.38
miR-499A > G	rs3746444	Cases	200	0.126	0.20
		Control	300	0.264	0.11

Abbreviations: HWE, Hardy–Weinberg equilibrium, MAF, minor allele frequency; miRNA, micro-RNA; SNP, single-nucleotide polymorphism.
[a] Number of cases and controls.

The control group consisted of 300 healthy parous women of the similar ethnic distribution as the patients with RM (Supplemental Table 1) with at least 2 live births and no history of miscarriages, preeclampsia, ectopic pregnancy, preterm delivery, or systemic diseases. All patients included in this study lived in Uttar Pradesh province of northern India. Blood samples (5 mL) from control and women with RM were collected in EDTA-coated collection vials and DNA was extracted with the use of Qiagen DNA extraction kits (Brand GMbH and Co KG, Cat # 51104). This study was approved by the ethics committees of SGPGIMS and Chhatrapati Shahuji Maharaj Medical University. Written informed consent was obtained from each participant before registering them for the study.

Genotype Analyses

Genetic variants of miR-146aC > G (rs2910164), miR-149T > C (rs2292832), miR-196a2T > C (rs11614913), and miR-499A > G (rs3746444) were analyzed in 200 patients with RM and 300 control participants. The genotyping of the single-nucleotide variants was based on polymerase chain reaction (PCR) amplification followed by restriction digestion-based assays.[20] All the amplification reactions were carried out in oil-free PTC-200 thermal cyclers (BioRad Laboratories India Pvt Ltd). In each reaction, 50 ng genomic DNA was amplified in 10 mL PCR buffer, 67 mmol/L Tris-HCl, pH 8.8, 16 mmol/L $(NH_4)2SO_4$, 2 mmol/L $MgCl_2$, 0.01% Tween-20, and 100 mmol/L deoxynucleotides, containing 0.5 U Taq DNA polymerase. We followed a double-blind genotyping analysis where the coded DNA samples were given to the person carrying out genotyping and decoding was done by another person. Wherever we found discrepancy, the genotyping was repeated.

Statistical Analysis

The power of this study was calculated using Quanto version 1.1 (http://hydra.usc.edu/gxe) with the variables set as follows: study design, case–control; significance level, <0.05 (2 sided); model of inheritance, log additive; minor allele frequency, 0.15; and genetic effect (odds ratio [OR]) 0.6 or 1.6. The study achieved 80% power, which was sufficient to consider an OR $\leq$0.6 or $\geq$1.6, with type 1 error = 0.05. Statistical analyses for the genotypic and allelic frequencies were performed with the use of Graphpad Prism (v3.0; Graphpad Software). Allele, genotype frequencies, heterozygosities, and likelihood ratio test for Hardy-Weinberg equilibrium were calculated using Popgen v16 (www.ualberta.ca/-fyeh/fyeh). Haplotypes were generated for miR-146aC > G (rs2910164), miR-149T > C (rs2292832), miR-196a2T > C (rs11614913), and miR-499A > G (rs3746444) from multilocus diploid data based on a Gibbs sampling strategy with the use of the Arlequin v3.5 software package. Allele, genotype, and haplotype frequency differences between the RM and the control groups were tested for significance with the use of Fisher exact test with Bonferroni correction. The magnitude of the effect was estimated by ORs and their 95% confidence intervals (CIs; SPSS). Logistic regression analysis was performed with different genotypes as well as alleles as the independent factors, with the risk of RM being the dependent variable: additive model comparing variant homozygous and heterozygous genotypes individually with wild-type homozygous genotypes; recessive model comparing variant homozygous genotype with wild-type homozygous and heterozygous genotypes taken together; and dominant model comparing variant homozygous and heterozygous genotypes taken together with wild homozygous genotype. P value of <0.05 was considered to be statistically significant. All statistical analyses were adjusted for age.

Results

Different alleles and their genotypes, recessive, dominant, and additive models for all miR-146aC > G (rs2910164), miR-149T > C (rs2292832), miR-196a2T > C (rs11614913), and miR-499A > G (rs3746444) in this study have been investigated. The minor allele frequencies and Hardy-Weinberg equilibrium of both cases and controls are presented in Table 1. Both cases and controls were in Hardy-Weinberg equilibrium. We analyzed the effect of the genotypes of 4 miRNA polymorphisms under dominant and recessive genetic models. The genotypes of 499 A > G were risk associated in additive, dominant, and recessive models, whereas 196a2C > T revealed significant association under recessive model (Table 2). The 149C > T and 146 A > G revealed no significance. The observed heterozygosities (ho) for SNPs of miR-146aC > G, miR-149T > C, miR-196a2T > C, and miR-499A > G were in the range of 0.004 to 0.31 for control participants and did not differ significantly from the expected heterozygosities (he) which were in the range of 0.01 to 0.20. The haplotypes were constructed for miR-146aC > G (rs2910164), miR-149T > C (rs2292832), miR-196a2T > C (rs11614913), and miR-499A > G (rs3746444). There were a total of 8 haplotypes (Table 3) among the RM and controls. The haplotype estimation of miR-146aC > G (rs2910164), miR-149T > C (rs2292832), miR-196a2T > C (rs11614913), and miR-499A > G (rs3746444) together showed that the haplotype consisting of wild-type alleles of all the 4 studied variables C-T-T-A was represented more often in the control group (35.5%) than in the patients group (22.5%), revealing a significant protective effect of this haplotype (OR = 0.53, 95% CI = 0.39-0.70; $P < .0001$). On the other hand, the haplotypes C-T-C-G and C-C-T-A were seen more frequently in the patients group (9.5% and 6.7%, respectively) than in the control group (1.8% and 3.1%, respectively) resulting in 5-fold and 2-fold risk association with RM.

We applied multifactor dimension reduction analysis and observed that the best interaction model was for miR-146aC > G, miR-149T > C, miR-196a2T > C, and miR-499 A > G with testing accuracy = 0.65, cross-validation consistency = 10/10, and permutation $P = 0.0001$ with RM.

Discussion

In the present study, we found miR-196a2CC, miR-499AG + GG and the miR-196a2CC/miR-499AG + GG along with some of the haplotypic combinations are associated with increased risk of RM. On haplotype analysis (prevalence >5%) of miR-146aC > G (rs2910164), miR-149T > C (rs2292832), miR-196a2T > C (rs11614913), and miR-499A > G (rs3746444) variants, we observed that the haplotype C-T-T-A showed a significant protective effect ($P < 0.0001$), whereas the haplotypes C-C-T-A and C-T-C-G were risk-associated haplotypes with 2- and 5-fold risks. The computational example using a stimulated data set of 500 individuals for 4 *miR* genes revealed the predictive ability with validation accuracy of $\sim$60.0% for all the 4 miRNAs. The permutation testing revealed significant P values. We have observed a low hetrozygosity rate among these miRNAs. This may be due to developmental regulation and evolutionary conservation, which imply functional importance of miRNAs. Our results indicate that these miRNAs may be important in the etiology of RM. Studied miRNAs have been reported to be associated with various types of malignancies. Ryan et al revealed that miR-146a, miR-149, miR-196a2, and miR-499 are closely associated with cellular proliferation and differentiation.[11]

It has been demonstrated that upregulation of miR-196a2 affects mRNA expression of the HOX family of genes and Akt signaling,[29] which are linked to endometriosis[30] and miscarriages.[20] Also, the important role of *HOX* genes has been implicated in implantation.[29] *HOXB8*, target of miR-196a2,[25] is essential for the myeloid differentiation and limb development.[31] The main target of miR-499 is the transcriptional repressor *SOX6*, which suppresses the expression of FGF-3.[26] The *SOX6*, FGF-3, and miR-499 are possibly functionally linked to breast cancer.[13] Our results are in concordance with Jeon et al where it has been suggested that miR-196a2T > C and miR-499A > G polymorphisms may be responsible for recurrent spontaneous miscarriage.[20] Renthal et al demonstrated that

Table 2. Distribution of miR Gene Variants Among Patients With RM and Controls.

Genotype	Patients, n = 200 (%)	Controls, n = 300 (%)	*P* Value	OR (95% CI)
miR-146aG > C (dbSNP ID rs2910164)				
Genotype frequency				
CC	63 (31.5%)	108 (36.0%)	1	–
CG (additive model)	107 (53.5%)	156 (52.0%)	.4813	1.17 (0.79-1.74)
GG (additive model)	30 (15.0%)	36 (12.0%)	.2379	1.42 (0.83-2.54)
GG + CG vs CC (dominant model)			.3361	1.22 (0.83-1.78)
GG vs CG + CC (recessive model)			.3473	1.29 (0.76-2.18)
Allele frequency				
C	233 (58.3%)	372 (62.0%)	.2356	0.85 (0.66-1.10)
G	167 (41.7%)	228 (38.0%)	.2356	1.16 (0.90-1.51)
miR-149T > C (dbSNP ID rs2292832)				
Genotype frequency				
TT	128 (64.0%)	207 (69.0%)	1	–
TC (additive model)	62 (31.0%)	84 (28.0%)	.4173	1.19 (0.80-1.77)
CC (additive model)	10 (5.0%)	9 (3.0%)	.2323	1.79 (0.71-4.54)
CC + TC vs TT (dominant model)			.2461	1.25 (0.85-1.82)
CC vs TC + TT (recessive model)			.3396	1.70 (0.67-4.26)
Allele frequency				
T	318 (79.5%)	498 (83.0%)	.1825	0.79 (0.57-1.09)
C	82 (20.5%)	102 (17.0%)	.1825	1.25 (0.91-1.73)
miR-196aT > C (dbSNP ID rs11614913)				
Genotype frequency				
TT	65 (32.5%)	104 (34.6%)	1	–
TC (additive model)	95 (47.5%)	158 (52.6%)	.9185	0.96 (0.64-1.43)
CC (additive model)	40 (20.0%)	38 (12.6%)	.0718	1.68 (0.97-2.89)
CC + TC vs TT (dominant model)			.6307	1.10 (0.75-1.61)
CC vs TC + TT (recessive model)			.0323[a]	1.72 (1.06-2.80)
Allele frequency				
T	225 (56.3%)	366 (61.0%)	.1487	0.82 (0.63-1.06)
C	175 (43.7%)	234 (39.0%)	.1487	1.21 (0.94-1.57)
miR-499A > G (dbSNP ID rs3746444)				
Genotype frequency				
AA	130 (65.0%)	237 (79.0%)	1	–
AG (additive model)	58 (29.0%)	57 (19.0%)	.0045[a]	1.85 (1.21-2.83)
GG (additive model)	12 (6.0%)	6 (3.0%)	.0109[a]	3.64 (1.33-9.94)
GG + GA vs AA (dominant model)			.0006[a]	2.02 (1.35-3.02)
GG vs GA + AA (recessive model)			.0258[a]	3.12 (1.15-8.47)
Allele frequency				
A	318 (79.5%)	531 (88.5%)	.0001[a]	0.50 (0.35-0.71)
G	82 (20.5%)	69 (11.5%)	.0001[a]	1.98 (1.40-2.81)

Abbreviations: CI, confidence interval; dbSNP, Single Nucleotide Polymorphism Database; NS, not significant; OR, odds ratio; RM, recurrent miscarriage.
[a]The difference in frequencies between the case and control groups was analyzed for statistical significance at the 95% confidence interval using Fisher exact test with Bonferroni correction under additive, recessive, as well as dominant models of inheritance. Odds ratios were calculated and reported within the 95% confidence limits. Logistic regression analysis was performed with different genotypes as well as alleles as the independent factors, with the risk of RM being the dependent variable; additive model: comparing variant homozygous and heterozygous genotypes individually with wild-type homozygous genotypes; recessive model: comparing variant homozygous genotype with wild-type homozygous and heterozygous genotypes taken together; dominant model: variant homozygous and heterozygous genotypes taken together compared with wild-type homozygous genotype.

certain miRNAs can modulate uterine quiescence and contractility during pregnancy and labor.[32] The evidences that support a role for this abnormal cell proliferation in RM comprise the increased risk of abortion associated with polymorphisms of the cell cycle-related genes *TP53* and *MDM2*.[33] The potential of miRNAs to act as signaling molecules, that is, miRNAs released by certain cells, can be taken up by other cells where they can elicit regulatory effects. However, no targeted miRNA expression profiling has been performed for RM-related issues. There are placenta-specific miRNAs capable of crossing the placental barrier and have been detected in maternal plasma and an altered profile of several miRNAs has been shown in pregnancy complications. One of the extensively studied is miR-146a, which binds to a site in the *FAS* mRNA 3′-UTR, regulating *FAS* expression.[23] The expression of miR-146a is regulated by the nuclear factor κB pathway and elevated miR-146a levels promote the viability of mesenchymal stem cells.[23] Another important target of miR-146a is SMAD4.[34]

Table 3. Haplotype Analysis of miR-146aC > G, miR-149T > C, miR-196a2T > C, and miR-499A > G gene Variants Among Patients With RM and Controls.[a]

Haplotypes	Patients, n = 400 (%)	Control, n = 600 (%)	OR	95% CI	*P* Value
CTTA[b]	90 (22.5%)	213 (35.5%)[c]	0.53	0.39-0.70	.0001[c]
GTCA[c]	61 (15.3%)	85 (14.1%)[c]	1.09	0.76-1.55	.6484
GTTA[c]	70 (17.5%)	86 (14.3%)[c]	1.26	0.89-1.78	.1829
GTTG	8 (2.0%)	22 (3.6%)	0.53	0.23-1.21	.1843
CCCA[c]	35 (8.7%)	56 (9.3%)[c]	0.93	0.59-1.45	.8227
CTTG	14 (3.5%)	8 (1.3%)	2.68	1.11-6.46	.0274[c]
CTCA[c]	26 (6.5%)	55 (9.1%)[c]	0.68	0.42-1.11	.1555
CTCG[c]	38 (9.5%)	11 (1.8%)	5.62	2.83-11.13	.0001[c]
CCTA[c]	27 (6.7%)	19 (3.1%)	2.21	1.21-4.03	.0128[c]
GCTA	6 (1.5%)	8 (1.3%)	1.12	0.38-3.27	1.0000
CCTG	2 (0.5%)	10 (1.6%)	0.29	0.06-1.36	.1384
GTCG	11 (2.7%)	18 (3.0%)	0.91	0.42-1.95	.8505
GCCA	3 (0.75%)	9 (1.5%)	0.49	0.13-1.84	.3806
GCTG	8 (2.0%)	–			
CCCG	1 (0.25%)	–			

Abbreviations: CI, confidence interval; NS, not significant; OR, odds ratio; RM, recurrent miscarriage.
[a]Haplotypes were generated from multilocus diploid data based on a Gibbs sampling strategy (Arlequin v3.5).
[b]Statistically significant protective haplotypes.
[c]The difference in haplotype frequencies between the case and control groups for the prevalent haplotypes (prevalence more than 5% in either cases or controls) was analyzed for statistical significance using Fisher exact test with Bonferroni correction, and ORs are reported within the 95% confidence limits.
[d]Statistically significant RM predisposing haplotypes.

The SMAD4 plays an essential role in stabilizing the undifferentiated state of human embryonic stem cells[34] by regulating the promoter of p21/WAF1/Cip1. The target genes of miR-149 are *Akt1* and *E2F1*, which are involved in promoting cell growth and cell cycle progression.[24]

These data suggest that miR-196a2T > C and miR-499A > G polymorphisms could contribute to RM and may be taken into consideration while evaluating these patients. Our study is the only study from Indian subcontinent and another study from Korea revealed the importance of these miRNAs[20]; therefore, follow-up studies in other populations, and of other pre-miRNA polymorphisms, may be important for understanding the role of miRNAs in patients with RM.

Conclusion

In conclusion, our findings demonstrate the susceptible role of miR-196aT > C (Single Nucleotide Polymorphism Database [dbSNP] ID rs11614913) and miR-499A > G (dbSNP ID rs3746444) among patients with RM. The main limitation of the present study is that we have not carried out the expression profile of these miRNAs; hence, further studies are required to clarify the associations between the miRNAs and RM. However, there exists increasing amount of evidence accumulated during the past few years which show that miRNAs are associated with numerous diseases. Moreover, noncoding RNAs have been proposed as therapeutic agents; given their silencing capabilities, this reveals the importance of our studied miRNAs.

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Declaration of Conflicting Interests

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Supplemental Material

The online supplementary tables are available at http://rs.sagepub.com/supplemental.

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