Potential role of circulating microRNAs as a biomarker for unexplained recurrent spontaneous abortion

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Objective: To compare circulating microRNA (miRNA) profiles between unexplained recurrent spontaneous abortion (URSA) and normal early pregnancies (NEP) and to evaluate the potential role of circulating miRNA as a biomarker for URSA.
Design: Laboratory study using human plasma samples.
Setting: Special hospital and research institutes.
Patient(s): From September 2012 to April 2013, samples of plasma were obtained from 27 URSA patients and 28 NEP patients at 6–10 weeks of gestation at the Department of Reproductive Immunology in Family Planning Special Hospital of Guangdong Province.
Intervention(s): Differential miRNA profiling analysis of plasma collected from URSA and NEP patients was performed with the use of microarray.
Main Outcome Measure(s): The circulating miRNA expression profile was assessed by means of microarray and real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis.
Result(s): Twenty-five circulating miRNAs were expressed differentially in URSA compared with NEP. Of these, nine were overexpressed and 16 down-regulated. Six differentially expressed circulating miRNAs were selected to validate the microarray results, and qRT-PCR data confirmed the reliability of the microarray results. Further analysis showed that four circulating miRNAs (miR-320b, miR-146b-5p, miR-221-3p, miR-559) were up-regulated. In URSA, one circulating miRNA (miR-101-3p) was down-regulated in other larger scale samples according to qRT-PCR. Based on target gene analysis, we speculate that these circulating miRNAs regulate URSA by targeting immune, apoptosis, and angiogenic gene functions.
Conclusion(s): Circulating microRNAs may be involved in URSA pathogenesis and provide a promising new diagnostic biomarker for URSA. (Fertil Steril® 2016;105:1247–54. ©2016 by American Society for Reproductive Medicine.)
Key Words: Circulating microRNA, biomarker, microarray, unexplained recurrent spontaneous abortion, normal early pregnancy

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Recurrent spontaneous miscarriage, defined as three or more consecutive pregnancy losses of clinically recognized pregnancies (ectopic, molar, and biochemical pregnancies are not included), occurs in 1%–5% of women of reproductive age and has a negative effect on human reproductive health (1). Although the causes of recurrent spontaneous miscarriage involve genetic, anatomic, endocrinologic, infectious, and

autoimmunologic abnormalities, in some cases no cause can be identified (2). These cases are termed unexplained recurrent spontaneous abortion (URSA). Currently, URSA still lacks a safe and effective therapy and a reliable early means of diagnosis and prediction. URSA is a major challenge in contemporary obstetrics, and resources are now focused on early pregnancy, where prophylactic strategies may help to reduce the incidence of pregnancy loss. It has been reported that mixed lymphocyte reaction blocking factor (MLR-Bf) is a potential biomarker of URSA (3). However, MLR-Bf is not definitive, and the use of MLR-Bf to identify URSA is limited by false-positive and false-negative rates. Therefore, additional markers to diagnose URSA during early pregnancy are necessary.

In the early 2000s, the term microRNA (miRNA) was first introduced. MicroRNAs are small noncoding RNAs 20–24 nucleotides long. These molecules have been shown to play critical regulatory roles in a wide range of biologic and pathologic processes involving cell differentiation, proliferation/growth, apoptosis, angiogenesis, and inflammation (4–6). MiRNAs mediate post-transcriptional regulation by binding to complementary sequences on target mRNAs and promoting mRNA degradation or translational repression. In both cases, the pairing is performed at the 3′-UTR (untranslated region) or 5′-UTR of mRNA (6). Recent studies have also proposed a role for miRNAs in cell-to-cell communication (7). Indeed, miRNAs have been detected in most extracellular fluids, particularly in plasma/serum (8, 9). Additionally, circulating miRNAs appear to be resistant to endogenous ribonuclease activity (9). Consequently, circulating miRNAs have been recently postulated as useful biomarkers for a variety of conditions, such as cancer, cardiovascular disorders, and immune inflammatory diseases (10). It has been demonstrated that miRNA has a critical regulatory role in endometriosis (11), pre-eclampsia (12, 13), infertility (14), and other reproductive system diseases. MiR-133a can induce recurrent spontaneous abortion (RSA) and is regulated by human leukocyte antigen HLA-G gene (15). Few studies have focused on miRNA implication and misregulation in early pregnancy loss. MiR-17 and miR-19a are strikingly decreased in the placenta when it comes to early spontaneous miscarriage (16). MiR-184 is up-regulated in the villus and decidua of RSA patients (17). It has been suggested that some single-nucleotide polymorphisms (SNPs) are associated with RSA.

Two recent studies examined the association of miRNA polymorphisms with RSA. One study compared 217 RSA patients and 431 unaffected control subjects and identified two variant alleles in primary miRNA-125a that lead to the altered production of miR-125a and correlate with an increased risk for RSA in a Han-Chinese population (18). With the use of a case-control study, another group identified an association between four pre-miRNA polymorphisms (miR-196a2CC, miR-499AG+GG, and the miR-196a2CC/miR-499AG+GG combination) and RSA in Korean women (19).

To the best of our knowledge, no study has evaluated the presence of circulating miRNAs in the plasma of URSA patients. In the present study, our aim was to compare the circulating miRNA profiles of URSA and NEP patients and to explore the potential of miRNAs as an early predictive noninvasive biomarker of URSA.

MATERIALS AND METHODS

Sample Collection

All participants were recruited at the Department of Reproductive Immunology at Family Planning Special Hospital of Guangdong Province (Guangzhou, People's Republic of China) from September 2012 to April 2013. The study enrolled URSA patients who had at least three consecutive spontaneous early miscarriages ranging from 6 to 10 weeks of gestation. In these patients, we excluded individuals with infections, endocrine or metabolic disorders, anatomic abnormalities, autoimmune diseases, and paternal or maternal chromosomal abnormalities. Laboratory tests found no abnormalities in the participants of this study.

The women in this study were then divided into two groups: the URSA group and the normal early pregnancy (NEP) group. The URSA group comprised 27 women aged 29.10 ± 4.22 years with URSA. The NEP group comprised 28 healthy women aged 28.72 ± 4.80 years who were in early pregnancy (mean gestational age 7.46 ± 2.62 wk). The venous blood/plasma samples from the URSA patients were taken after a miscarriage, and the plasma samples from NEP group were collected before elective termination of early pregnancy. All samples were collected in accordance with approved guidelines and relevant regulations. All samples were obtained after receiving informed written consent, and the Institutional Review Board for Ethical, Legal and Social Issues of the Family Planning Special Hospital of Guangdong Province approved the study protocol.

Total RNA Extraction and Enrichment Procedure for miRNAs

To harvest cell-free plasma, whole blood samples were centrifuged twice at 1,200*g* for 10 minutes at room temperature. All samples were stored at −80°C until further processing. Total RNA was extracted from 1 mL plasma samples derived from the participants. Extraction was then followed by an enrichment procedure for small RNAs according to the manufacturer's instructions with the use of an Amirvana microRNA isolation kit (Ambion). Regarding plasma samples, Trizol LS reagent and the manufacturer's protocol for total RNA extraction from biologic fluids (Invitrogen) preceded the small RNA enrichment procedure. To minimize DNA contamination, we treated the eluted RNA with 5 μL DNase I (Fermentas Internationala) for 30 minutes at 37°C. The sample quality control data file from a Nanodrop 1000 spectrophotometer and standard denaturing agarose gel electrophoresis were used.

miRNA Microarray Analysis

miRNA microarray analysis was performed on plasma from cases of URSA (n = 3) and NEP (n = 3). Total RNA from each sample was used. MiRNA was labeled with the Mircury Hy3/Hy5 Power labeling kit (Exiqon) according to instructions in the manual. The hybridization was carried out

overnight on the Mircury LNA Array chip (v. 18.0) (Exiqon), which is a sensitive array for miRNA expression profiling that is based on locked nucleic acids. The microarray contains 3,100 capture probes covering all human, mouse, and rat miRNAs annotated in miRBase 18.0 as well as all viral microRNAs related to these species. In addition, this array contains capture probes for 25 miRPlus human microRNAs. These are proprietary miRNAs not found in miRBase. Hybridization images were collected with the use of a Genepix 4000B laser scanner and digitized with the use of Genepix Pro 6.0 software (Axon Instruments). Only those genes with significant differential expression ($\geq$2.0-fold or $\leq$0.5-fold) were reported. With the use of Cluster 3.0, unsupervised hierarchic clustering was carried out with average linkage and uncensored correlation as the similarity metrics. Heat maps were generated in Java Treeview. Data from each raw probe from the microarrays of all samples were averaged, and then the respective data from the samples were transformed as the provider divided by the average (mean). The relative expression of each miRNA was calculated via the ratio between the sample microarrays and the average of all microarrays. To draw a simple and perspicuity figure with the software, the relative expression of each gene was described as the log10 (ratio) in the heat map figures from Cluster 3.0. To identify differentially expressed miRNAs with statistical significance, a Volcano Plot filtering between the two groups from the experiment was performed.

Real-time Quantitative Reverse-transcription Polymerase Chain Reaction

In accordance with the microarray results and literature, candidate miRNAs were chosen for further validation with real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Blood samples from 27 URSA patients and 28 NEP patients were used to screen the findings from miRNA profiling. Total RNAs were isolated from plasma with the use of Trizol reagent (Invitrogen) for quantitative detection of miRNA. Complementary DNA was synthesized with the use of the reverse transcription Taqman. All miRNA reverse transcription kit and miRNA-specific primers were purchased from Taqman microRNA Assays (Life Technologies). Each RT reaction consisted of 5 μL total RNA, 0.15 μL dNTPs with dTTP (100 mmol/L), 1.00 μL multiscribe RT enzyme (50 U/μL), 1.5 ìL 10$\times$ RT buffer, 0.19 ìL RNase inhibitor (20 U/μL), 4.16 μL nuclease-free water, and 3 μL 5$\times$ RT primer, in a total volume of 15 μL. Reactions were performed with the use of the following conditions: 30 minutes at 16°C, 30 minutes at 42°C, 5 minutes at 85°C, and held at 4°C. qRT-PCR was performed with the use of Applied Biosystems 7900HT Real-Time PCR System, with 10 μL PCR reaction mixture that included 2 μL of the cDNA, 5 μL 2$\times$ Taqman Universal PCR Master Mix II (Applied Biosystems), 0.5 μL 20$\times$ miRNA-specific PCR primer/probe mix (Applied Biosystems), and 2.5 μL nuclease-free water. The primer pairs used for miRNAs and U6 are presented in Supplemental Table 1 (Supplemental Tables 2–4). PCR reactions in a 384-well plate were run at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Each sample was run in triplicate for analysis. The 5$\times$ RT primers (miRNA-specific stem-loop primers) and 20$\times$ miRNA-specific PCR primer/probe mix were supplied by the TaqMan MicroRNA Assays (Life Technologies) based on the miRNA sequences obtained from the miRBase database. Data were collected with the use of the SDS 2.3 software (Applied Biosystems). After normalization to U6, the expression levels of miRNAs were calculated by means of the C_T method (20).

miRNA Target Prediction and Function Analysis

Differentially expressed miRNAs were identified with the use of the significance analysis of microarrays program. The miRNAs with $P<.05$ and fold change $\geq$2.0 or $\leq$0.5 were considered to be significantly differentially expressed. With the use of miRbase (www.microrna.org/microrna/home.do), miRanda (www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/), and Targetscan (www.targetscan.org/vert_60/). We predicted the target genes of significant differentially expressed miRNAs and then performed gene ontology (GO) and pathway analysis. The $-$log10 (P value) indicating the GO and pathway result was used in the histogram and line chart by Sigmaplot (Systat Software).

The genes that were coidentified by three programs were considered to be potential target genes regulated by a given miRNA. For GO terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analysis, we used the transcriptional networks analysis tool Funnet (www.funnet.ws/) with default settings. $P<.01$ and the false discovery rate (FDR) $<$ 0.05 were used as the threshold of significant GOs regulated by miRNA.

Statistical Analysis

Demographic variables were compared between patients and matched control subjects with the use of a Fisher exact test for qualitative variables and a t test for quantitative variables. Expression levels of miRNAs were compared with the use of the Mann-Whitney U test. MiRNA data are presented as fold change relative to the control group. All statistical analyses were carried out with the use of DataAssist version 3.0 software, and SPSS version 17.0 software, and Graphpad Prism 5.01 (Graphpad Software). $P<.05$ was considered to be statistically significant.

RESULTS

Clinical Characteristics of the Participants

The clinical characteristics of the study groups are presented in Supplemental Table 2 where the mean ($\pm$SD) ages of patients in the URSA and NEP (control) groups were 29.10 $\pm$ 4.22 years and 28.72 $\pm$ 4.80 years, respectively. All of the patients and control subjects were of Han nationality, and there were no differences in age, gestational age, miscarriages, or body mass index between URSA patients and control subjects.

miRNA Microarray Analysis

After the raw data were normalized, and with the use of the R package from Service & Asset Management (SAM) to screen

the differential expression of miRNAs by means of the criteria $P<.05$ and fold change ≥ 2.0 or ≤ 0.5, we identified 25 miRNAs that expressed differentially according to miRNA microarray, of which nine miRNAs were up-regulated and 16 miRNAs were down-regulated in plasma of URSA compared with NEP (Table 1). The significance was maintained after correction for multiple testing. In unsupervised hierarchic clustering analysis, the normalized microarray expression data for the 25 miRNAs showing differential expression were used to generate a heat map. Hierarchic clustering was performed with the use of the Euclidean metric and complete linkage rule. The samples were self-segregated into the URSA patients and control clusters (Fig. 1A). We could intuitively observe this phenotype in log-log scatter plot (Fig. 1B) and volcano plot (Fig. 1C).

miRNA Expression Validation

According to the microarray profiling outcome, we compared the expression of miR-320b, miR-146b-5p, and miR-221-3p (up-regulated) and miR-559, miR-22-5p, and miR-204-3p (down-regulated) in plasma from URSA with that from NEP. These particular miRNAs were subsequently reanalyzed in each of the individual case and control samples (validation set) with the use of qRT-PCR. Relative expression of miRNA was calculated by normalization with U6 small nuclear RNA expression by means of the comparative C_T method (20). The tendencies among the six miRNAs analyzed were conserved between microarray and the qRT-PCR assays (Fig. 2). Moreover, differential levels obtained by means of qRT-PCR were similar to, though slightly lower than, those found by means of microarray analysis, suggesting that qRT-PCR data could confirm the reliability of microarray results.

TABLE 1

MicroRNAs that were up-regulated and down-regulated in unexplained recurrent spontaneous abortion compared with normal early pregnancies.

miRNA name	Fold change	*P* value[a]
Up-regulated		
Hsa-miR-320b	2.637	.00218
Hsa-miR-146b-5p	5.108	.00869
Hsa-miR-101-3p	3.614	.01932
Hsa-miR-92a-3p	2.662	.03084
Hsa-miR-148b-3p	4.595	.04343
Hsa-miR-221-3p	7.409	.00704
Hsa-miR-30d-5p	4.361	.02854
Hsa-miR-99b-5p	9.743	.00842
Hsa-miR-23b-3p	3.430	.02100
Down-regulated		
Hsa-miR-204-3p	0.394	.03839
Hsa-miR-559	0.390	.01504
Hsa-miR-22–5p	0.337	.01717
Hsa-miR-365a-3p	0.321	.03177
Hsa-miR-3201	0.154	.04234
Hsa-miR-4653-3p	0.318	.02828
Hsa-miR-4468	0.425	.03839
Hsa-miR-4264	0.113	.00113
Hsa-miR-1973	0.178	.03137
Hsa-miR-4678	0.251	.01279
Hsa-miR-1182	0.238	.01855
Hsa-miR-4536-3p	0.245	.01276
Hsa-miR-4474–5p	0.196	.03994
Hsa-miR-4284	0.428	.00789
Hsa-miR-4300	0.246	.02629
Ebv-miR-BART8	0.418	.03950

Note: Hsa = Homo sapiens.
[a] If the fold change was ≥ 2.0 or ≤ 0.5, the difference was recognized to be significant; the significance was maintained after correction for multiple testing.

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Target and Function Prediction of Differentially Expressed miRNAs

If a gene can be predicted as a target gene by these three databases simultaneously, we conclude that the target gene is effective. We then used these target genes (Supplemental Table 3) and differentially expressed miRNAs to establish the miRNA-gene Venn diagram (Supplemental Fig. 1; Supplemental Figs. 1–3 available online at www.fertstert.org). A network of 947 target genes of differentially expressed miRNAs is shown in Supplemental Figure 2.

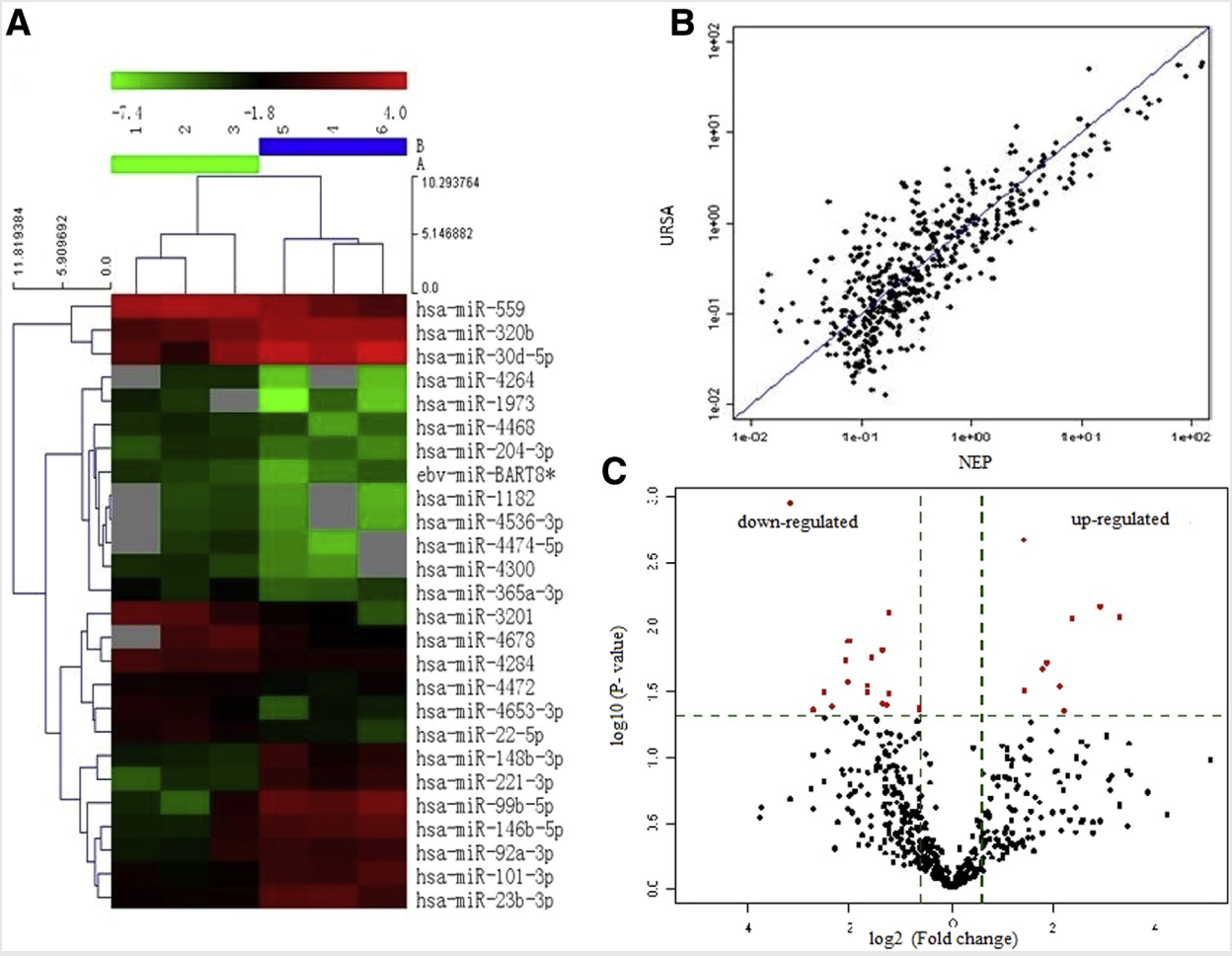
The target genes, which can be the target of two or more miRNAs simultaneously, were used to perform GO and KEGG pathway analysis. The ontology covers three domains: biologic process, cellular component, and molecular function. The most significant biologic processes of these target genes of differentially expressed miRNAs in plasma of URSA patients were regulation cellular process and regulation of biological process (Supplemental Fig. 3A). The most significant cellular component of these target genes of differentially expressed miRNAs in plasma of URSA patients were intracellular part and intracellular organelle (Supplemental Fig. 3B). The most significant molecular function of these target genes of differentially expressed miRNAs in plasma of URSA patients were protein binding and enzyme binding (Supplemental Fig. 3C). Similarly, the KEGG pathway analysis showed that the predicted target genes were involved in FoxO signaling pathway, estrogen signaling pathway, retrograde endocannabinoid signaling, MAPK signaling pathway, and focal adhesion (Supplemental Fig. 3D).

Potential Biomarker for URSA According to qRT-PCR

To further screen the potential URSA biomarker, qRT-PCR was used to screen differentially expressed circulating miRNAs in the larger sample of 24 URSA and 25 NEP participants. The primer pairs used for miRNAs and U6 are presented in Table 4. Relative expression of miRNA was calculated with the use of normalization with U6 small nuclear RNA expression by means of the comparative C_T method (20). With the use of qRT-PCR, we screened the differential expression of miR-320b (3.06-fold increase in URSA; $P<.05$), miR-146b-5p (4.03-fold increase in URSA; $P<.05$), miR-221-3p (4.75-fold increase in URSA; $P<.05$), miR-559 (4.79-fold increase in URSA; $P<.05$), and miR-101-3p (0.21-fold decrease in URSA; $P<.05$) in a set of experiments (Fig. 3).

Five miRNAs (miR-320b, miR-146b-5p, miR-221-3p, miR-559, and miR-101-3p) were analyzed, and chromosomal

FIGURE 1



The expression profiles of global miRNAs in the plasma of unexplained recurrent spontaneous abortion (URSA) and normal early pregnancy (NEP) patients. (**A**) Heat map of global miRNAs in plasma from three URSA patients (1, 2, 3) and three NEP patients (4, 5, 6). (**B**) Log-log scatter plot comparing global miRNA expression profiles in plasma of URSA patients with those of NEP control subjects. (**C**) Volcano plot of differentially expressed miRNAs. The *vertical lines* correspond to 2.0-fold up and 0.5-fold down, and the *horizontal line* represents a *P* value of 0.05. The *red point* in the plot represents the differentially expressed miRNAs with statistical significance.

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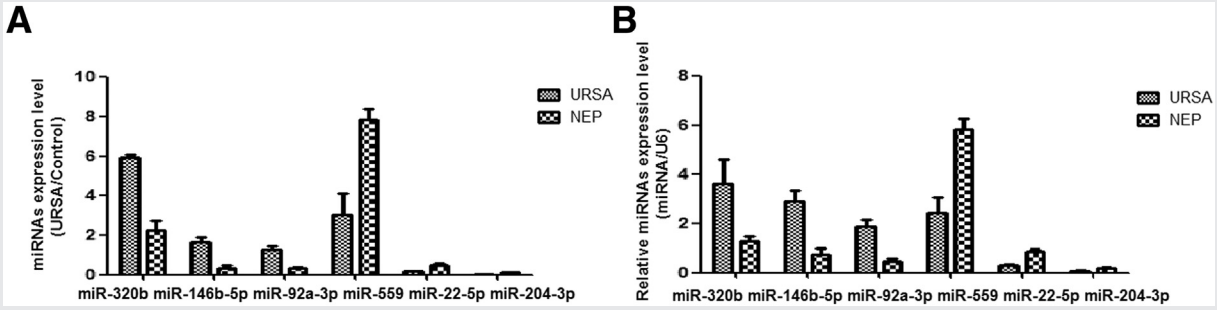
location and target gene were predicted with the use of Targetscan, miRanda, and miRbase. Notably, two miRNA clusters on human chromosomes 9 (miR-101-3p, miR-23b-3p, and miR-204-3p) and 1 (miR-320b and miR-101-3p) were expressed differentially in the URSA group. The top four putative targets with the highest score that were identified with the use of Targetscan are presented in Supplemental Table 4.

DISCUSSION

Currently, the diagnosis of URSA is primarily made after excluding any verifiable causes. Diagnosis of URSA is often difficult owing to the fact that there is no effective diagnostic biomarker available. Despite decades of effort on peripheral/serum growth factors, cytokines, hormones, and metabolic markers, a noninvasive quantitative clinical test to aid the diagnosis of URSA remains elusive. However, discovery of circulating miRNAs in maternal blood has not only facilitated the understanding of their role in normal pregnancy, but also paved new avenues for biomarkers to detect pregnancy-associated complications such as preeclampsia, ectopic pregnancy, gestational diabetes mellitus, fetal growth restriction, and preterm delivery (21). Unfortunately, the research on miRNAs in blood as biomarkers for reproductive diseases, specifically for URSA, is still in its infancy. To the best of our knowledge, this is the first report on the investigation of circulating miRNA profiling in URSA patients. In this study, we screened circulating miRNAs from plasma of URSA patients with the use of a miRNA microarray analysis. We identified 25 differentially expressed miRNAs in plasma, of which nine miRNAs were up-regulated and 16 others down-regulated. Then qRT-PCR was used to confirm the expression levels of four out of the 25 miRNAs that were differentially expressed in the microarray. The potential

FIGURE 2



qRT-PCR validation of circulating miRNAs. (**A**) Differential expression results of circulating miRNAs according to microarray. (**B**) Differential expression results of circulating miRNAs according to quantitative reverse-transcription polymerase chain reaction. Abbreviations as in Figure 1.

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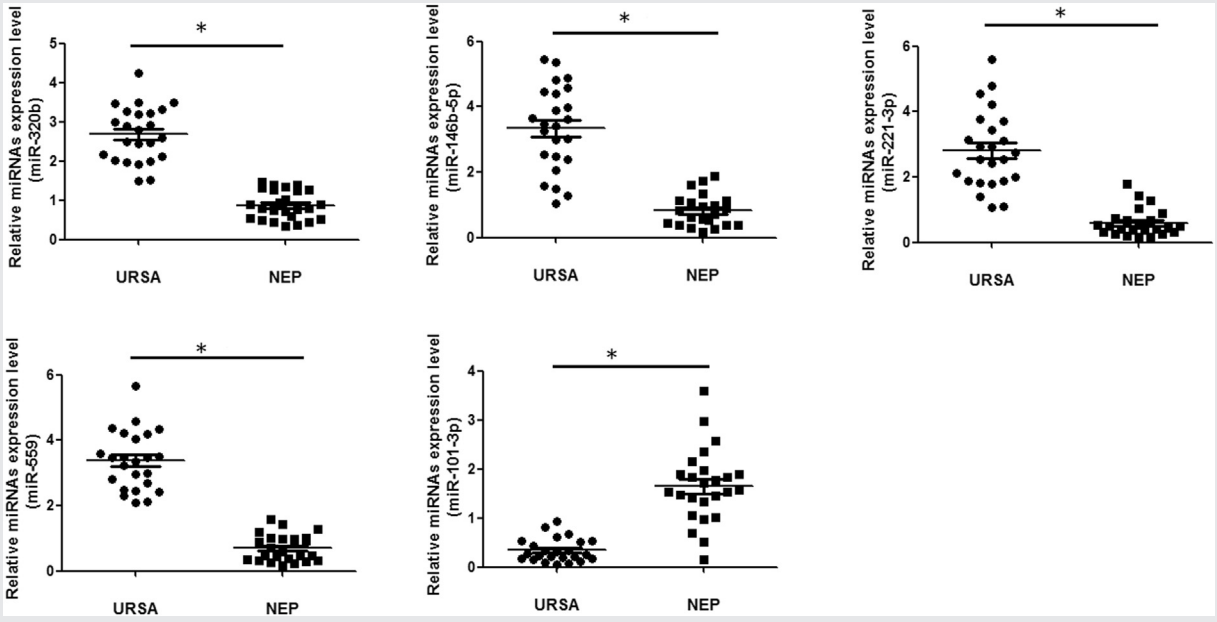
biomarker miRNAs for URSA were further screened by means of real-time PCR in larger-scale samples (24 URSA patients and 25 NEP). The results indicated that four miRNAs (miR-320b, miR-146b-5p, miR-221-3p, and miR-559) were up-regulated and one miRNA (miR-101-3p) was down-regulated in URSA. Therefore, our results indicate that the five circulating miRNAs might serve as candidate biomarkers for URSA diagnosis. Although the sample size of our study was small, the results could provide the rationale for future investigations of circulating miRNAs for diagnostic and prognostic purposes in URSA. Therefore, a comprehensive study in humans with a larger sample size should be conducted in the future to further demonstrate the diagnostic ability of the five miRNAs for URSA.

To analyze the circulating microRNAs that might be involved in URSA pathogenesis, the target genes of these differentially expressed miRNAs were predicted with the use of miRanda, miRbase, and Targetscan, and we speculate that these miRNAs regulate URSA by targeting immune, adhesion, and angiogenesis gene functions. In this current study, we used an integrated analysis of miRNA expression profiling combined with bioinformatics analysis. A list of predicted miRNA gene targets was curated from the Targetscan database. The intersection in the Venn diagram among the

FIGURE 3



The potential biomarker miRNAs according to quantitative reverse-transcription polymerase chain reaction. Relative miRNA expression levels are normalized against the amount of U6; the results are shown in the *graphs*. Significant overexpression of miR-320b, miR-146b-5p, miR-221-3p, and miR-559 was found in URSA versus NEP. And significant underexpression of miR-101-3p was found in URSA versus NEP. *Horizontal lines* represent the median. *P* value was calculated with the use of the nonparametric Mann-Whitney U test. Abbreviations as in Figure 1. *$P<.05$.

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three lists identified 947 common genes that showed a strong enrichment for genes involved in regulating the cell cycle and cell differentiation. The GO analysis showed that the most significant biologic processes of these target genes of differentially expressed miRNAs in URSA patient plasma were regulation of cellular process, intracellular part, and protein binding. Similarly, the KEGG pathway analysis showed that the predicted target genes were involved in FoxO signaling pathway, estrogen signaling pathway, retrograde endocannabinoid signaling, MAPK signaling pathway, and focal adhesion.

Ouyang et al. showed FoxO1 to be a pivotal regulator of Treg cell function. Treg cells expressed high amounts of FoxO1 and displayed reduced T-cell receptor–induced Akt activation, FoxO1 phosphorylation, and FoxO1 nuclear exclusion (22). Otherwise, several studies showed recurrent spontaneous abortion was related to the absence of a number and function of Treg cell (23–25). Taylor et al. found that endocannabinoid signaling was known to have adverse effects on various aspects of reproduction, including ovulation, spermatogenesis, implantation, and pregnancy duration (26).

The five potential biomarker miRNAs in our study have never before been reported to be involved in URSA. MiR-146b-5p can inhibit glioma migration and invasion by targeting matrix metalloproteinase 16 (27), which is involved in promoting the invasiveness of trophoblasts and decidual stromal cells. Abnormal low expression of matrix metalloproteinase would lead to the deficiency of trophoblast invasion and further contribute to the origin of RSA (28, 29). Overexpression of miR-221-3p promotes the growth, metastasis, and invasion of a variety of malignant tumors, including breast cancer, lung cancer, and prostate cancer (30). miR-221-3p may provide crucial information about molecular mechanisms of the disease on PAK1 activity or different mechanisms regarding histopathology and severity of breast cancer (31). miR-559 and miR-548d-3p interact with a specific target sequence localized in the v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2) 3′-UTR and play an important role in oncogenesis (32). The reported functions of miRNA-320b are largely associated with human cancers. For example, a recent report suggests that miRNA-320b targets β-catenin, neuropilin-1, and Rac-1 as a facilitator of colorectal cancer proliferation and invasion and therefore may also become a target for colorectal cancer therapies (33). The miR-320 family (miR-320a, b, c, d, e) promotes adipogenesis by blocking the mesenchymal stem cell differentiation pathway (34). These results suggested that miR-221-3p, miR-559, and miR-320 may play an important role in the development of cancer. Embryo development shares similar phenomena and mechanisms with tumor invasion (35). Abnormal expression of these miRNAs would cause the deficiency of trophoblast invasion, and may be associated with URSA. Breakdown of the epithelial sodium channel or cAMP-response element binding protein (CREB) in the human endometrial surface epithelial (HES) cells or in mouse uterus in vivo results in increasing in miR-101 and miR-199a-3p accompanied with decreases in COX2 protein levels and reduction in implantation rate (36). Loss of miR-101 expression could contribute to endoplasmic reticulum (ER) stress-induced trophoblast cell apoptosis by targeting ERp44 (37), and may contribute to development of URSA. From the above results, we can speculate circulating miRNAs that might be involved in URSA pathogenesis by targeting immune, apoptosis, and angiogenesis gene functions.

CONCLUSION

The main focus of this study was analysis of the circulating miRNA profiles between URSA and NEP women and evaluation of the potential role of circulating miRNAs as a biomarker for URSA. We found 25 circulating miRNAs that were expressed differentially in URSA compared with NEP. Of these, nine were overexpressed and 16 down-regulated. Further analysis showed that four circulating miRNAs (miR-320b, miR-146b-5p, miR-221-3p, and miR-559) were up-regulated and one circulating miRNA (miR-101-3p) demonstrated potential as a biomarker for URSA. Based on target gene analysis, we hypothesize that these circulating miRNAs regulate URSA by targeting immune, apoptosis, and angiogenic gene functions.

There are several limitations to this study, however. First, the development of miRNAs as noninvasive diagnostic markers is in its infancy. Further study should confirm whether circulating miRNAs are useful for URSA diagnosis. Second, the sample size in our study was relatively small; a larger randomized study is needed to validate this potential biomarker of URSA.

We plan to investigate the target genes or proteins regulated by these altered miRNAs and to analyze the relationship between these target proteins and URSA in a future study.

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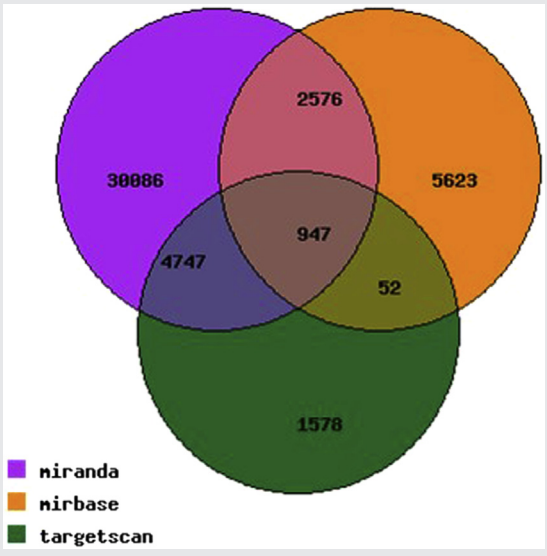
SUPPLEMENTAL FIGURE 1



Venn diagram of differentially expressed miRNAs. Target gene can be predicted as a target gene by these three databases (Targetscan, miRbase, and miRanda) simultaneously. These differentially expressed miRNAs have 947 target genes.

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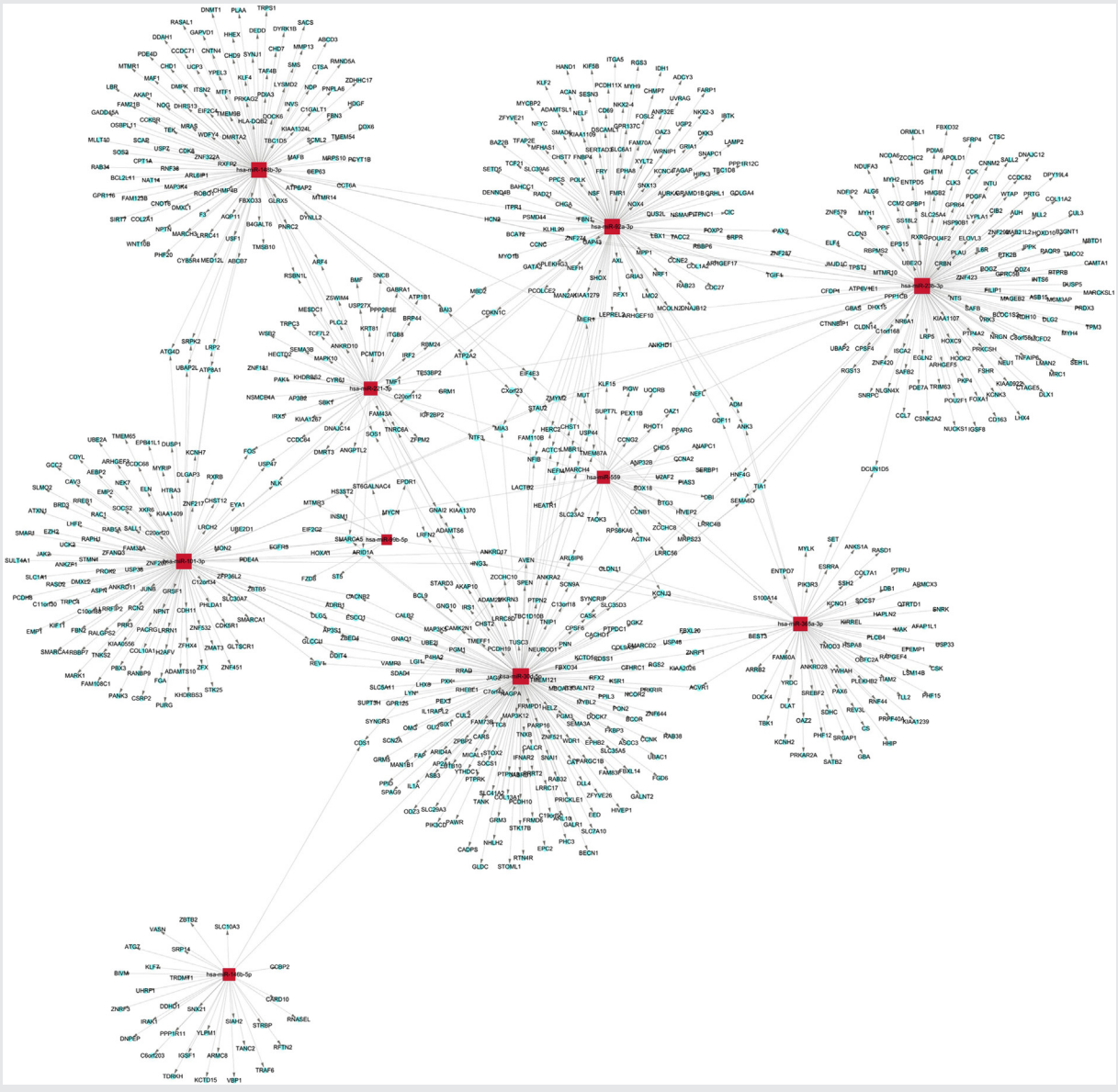
SUPPLEMENTAL FIGURE 2



Target genes network of differentially expressed miRNAs. *Blue dots* are target genes; *red squares* are miRNAs.

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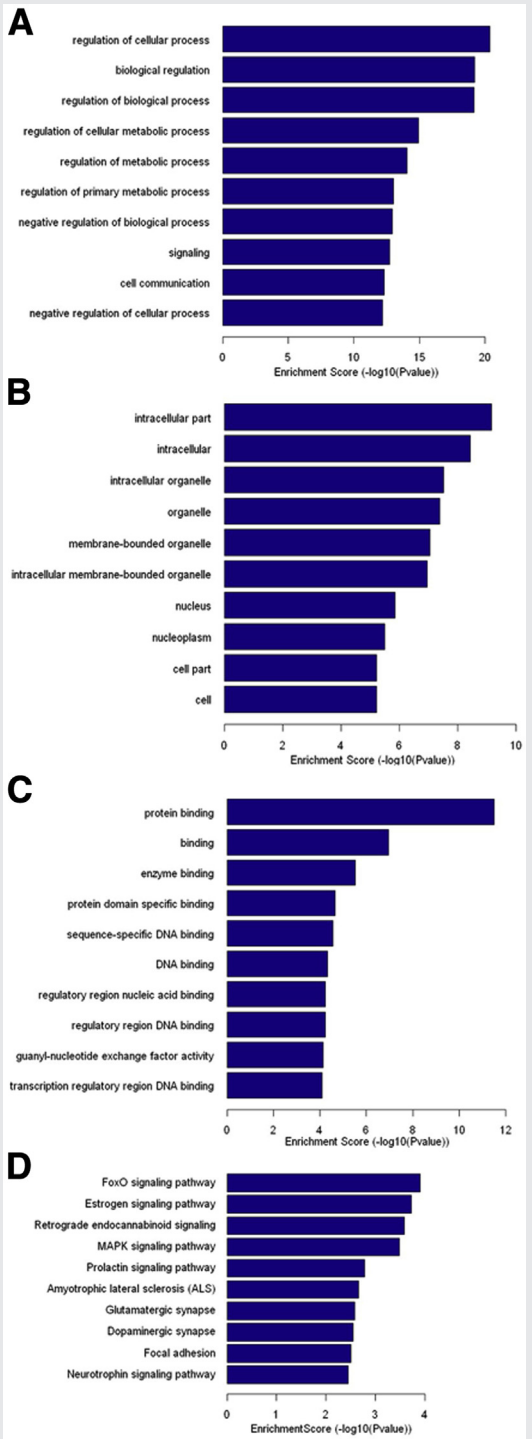
SUPPLEMENTAL FIGURE 3



Result of the gene ontology (GO) and pathway analysis of the target genes predicted by differentially expressed miRNAs. The ontology covers three domains: biologic process, cellular component and molecular function. −Log10 (*P*) indicates the GO score related to genes with the biologic process by *P* value. (**A**) Main biologic process of differentially expressed miRNAs. (**B**) Main cellular component of differentially expressed miRNAs. (**C**) Main molecular function of differentially expressed miRNAs. (**D**) Results of pathway analysis the target genes predicted by differentially expressed miRNAs.

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