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A novel platform for discovery of differentially expressed microRNAs in patients with repeated implantation failure

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Objective: To identify predictor microRNAs (miRNAs) from patients with repeated implantation failure (RIF).
Design: Systemic analysis of miRNA profiles from the endometrium of patients undergoing in vitro fertilization (IVF).
Setting: University research institute, private IVF center, and molecular testing laboratory.
Patient(s): Twenty five infertile patients in the discovery cohort and 11 patients in the validation cohort.
Interventions(s): None.
Main Outcome Measure(s): A signature set of miRNA associated with the risk of RIF.
Result(s): We designed a reproductive disease-related PanelChip to access endometrium miRNA profiles in patients undergoing IVF. Three major miRNA signatures, including hsa-miR-20b-5p, hsa-miR-155-5p, and hsa-miR-718, were identified using infinite combination signature search algorithm analysis from 25 patients in the discovery cohort undergoing IVF. These miRNAs were used as biomarkers in the validation cohort of 11 patients. Finally, the 3-miRNA signature was capable of predicting patients with RIF with an accuracy >90%.
Conclusion(s): Our findings indicated that specific endometrial miRNAs can be applied as diagnostic biomarkers to predict RIF. Such information will definitely help to increase the success rate of implantation practice. (Fertil Steril® 2021;■:■–■. ©2021 by American Society for Reproductive Medicine.)
Key Words: Endometrial receptivity, infertility, microRNA, PanelChip, repeated implantation failure

In recent years, advances in assisted reproductive technologies (ART) have increased the overall success rate of implantation during in vitro fertilization (IVF) treatment cycles. Despite the improvements, a significant number of couples still experience repeated implantation failures (RIF) (1). The precise definition of RIF is debatable (2, 3), but in this study, we considered ≥2 unsuccessful cycles of IVF as RIF. There are many contributing factors to RIF, one of which is a shift in endometrial receptivity (4, 5). The receptivity of an endometrium to blastocyst implantation occurs during a tightly controlled interval known as the window of implantation (WOI), which often is restricted to a period within days 16–22 of a 28-day normal menstrual cycle (6). In the majority of women, the WOI normally appears 7 days after the luteinizing hormone surge or human chorionic gonadotropin administration in a natural cycle (7–9), which correlates to 5 days after progesterone administration during hormone replacement therapy in IVF treatment cycles (8, 10). A number of studies have shown that a displaced WOI contributes to RIF (11, 12).

First discovered in 1993, microRNAs (miRNAs) are 18–25 nucleotides

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long, noncoding ribonucleic acids (RNAs) involved in gene regulation that have been found in humans and other multicellular organisms (13, 14). More than 2,500 miRNAs have been discovered in the human genome (15), some of which are implicated to play important roles in reproductive cycles (16–18). Emerging literatures have demonstrated that miRNAs regulate the expression of the genes involved in the establishment of the WOI (19–25). Therefore, a dysregulation of miRNAs could contribute to the RIF experienced by infertile patients.

In the present study, we utilized a novel multigene expression profiling platform, PanelChip (Quark Biosciences, Inc., Hsinchu, Taiwan) (26, 27), to explore miRNAs that are differentially expressed in patients with RIF. We identified 6 miRNAs that could contribute to RIF. Further target analyses indicated that these miRNAs targeted the genes that regulated the establishment of WOI. Finally, a signature of 3 miRNAs was identified from the 6 candidates that could potentially be used as biomarkers to determine whether or not the patients have a displaced implantation period.

MATERIALS AND METHODS

Subjects

Endometrial tissue samples were obtained from 36 infertile patients undergoing assisted reproductive technology treatment. Biopsies were collected from the uterine cavity using a Pipelle catheter (Gynétics Medical products, Lommel, Belgium) after 120 hours of progesterone administration in a hormone replacement therapy cycle for endometrial receptivity analysis (ERA) from Igenomix Group (Valencia, Spain). Residual endometrial tissues were used for the identification of differentially expressed miRNAs. Approval of the study was obtained from the Joint Institutional Review Board (JIRB No.19-S-011-1) and written informed consent was obtained from all participants (NTHU_TIG_TYGH_QBs_002).

Study Design

For the discovery cohort, 25 patients undergoing hormone replacement therapy for IVF treatment were enrolled in the study. Endometrial biopsy tissues were collected 120 hours after progesterone administration (P+5). Two groups of women, RIF (n = 8) and nonRIF (n = 17), in the discovery cohort were recruited at Taiwan IVF Group, a private, multiphysician group practice IVF center in Taiwan. The RIF group included 8 women who had ≥2 unsuccessful IVF cycles and had a nonreceptive endometrium as determined by the ERA test. All of the women in the RIF group subsequently had successful implantation with a personalized embryo transfer on the basis of ERA test results. The nonRIF group, which served as a control group, included 17 infertile women who had successful implantation after the first embryo transfer attempt in IVF. All 17 women in the nonRIF group had a receptive endometrium as determined by the ERA test.

To validate our findings of RIF-related miRNAs, miRNA expression profiling using PanelChip analysis was performed on endometrial samples from 5 RIF patients and 6 nonRIF patients in the validation cohort. All 11 women subsequently had successful implantation with a personalized embryo transfer on the basis of ERA test results.

RNA Extraction and miRNA Enrichment

Total RNAs were isolated from the endometrial tissue using miRNeasy Micro Kit (QIAGEN, Germantown, MD) as described in the manufacturer's instruction manual. Briefly, 5 mg of endometrial tissue was disrupted and homogenized in liquid nitrogen with a mortar and pestle. Then 700 μL of QIAzol Lysis Reagent (QIAGEN) was added to the homogenized tissue, which was incubated at room temperature for 5 minutes to promote the dissociation of nucleoprotein complexes. After incubation, the sample was transferred to a tube followed by the addition of 140-μL chloroform. The tube was shaken vigorously for 15 seconds and incubated at room temperature for 3 minutes. The sample was then centrifuged at 12,000 × g for 15 minutes at 4°C, with the resulting upper aqueous phase transferred to a new tube. One volume, equal to the amount of the upper aqueous phase, of 70% ethanol was added to the tube and vortexed thoroughly. The sample was added to an RNeasy MinElute spin column (Qiagen Sciences, Hilden, Germany) and centrifuged at 8,000 × g for 15 seconds at room temperature. The flow-through was pipetted to a 2-mL tube and vortexed thoroughly after the addition of 100% ethanol at 0.65 volume of the flow-through . The mix was transferred to an RNeasy MinElute spin column and centrifuged at 8,000 × g for 15 seconds at room temperature. The following column washing process with centrifugation was performed: 700-μL Buffer RWT (Qiagen, Hilden, Germany); 500-μL Buffer RPE (Qiagen, Victoria, Australia); and 500-μL 80% ethanol. Finally, the column was centrifuged for 5 minutes at 8,000 × g and miRNAs were eluted with 14–20 μL nuclease-free water for 1 minute at 8,000 × g. The miRNA-enriched fraction was stored at −80°C for future analysis.

Complementary Deoxyribonucleic Acid Synthesis

More than 2 ng of miRNA-enriched fraction from the endometrial tissue was used to synthesize complementary deoxyribonucleic acid (cDNA) in a 20-μL reverse transcription reaction. Reverse transcription was performed using the microRNA Universal RT Kit (Quark Biosciences, Inc., Hsinchu, Taiwan) according to the manufacturer's instructions. Briefly, Poly-A tail was added to the miRNA using Poly-A polymerase, which was performed according to the following program: 42°C for 60 minutes, 95°C for 5 minutes, and 4°C forever. The cDNA was stored at −20°C for future analysis.

miRNA Expression Analysis with PanelChip

A reproductive disease-related PanelChip was designed to analyze miRNA expression in patients undergoing IVF. The panel was constructed by first selecting genes involved in reproductive diseases from the Human Disease Ontology database (28) and subsequently utilizing miRTARBase (29), TargetScan (30), and miRDB (31) to select for potential regulator miRNAs (Supplemental Table 1, available online). For each miRNA target, 9 technical replicates were included in

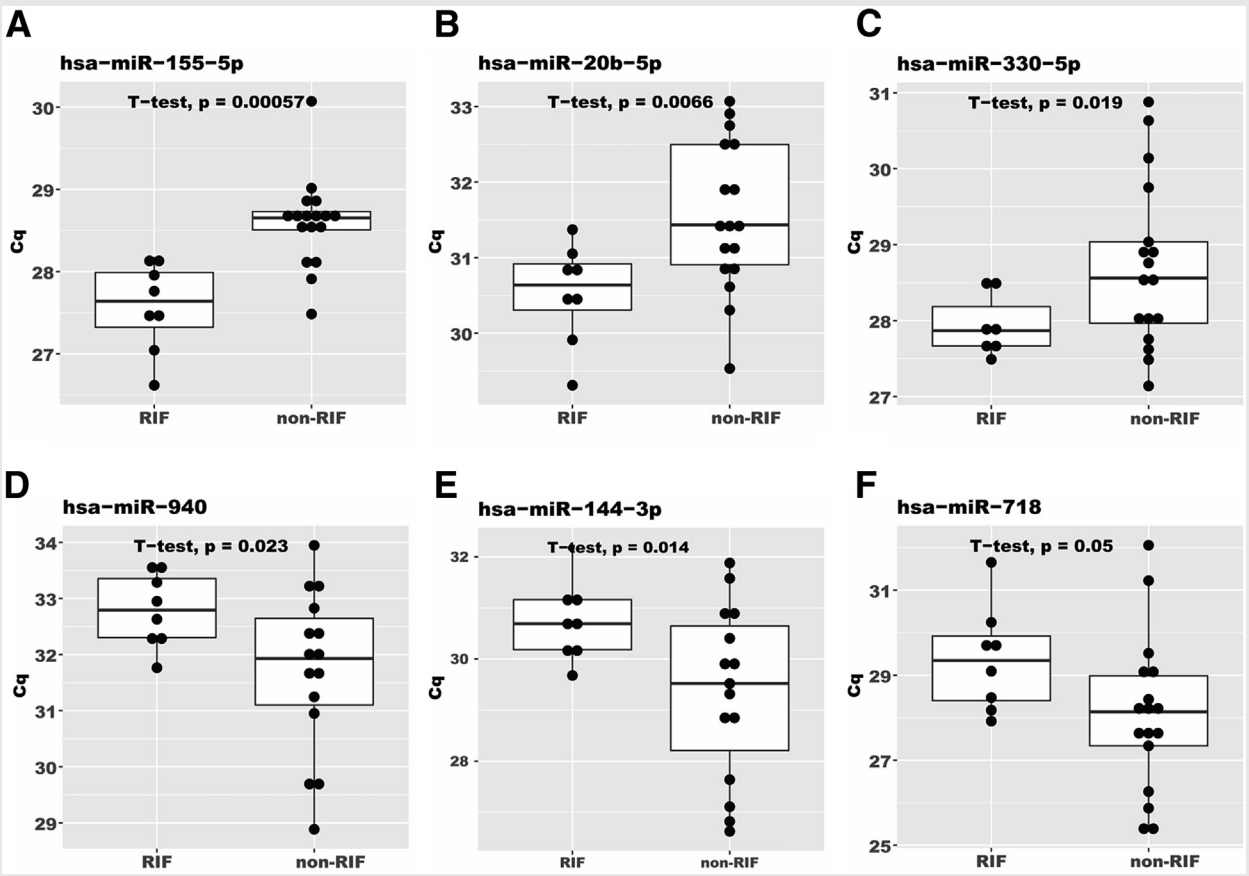
the customized reproductive disease-related PanelChip. Each technical replicate represented 1 real-time polymerase chain reaction for the target miRNA.

For the analysis of sample data and the quality of the experiment, 3 endogenous controls (RNU6B, RNU43, and 18s rRNA) and 3 exogenous spike-in controls were also included in the panel to monitor the extraction, cDNA synthesis, and quantitative polymerase chain reaction. First, 30 μL of 2X SYBR Master Mix (Quark Biosciences, Inc.) was added to the cDNA (equal to 0.1 ng of miRNA-enriched fraction) followed by the addition of nuclease-free water for a final volume of 60 μL. The master mix was mixed thoroughly and spun down. 60 μL of the mixture was dispensed along the edge of the chip, and a glass slide was used to scrape the mixture across the entire surface of the chip. The chip was then submerged into a tray containing mineral oil, with the reaction wells on the surface of the chip facing down. The trays were then placed into a proprietary thermocycler, Q Station (Quark Biosciences, Inc., Hsinchu, Taiwan), for the amplification and detection of the templates and its corresponding signals. The following cycling program was performed for quantitative polymerase chain reaction: 95°C for 36 seconds and 60°C for 72 seconds for 40 cycles.

Data Processing and Analysis

Data obtained from at least 2 PanelChips per sample were preprocessed (Supplemental Table 2, available online). A total of 92 expression profiles were normalized using the quantile normalization method (32). After averaging the replicates, 36 profiles remained. For further data preprocessing, miRNAs without amplification signals across all profiles were removed; the missing miRNA values for individual profiles were replaced with the maximum ΔCq of all profiles. Data were then separated into a discovery and a validation set. To identify the differentially expressed miRNAs in the discovery cohort of 25 patients, 2 criteria, fold change $\geq \pm 1.5$ and adjusted P value $\leq .05$, were applied. The fold changes were measured between the 2 groups of patients, with the significance (adjusted P value) calculated using the Benjamini-

FIGURE 1



Expression level in Cq values of differentially expressed miRNA between the RIF and nonRIF groups. Box plots of hsa-miR-155-5p (**A**), hsa-miR-20b-5p (**B**), hsa-miR-330-5p (**C**), hsa-miR-940 (**D**), hsa-miR-144-3p (**E**), and hsa-miR-718 (**F**). The box represents the range of miRNA expression distribution in Cq values between the 25th and 75th percentile. The horizontal line within the box represents the median Cq value of miRNA. The *P* values were determined by *t*-test. Cq = quantification cycle; miRNA = microribonucleic acid; nonRIF = non repeated implantation failure; RIF = repeated implantation failure.

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Hochberg method. Here we excluded low abundant miRNAs that were not detectable in >1 sample as they challenged the detection limit in our analysis (19). The infinite combination signature (ICS) search algorithm was designed to identify the significant biomarkers with Cq cutoffs. The ICS search algorithm searched for the variation combination of miRNA biomarkers that best categorized the RIF patients on the basis of statistically significant Cq cutoffs. We evaluated all combinations of Cq cutoffs (from 0 to 40) in the ICS search algorithm to find the best one as a combination of miRNA biomarkers (Supplemental Tables 3–7). Out of the 6 differentially expressed miRNAs, 3 biomarkers were selected to build a rule-based classifier.

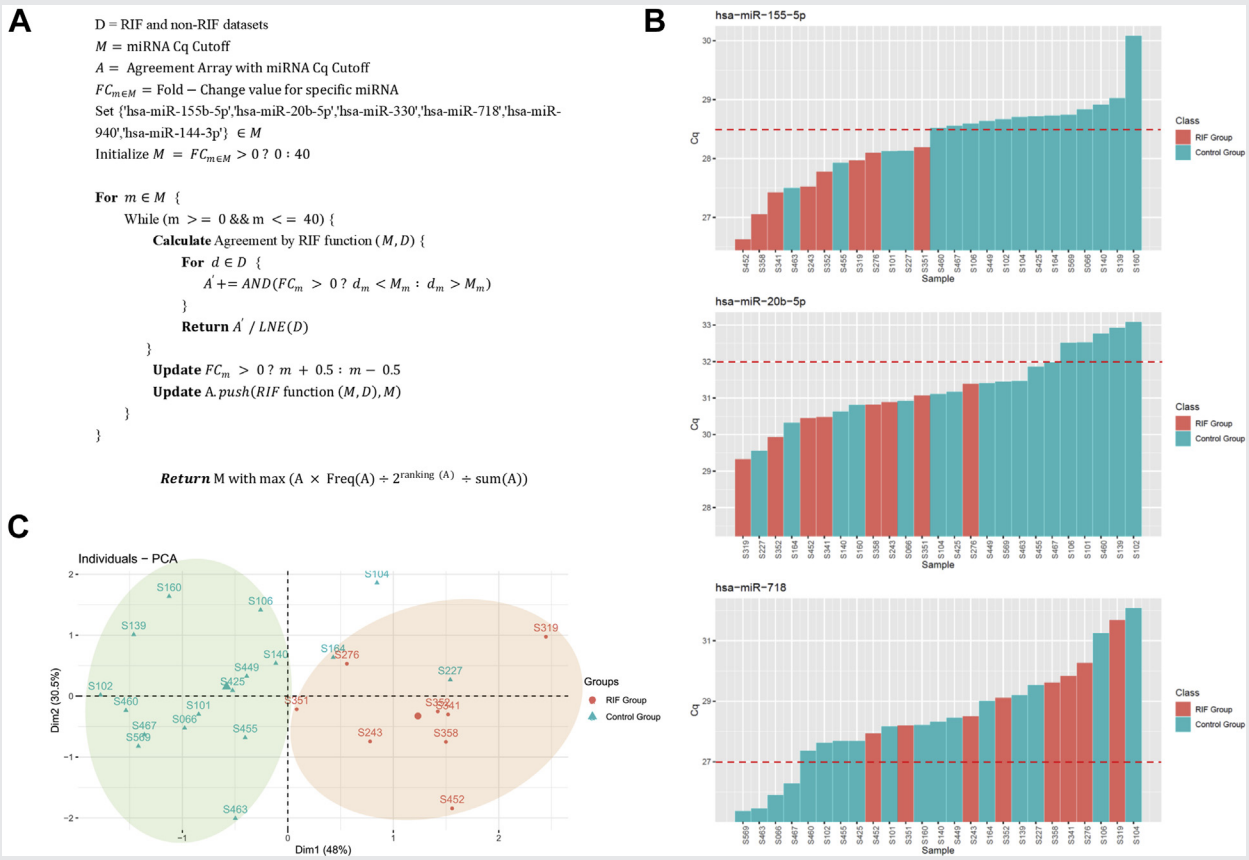
Principal components analysis was calculated in R version 3.5.2 using the prcomp package on the basis of the biomarkers found using the ICS search algorithm to distinguish between RIF and nonRIF signals. For in silico network analysis, the miRNA and messenger RNA regulatory network was confirmed using miRTarBase (29) and manually curated for genes related to RIF (33) and ERA (34). The miRNA-messenger RNA networks were visualized using Cytoscape version 3.8.0 (35).

RESULTS

Identification of Differentially Expressed miRNAs

To discover differentially expressed miRNAs, we designed a PanelChip of miRNA candidates that were previously shown to be involved in reproductive diseases, and more specifically for this study, regulate RIF-related genes (Supplemental Table 1). Of the 25 patients, 8 women had ≥2 unsuccessful implantations. The nonRIF control group consisted of 17 women who all had successful implantation after the first IVF treatment attempt. Additional clinical information for

FIGURE 2



Determination of RIF prediction signature. (**A**) The infinite combination signature search algorithm was developed to search for a variation combination of miRNA biomarkers that best categorized RIF patients on the basis of statistically significant Cq cutoff values. (**B**) A signature of 3-miRNAs with specific Cq cutoff values defining the RIF group. The expression level of hsa-miR-155-5p, hsa-miR-20b-5p, and hsa-miR-718 in the RIF group (n = 8) or the nonRIF group (n = 17). Each bar represents an endometrial tissue sample from the RIF group or the nonRIF group. *Red* color indicates the RIF group, and *green* color indicates the nonRIF group. The *red dashed lines* indicate the Cq cutoff level. (**C**) Principal component analysis of the 3-miRNA signature. Each individual shape, circle or triangle, and sample ID represent one sample. The *red dots* represent patients from the RIF group whereas the *green triangles* represent patients from the nonRIF group. Cq = quantification cycle; miRNA = microribonucleic acid; nonRIF = non repeated implantation failure; RIF = repeated implantation failure.

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TABLE 1

Differentially expressed microribonucleic acid in the endometrial tissue of repeated implantation failure patients compared with that in endometrial tissue of non repeated implantation failure patients.

miRNA	No. of RIF	No. of nonRIF	Fold change	*P* value	Adjusted *P* value
Up-regulation in RIF					
hsa-miR-155-5p	8	17	2.04	.0006	.0083
hsa-miR-20b-5p	8	17	2.02	.0066	.0167
hsa-miR-330-5p	7	17	1.71	.0194	.0333
Down-regulation in RIF					
hsa-miR-718	8	17	0.41	.05	.05
hsa-miR-940	8	15	0.48	.0235	.0417
hsa-miR-144-3p	8	15	0.38	.0138	.0250

Note: *P* values were determined by *t*-test; adjusted *P* values were determined by the Benjamini-Hochberg method. miRNA = microribonucleic acid; nonRIF = non repeated implantation failure; RIF = repeated implantation failure.

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each patient involved in the study can be found in Supplemental Table 2. Despite similar implantation conditions, the absence of uterine abnormalities and no significant differences in age, 6 miRNAs were differentially expressed between the 2 groups. The miRNAs were selected based on the fold change of $\geq \pm 1.5$ and whether or not the changes were significant based on adjusted *P* value $\leq .05$. As shown in Table 1, 3 miRNAs, hsa-miR-155-5p, hsa-miR-20b-5p, and hsa-miR-330-5p, were up-regulated in the RIF group, whereas hsa-miR-718, hsa-miR-940, and hsa-miR-144-3p were down-regulated in the RIF group (Table 1). Furthermore, box plots with median and interquartile ranges illustrate that the 6 miRNAs were statistically differentially expressed in the RIF group (Fig. 1).

All patients in the discovery cohort underwent ERA testing (Igenomix, Spain). The results of the ERA test indicated a displaced WOI in all 8 patients in the RIF group and a normal WOI in all patients in the nonRIF control group (Supplemental Table 2). The 8 patients in the RIF group subsequently had successful implantation after personalized embryo transfer on the basis of the results of ERA, which suggested that the identified miRNAs might be involved in regulating endometrial receptivity.

Determination of RIF Prediction Signature

On the basis of the expression data of the 6 differentially expressed miRNAs, we next sought a biomarker signature combination that could best distinguish patients with RIF from those in the nonRIF group. Using the ICS search algorithm (Fig. 2A), we identified a 3-miRNA signature (RIF prediction signature) with the Cq cutoff values—hsa-miR-155-5p $\leq$ 28.5, hsa-20b-5p $\leq$ 32, and hsa-miR-718 $\geq$ 27—that best categorized the RIF patients in the discovery cohort (Fig. 2B). To further evaluate the 3-miRNA biomarker combination, principal components analysis revealed that the chosen signature was able to distinguish between the 2 groups (Fig. 2C).

Validation of RIF Prediction Signature

To validate the performance of the RIF prediction signature, 11 additional patients were recruited. Of the 11 patients, 5 were patients with RIF and the other 6 patients had successful implantation after the first attempt (nonRIF group). The performance of the prediction signature is illustrated in Table 2. The RIF prediction signature had a specificity of 100%, a sensitivity of 80%, a positive predictive value of 100%, a negative predictive value of 85.7%, and an accuracy of 90.9%.

TABLE 2

Specificity, sensitivity, positive prediction value, negative predictive value, and accuracy of the prediction signature in repeated implantation failure prediction.

Statistical data	Discovery cohort (n = 25)	Validation cohort (n = 11)
Specificity[a]	88.2%	100.0%
Sensitivity[b]	100.0%	80.0%
Positive prediction value[c]	80.0%	100.0%
Negative prediction value[d]	100.0%	85.7%
Overall accuracy[e]	92.0%	90.9%

Note: RIF = repeated implantation failure.
[a] Specificity = True negatives/(True negatives + False positives).
[b] Sensitivity = True Positives/(True positives + False negatives).
[c] Precision or positive predictive value = True positives/(True positives + False positives).
[d] Negative predictive value = True negatives/(True negatives + False negatives).
[e] Accuracy = (True positives + True negatives)/(True positives + False positives + False negatives + True negatives).

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miRNA-Gene Regulatory Pathway Analysis

We next investigated the RIF-related gene targets of the 6 differentially expressed miRNAs. Because all of the patients with RIF had a displaced WOI on the basis of the results of ERA results, we also included the target genes involved in establishing endometrial receptivity in our network analysis. The genes involved in RIF and endometrial receptivity were based on 2 previous studies (33, 34). In silico analysis revealed that the 6 miRNAs potentially regulated 164 out of 304 RIF-related genes and 106 out of 238 endometrial receptivity-related genes. From the analysis, we discovered only 1 gene (*DKK1*) that was involved in both RIF and endometrial receptivity (Supplemental Fig. 1, available online).

DISCUSSION

Endometrial receptivity plays an important role in dictating the successful implantation of blastocyst during IVF treatment cycles. The dysregulation of gene expression in the endometrium of infertile patients could cause a displaced WOI, resulting in unsuccessful implantation. Utilizing, PanelChip, a novel biomarker discovery platform, we discovered 6 differentially expressed miRNAs in patients with RIF. The analysis of endometrial receptivity indicated that these patients had displaced WOI, suggesting that the irregular expression of the miRNAs caused a shift in WOI.

Our in silico analysis illustrates that the 6 miRNAs regulated 270 genes involved in RIF and endometrial receptivity, as discovered from previous studies (33, 34). Note that the 90.9% prediction accuracy of the final 3-miRNA signature was estimated from the validation cohort of 11 patients in this study. The actual prediction power of the 3-miRNA signature in the real-world population should be further evaluated in future large cohort studies enrolling more patients. To our knowledge, this is the first report of the association between 6 miRNAs and endometrial receptivity. We examined a number of genes and how they might be regulated by miRNAs during the menstrual cycle. MPP5, also known as Stardust, is a protein tightly regulated in a meticulous manner during the menstrual cycle, with its expression progressively being lowered from the proliferative to the late secretory phases (36). It has also been shown to be down-regulated in patients with RIF (33). Our analysis indicates that hsa-miR-155-5p, up-regulated in patients with RIF, targets MPP5, potentially disrupting the function of the protein during the menstrual cycle. Similarly, hsa-miR-155-5p also down-regulates IGF2, which is a well-documented protein within the insulin-like growth factor family that needs to be highly expressed during the WOI (37). Interestingly, our analysis also shows that IGF2 is targeted by another miRNA, hsa-mir-940, that is down-regulated in RIF patients, playing the opposite role of hsa-miR-155-5p. Therefore, further functional study needs to be conducted to confirm which miRNAs repress IGF2. DKK1, targeted by 2 down-regulated miRNAs, hsa-miR144-5p and hsa-miR940, is involved in both RIF and endometrial receptivity (30, 38). Aberrant expression of DKK1 might impair embryo attachment and implantation (39); therefore, tight regulation by miRNAs could play a critical role in affecting implantation. Although our in silico analysis indicated an intricate network of regulation by miRNAs, further investigation of the mechanisms of miRNA regulation in the menstrual cycle will still need to be performed. In addition, it is very likely that many more miRNAs are involved in the regulation of WOI.

MicroRNAs are involved in the regulation of biologic pathways, thus making them potential diagnostic and prognostic biomarkers (40, 41). We discovered a signature of 3-miRNA biomarkers that is capable of predicting patients with RIF with 90% accuracy. Both tissue and circulating miRNAs have also been shown to predict endometrial receptivity in previous studies (16–19). Most studies used a screening technology such as next-generation sequencing or microarray to identify miRNA biomarkers (42–44), which sometimes led to biased, contradictory results (45–49). The use of next-generation sequencing-based small RNA sequencing is the leading approach to identify new miRNA biomarkers at this stage, with the advantage of unbiased sequencing. Small RNA sequencing can query thousands of small RNA and miRNA sequences with unprecedented sensitivity and dynamic range. However, the power of small RNA sequencing largely depends on the quantity and quality of the sample preparation, especially when clinical samples are scarce or have a high chance of contamination. The use of PanelChip can quickly determine the expression profiles of a defined set of miRNAs in the given samples but with the loss of potential information on important miRNAs if they are not included in the PanelChip. It would be of great interest to use PanelChip, a precise expression profiling tool, to systematically determine biomarkers for endometrial receptivity that can be applied clinically (27). Further, both miRNAs and PanelChip can be used to discover biomarkers, such as the imprinted primate- and placenta-specific miRNAs on chromosome 19, in other topics related to reproductive diseases (50, 51). In summary, the 6 differentially expressed miRNAs potentially regulate a network of 270 genes involved in the menstrual cycle (33, 34, 52), again demonstrating miRNAs as powerful diagnostics biomarkers.

CONCLUSION

In conclusion, our finding indicated that specific endometrial miRNAs can be used as diagnostic biomarkers to predict RIF. Such information will definitely help to increase the success rate of implantation practice.

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