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Circulating and Endometrial Profiles of miR-145, miR-155-5p, miR-224, MPP-5, and PECAM-1 Expression in Patients with Repeated Implantation Failure: A Case Control Study

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Abstract

Objective: An association between microRNAs (miRNAs) and adhesion proteins expression with repeated implantation failure (RIF) has been recently reported; however, these findings are controversial. This study aims to evaluate the endometrial and circulating expressions of miR-145, miR-155-5p, and miR-224 in addition to the endometrial expressions of membrane protein palmitoylated-5 (*MPP-5*) and endothelial cell adhesion molecule-1 (*PECAM-1*) in patients with RIF compared to control subjects.

Materials and Methods: This case-control study was carried out between June 2021-July 2022. Subjects included 17 patients with RIF and 17 control subjects, who had previous spontaneous term pregnancy with a live birth, who referred to the Medical Centre of Arash Hospital, Tehran, Iran. Endometrial tissue samples were obtained via hysteroscopy and Pipelle catheter in the RIF and control subjects, respectively. Plasma samples were collected after ovulation in all subjects. The expression levels of *MPP5, PECAM-1*, miR-224, miR-145, and miR-155-5p were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). The student's t test, chi-square, Mann-Whitney U, and analysis of covariance (ANCOVA) were used for data analyses.

Results: RIF patients had less endometrial miR-155-5p expression, and higher endometrial and circulating expressions of miR-145 and miR-224 compared to control subjects. Endometrial *PECAM-1* and *MPP5* expression significantly decreased in patients with RIF compared to the control group. There was a positive correlation between circulating miR-224 and endometrial miR-155-5p, and between circulating miR-155-5p and endometrial *PECAM-1* expression levels in patients with RIF.

Conclusion: The present study suggests that circulating miR-224, endometrial miR-145, and *PECAM-1* can be reliable, novel biomarkers for diagnosis of RIF.

Keywords: Embryo Implantation, MiR-145, MiR-224, *PECAM-1*

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Introduction

Infertility is a common problem that affects 8-12% of reproductive-age couples. Over recent decades, *in vitro* fertilisation-embryo transfer (IVF-ET) has become an efficient therapeutic approach for improving fertility rates (1). Although some cases of IVF experience repeated failures, IVF-ET has remarkably increased the chance of successful pregnancy (2). Clinically, repeated implantation failure (RIF) is characterized by an inability to conceive after transferring at least four good quality embryos in a minimum of three fresh or frozen cycles, and it is a challenging issue in clinical medicine (1, 3). Although the precise definition of RIF is controversial, it commonly refers to cases that have three failed IVF attempts with good quality embryos. The main causes of RIF are structural and chromosomal abnormalities such as abnormal uterine cavity, hydrosalpinx, abnormal karyotype, thrombotic events, gene mutation, and autoimmune diseases (4). Endometrium function and receptivity are determining factors for successful IVF. The receptivity of the endometrium to blastocyst implantation occurs in a tightly controlled interval known as the

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window of implantation (WOI), which is restricted to a period within days 16-22 of a 28-day normal menstrual cycle. Displaced WOI appears to have a role in RIF (5).

Several biofactors play a role in endometrial receptivity (ER), including endothelial cell adhesion molecule-1 (PECAM-1). PECAM-1 is a 130-kD transmembrane glycoprotein that plays a key role in ER, in part, by acting on leukocyte migration, inflammatory and immune responses, and regulation of tumour growth factor-β1 (TGF-β1) expression and function (6). *PECAM-1* expression levels are lowest during the early proliferative phase and highest during the mid-secretory phase (6, 7). Women with RIF and embryo implantation failure appear to have reduced circulating levels of *PECAM-1*. Low expression or mutations in *PECAM-1* are associated with pregnancy complications such as preeclampsia, endothelial dysfunction, recurrent pregnancy loss and unexplained spontaneous miscarriages (8-10).

The precise interaction between maternal and embryo cells is important in successful implantation. During implantation, extravillous cytotrophoblasts gain the capacity to migrate, invade, and remodel the maternal spiral arterioles through the epithelial-mesenchymal transition (EMT) process (11). A number of genes, including membrane protein palmitoylated-5 (*MPP-5*), are involved in the EMT process and failure in this process is associated with pregnancy complications such as preeclampsia and foetal growth restriction (12). *MPP-5* is expressed in many tissues - the brain, heart, fallopian tube, endometrium, placenta, and epididymis (13). It interacts with nectins and facilitates the surface expression of nectin-1α, nectin-2α, and nectin-3α to improve cell trafficking and adhesion (14, 15). Patients with RIF appear to have decreased *MPP-5* expression (16). Although *MPP-5* expression levels gradually decrease from the proliferative to the late secretory stages (17), reduction in *MPP-5* gene expression in trophoblasts can damage angiogenesis, migration, and vascular invasion (18). *MPP-5* in contribution to other proteins can determine cell polarity and migration (19).

The association of microRNAs (miRNAs) with infertility has been widely studied since their discovery in 1993. miRNAs are small non-coding RNAs of approximately 19-25 nucleotides in length. miRNAs in the human genome, including miR-145, miR-155-5p, and miR-224 play a central role in the reproductive system and ER (16). It has been reported that miRNAs regulate the expressions of genes involved in the establishment of WOI and human endometrial disorders. miR-145, miR-155-5p, miR-20b-3p, and miR-330-5p appear to downregulate in women with RIF (20, 21). miRNA profiles determine ER in the late proliferative and midsecretory phases in fertile women (14). While miRNAs impact adhesion protein expressions in the reproductive system (16), the association of miR-145, miR-155-5p, and miR-224 with *PECAM-1* and *MPP-5* expression is unclear.

PECAM-1 and *MPP-5* in conjunction with miR-145, miR-155-5p, and miR-224 play keys roles during fertility and successful implantation. To date, only a few studies have investigated the association of RIF with *PECAM-1*, *MPP-5,* miR-145, miR-155-5p, and miR-224 in the Iranian population. Therefore, the present study aims to evaluate the circulating and endometrial expression levels of miR-224, miR-145, miR-155-5p, *PECAM-1,* and *MPP-5* in Iranian RIF patients compared with control subjects. The findings of this study may further provide a good predictor for differentiation of discrepant ER and RIF.

Materials and Methods

Study population

This case control study was approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran (IR.TUMS.Medicine.REC.1400.1207) and carried out between June 2021-July 2022. All participants provided written informed consent for study participation. Sample size was calculated using the confidence interval method and we enrolled 34 women - 17 women with a history of RIF and 17 women who had previous spontaneous term pregnancy with a live birth (control group).

Inclusion and exclusion criteria

Seventeen women less than 42 years of age who referred to the Medical Centre of Arash Hospital, Tehran, Iran with RIF were included in this study. The RIF patients previously underwent IVF/intracytoplasmic sperm injection (IVF/ICSI) and had at least three foetal transmission failures with at least four morphological high-grade embryos. The control group comprised 17 women with secondary infertility who had no prior history of IVF. The causes of their infertility included male infertility, tubal factors, or unexplained reasons, and they had successfully achieved a spontaneous term pregnancy resulting in a live birth.

Patients were assessed via vaginal ultrasound, hysteroscopy, laparoscopy, karyotype, or hormonal and immunological tests and excluded if they did not meet the eligibility criteria. Subjects with poor embryo quality (less than eight cells on day 3 after oocyte retrieval or less than 12 cells after oocyte retrieval on day 4 of the embryonic stage); poor ovarian response (less than four oocytes on adequate ovarian stimulation); known disorders of the uterus or endometrial pathology, such as uterine malformation, hydrosalpinx or endometriosis (diagnosed by ultrasound or laparoscopy); hereditary or acquired thrombophilia; diabetes or thyroid diseases; and all subjects with intrauterine pathologies (congenital uterus disorders, fibroids, polyps, intrauterine adhesion); polycystic ovary syndrome; endometriosis; adenomyosis; couples with abnormal chromosomal karyotypes; history of miscarriage; positive anti-lupus anticoagulant; infectious diseases; endocrine diseases (abnormal blood glucose or thyroid dysfunction); or use of contraceptives were excluded from the study.

Inclusion criteria for both the patient and control groups

consisted of a regular ovulation period (28–32 days) and normal endocrine profile.

Sampling

In accordance with a recent study (22), endometrial tissue samples were collected from both RIF and control subjects 5-7 days after ovulation. In RIF subjects, the samples were obtained via hysteroscopy, while in control subjects, the samples were collected using a Pipelle catheter. Plasma sampling was performed within 5 to 7 days after ovulation in all subjects. We chose hysteroscopy because it can detect intrauterine pathologies that are commonly missed by other investigative modalities. Ovulation time was determined by assessing morning luteinising hormone (LH) and transvaginal ultrasound (Phillips Afinity 70). The window period was defined as LH+7 days. The day of LH surge was considered to be LH 0. Endometrial tissue and plasma samples were sent to the laboratory immediately after sampling and frozen at -80°C for total RNA extraction. IVF/ICSI was performed using local protocols. All subjects had indications for IVF/ICSI treatment and underwent routine fertility tests and ovarian hyperstimulation with recombinant follicular stimulating hormone (Cinnal-F, Cinnal-F, Iran) or human menopausal gonadotropin (Menotropin, Pooyesh Darou Biopharmaceutical Co., Iran). In order to prevent LH surge, the pituitary gland was suppressed by a gonadotropin-releasing hormone antagonist (Cetronax, Ronak, Iran/ Cetrotide, Merck Serono, Germany). Final follicle maturation was triggered using 10 000 international units of human chorionic gonadotropin (HCG, Gonarx, Ronak, Iran). Oocyte retrieval was performed 36 hours after the HCG injection via guided ultrasound. In the control group, a single fresh blastocyst embryo with grade A quality was transferred using a catheter (Rada, Behrad, Iran). On the other hand, in the RIF group, two fresh blastocyst embryos with grade A quality were transferred using the same catheter.

Intravaginal progesterone (800 mg/day, Fertigest, Aburayhan, Iran) was administered for luteal phase support in both groups. Among the initial 37 patients enrolled in the RIF group, 20 patients with Müllerian malformation, hypothyroidism, chromosomal abnormalities, and antiphospholipid syndrome were excluded and 17 RIF patients who met the inclusion criteria were selected for the study.

Peripheral blood samples

Peripheral blood samples (5 mL) were obtained from patients and control subjects during WOI, at the same time as endometrial sampling before IVF. The blood samples were collected in EDTA tubes, stored on ice, and processed within 30 minutes. Each specimen was centrifuged at 1500 g for 15 minutes at 4°C to enable plasma separation. The supernatant was collected from each tube and stored at -80°C until miRNA isolation. Quantification of plasma exosomes miR-224, miR-145, and miR-155-5p was carried out using an miRNeasy Mini kit (Qiagen, USA) according to the manufacturer's protocol. The circulating miRNAs were detected in small vesicles (exosomes) in plasma.

Quantification of endometrial biopsies

An obstetrician-gynecologist obtained a small sample of endometrial tissue from each participant in the study.

RNA extraction and quantitative real time-polymerase chain reaction

Endometrial tissue lysates were used for total RNA extraction with an miRNeasy Mini kit (Qiagen, USA) according to the manufacturer's instructions. A NanoDrop spectrophotometer (Shimadzo, UV 160) and 3% agarose gel electrophoresis were used to assay the concentration and purity of the extracted RNA. A total of 300 ng total RNA was used for first strand cDNA synthesis and miRNA expression profiling with the MI Script II RT Kit (Qiagen, Germany).

The MI Script SYBR® Green PCR Kit (Qiagen, Germany) and specific primers were used to determine the expressions of miR-224, miR-145, miR-155-5p, *PECAM-1*, *MPP-5*, *U6 snRNA*, and *β-actin* genes (Qiagen, Germany) via quantitative real time-polymerase chain reaction (qRT-PCR). The primers were:

MPP5-

F: 5′-AGGCACCAAACCCAACATCT-3′
R: 3′-GCCAGAACCAGCGATCCTTA-5′

PECAM-1-

F: 5′-ACGTGCAGTACGGAAGTT-3′
R: 3′-GGAGCCTTCCGTTCTAGAGT-5′

miR-224-

F: 5′-GCGAGGTCAAGTCACTAGTGGT-3′
R: 5′-CGAGAAGCTTGCATCACCAGAGAACG-3′

miR-145-

F: 5′-GTCCAGTTTTCCCAGGAATCC-3′
R: 5′-CAGTGCAGGGTCCGAGGTAT-3′

miR-155-5p-

F: 5′-UAAUACCGUCUUAAAACCGU-3′
R: 5′-UUCUGGGAACGUGAAACCT-3′

β-Actin-

F: 5′-TTCCAGCCTTCCTTCTTG-3′
R: 3′-GGAGCCAGAGCAGTAATC-5′

U6 snRNA-

F: 5′-CTCGCTTCGGCAGCACATATACT-3′
R: 5′-ACGCTTCACGAATTTGCGTGTC-3′

All the reaction mixtures were incubated in a 96-well plate at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and at 60°C for 40 seconds. ABI-Step-One (Applied Biosystems, USA) was used for

PCR amplification. *MPP5, PECAM-1*, and the miRNA expression levels were estimated by the $2^{-\Delta\Delta Ct}$ method and normalized to *β-actin* (for *MPP5* and *PECAM-1)* and *U6 snRNA* for the miRNAs.

Statistical analysis

We used the SPSS Software Package version 18.0 (IBM Corporation, USA) for statistical analyses. Normality was assessed by the Shapiro-Wilk test. Categorical data were presented as frequency and continuous data as mean ± standard error of the mean or standard deviation (SD). The t test, chi-square and Mann -Whitney U tests were used to compare continuous data between groups, depending on the normality of the data. In addition, Pearson correlation coefficient analysis was performed to determine the correlation between miRNA expression levels and biochemical parameters. Analysis of covariance (ANCOVA) was performed to eliminate the possible effect of covariance on the miRNA expression levels. In addition, we performed linear logistic regression to assess the risk of failed implantation according to miRNA expression. One-way analysis of variance was performed to evaluate the number of variables followed by Tukey's post-hoc test. $P<0.05$ indicated statistical significance.

Results

Demographic characteristics

Table 1 lists the clinical characteristics of RIF patients and the control group. There were no significant differences in terms of age, body mass index (BMI), anti-Mullerian hormone (AMH), and cause of infertility between control subjects and RIF patients. Also, there was no significant difference between the average number of retrieved oocytes and embryos between the two groups. According to Table 1, the RIF group had a significantly lower implantation rate (5.9%) compared to the control group (29.4%, P=0.17). One woman from the RIF group became pregnant after endometrial scratching, which resulted in a live birth, and 5 women from the control group continued to pregnancy after 20 weeks.

Table 1: Demographic characteristics of study participants

Characteristics	Value	RIF patients (n=17)	Control subjects (n=17)	P value
Age at biopsy (Y)	Mean (SD)	34.05 (5.51)	34.11 (4.24)	0.97[1]
BMI (kg/m^2)	Mean (SD)	26.29 (3.35)	26.41(2.57)	0.43[2]
	Median (IQR)	25.14 (3.71)	26.07 (2.63)	
AMH (ng/dL)	Mean (SD)	3.76 (1.98)	3.10 (2)	0.34[1]
Cause of infertility, n (%)	Anthological infertility	5 (29.4)	5 (29.4)	0.71[3]
	Unexplained subfertility	8 (41.7)	6 (35.3)	
	Tubal pathology	4 (23.5)	6 (35.3)	
Treatment findings	Oocytes			
	Mean (SD)	15.71 (10.84)	10.47 (6.79)	0.23[2]
	Median (IQR)	14 (17)	9 (6.5)	
	Embryos			
	Mean (SD)	8.41 (5.37)	5.71 (3.38)	0.21[2]
	Median (IQR)	8 (9)	5 (3)	
Implantation rate, n (%)	Positive	1 (5.9)	5 (29.4)	0.17[3]
	Negative	16 (94.1)	12 (70.6)	

[1]; Independent sample t test, [2]; Mann-Whitney U test, [3]; Chi-square test, RIF; Repeated implantation failure, SD; Standard deviation, BMI; Body mass index, IQR; Interquartile range, and AMH; Anti-Mullerian hormone.

miRNA expression levels in the repeated implantation failure and control groups

The endometrial expression level of miR-155 in patients with RIF was significantly lower than the control group (P=0.03, Fig.1A). Endometrial miR-224 and miR-145 had significantly higher expression levels in RIF patients compared to the control group (P<0.0001, Fig.1B, C, respectively). There was no significant difference between circulating miR-155-5p expression levels of RIF and control subjects (P=0.547, Fig.1D). Circulating miR-224 had significantly higher expression levels in RIF patients compared to the control group (P<0.0001, Fig.1E). The circulating miR-145 expression levels were also higher in patients with RIF compared to control subjects (P<0.0001, Fig.1F). Both endometrial PECAM-1 and MPP-5 had significantly lower expressions in patients with RIF than control subjects (P<0.001 and P<0.05, respectively, Fig.1G, H).

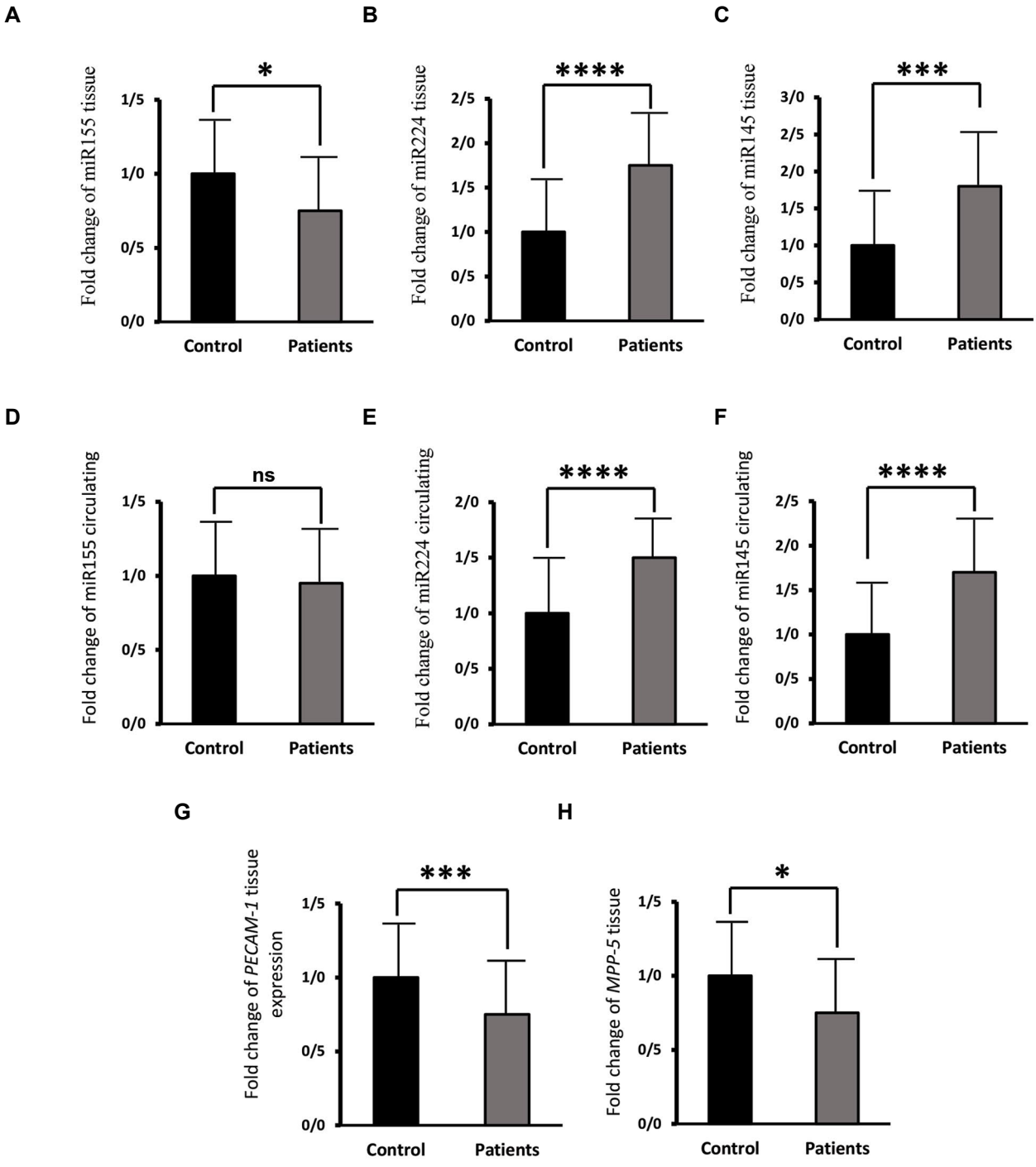

Fig.1: The expression levels of endometrial and circulating miR-155-5p, miR224, miR145, *PECAM-1* and *MMP-5* in RIF patients and control subjects. **A-C.** The expression levels of endometrial miR-155-5p, miR224, and miR145, respectively. **D-F.** The expression levels of circulating miR-155-5p, miR224, and miR145, respectively. **G.** Endometrial endothelial cell adhesion molecule-1 (*PECAM-1*) expression levels. **H.** Endometrial membrane protein palmitoylated-5 (*MPP-5*) expression levels. ****; P<0.0001, ***; P<0.001, *; P<0.05, and ns; Not significant.

Correlation of variables

Significant positive correlation was observed between circulating and endometrial miR-155-5p expression levels (P<0.001, Fig.2A) in the control group. There was also a significant positive correlation between expression levels of endometrial miR-145 and circulating miR-155-5p (P<0.05, Fig.2B); however, the correlation between endometrial and circulating miR-224 expression levels was significantly negative (P<0.05, Fig.2C). We observed a significant positive correlation between expression levels of endometrial and circulating miR-155-5p in the RIF group (P<0.05, Fig.3A). The correlation between expression levels of circulating miR-224 and endometrial miR-155-5p was significantly positive (P<0.01, Fig.3B). We also found a positive significant correlation between expression levels of miR-155-5p and endometrial *PECAM-1* (P<0.01, Fig.3C). There was no significant correlation between the other variables.

Receiver operator characteristic curve analysis

Receiver operator characteristic (ROC) curve analysis (Fig.4) was used to examine the probability of *MPP5*, *PECAM-1*, miR-224, miR-145, and miR-155-5p for RIF. Circulating miR-155-5p [area under the curve (AUC): 0.577, (95% confidence interval (CI): 0.378, 0.770), P=0.425] showed a poor discriminatory power; however, endometrial miR-224 [AUC: 0.878, (95% CI: 0.753,1), P<0.01], circulating miR-224 [AUC: 0.872, (95% CI: 0.753,1), P<0.01], endometrial miR-145 [AUC: 0.854, (95% CI: 0.708,1), P<0.01], and endometrial *PECAM-1* [AUC: 0.850, (95% CI: 0.718,0.981), P<0.01] showed high discriminatory power to detect RIF. Endometrial miR-155-5p [AUC: 0.686, (95% CI: 0.512,0.860), P<0.05] and endometrial MPP5 [AUC: 0.719, (95% CI: 0.553,0.885), P=0.024] had moderate power as a biomarker of RIF.

Table 2 shows the true positive (TP), true negative (TN), false positive (FP), and false negative (FN) values associated with *MPP5*, *PECAM-1*, miR-224, miR-145, and miR-155-5p that can be used to assess the performance of each biomarker in predicting RIF disease status. Circulating miR-224 and miR-145, and endometrial miR-145 had the same TN (16), TP (18), FN (3), and TN (3) values. Endometrial *MPP5* had higher FN (6) and FP (8) compared to *PECAM-1* and the miRNAs.

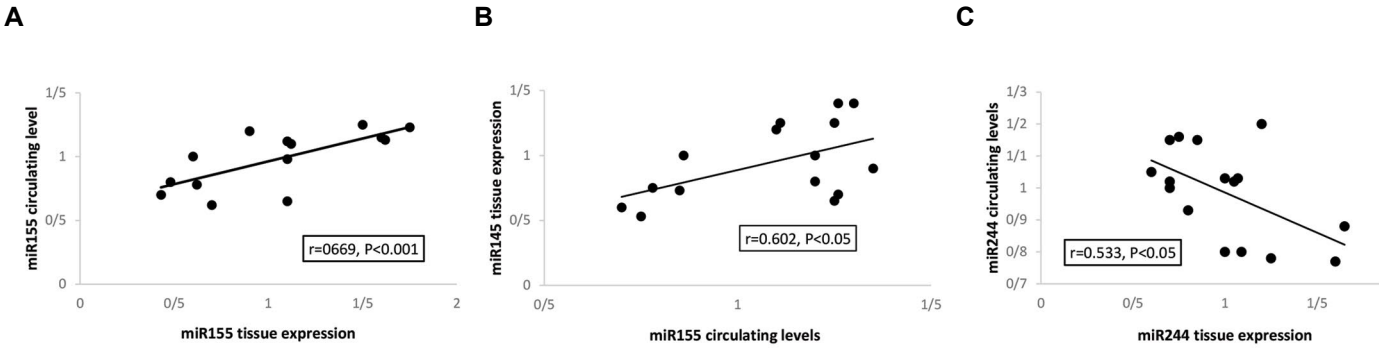

Fig.2: Pearson's correlation coefficient analysis for correlations between expression levels of miR-155-5p, miR-145 and miR-224 in the control group. **A.** Endometrial miR-155-5p versus circulating miR-155-5p, **B.** endometrial miR-145 versus circulating miR-155-5p, and **C.** endometrial miR-224 versus circulating miR224.

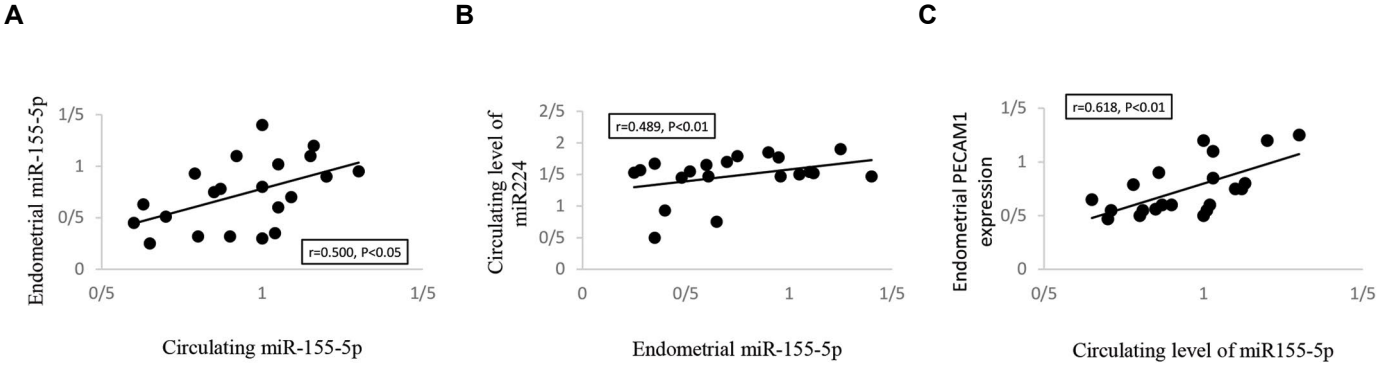

Fig.3: Pearson's correlation coefficient analysis for correlations between expression levels of miR-155-5p, miR-224, and *PECAM-1* in RIF patients. **A.** Endometrial miR-155-5p versus circulating miR-155-5p, **B.** Endometrial miR-155-5p versus circulating miR-224, and **C.** Circulating miR-155-5p versus endothelial cell adhesion molecule-1 (*PECAM-1*) in the repeated implantation failure (RIF) group.

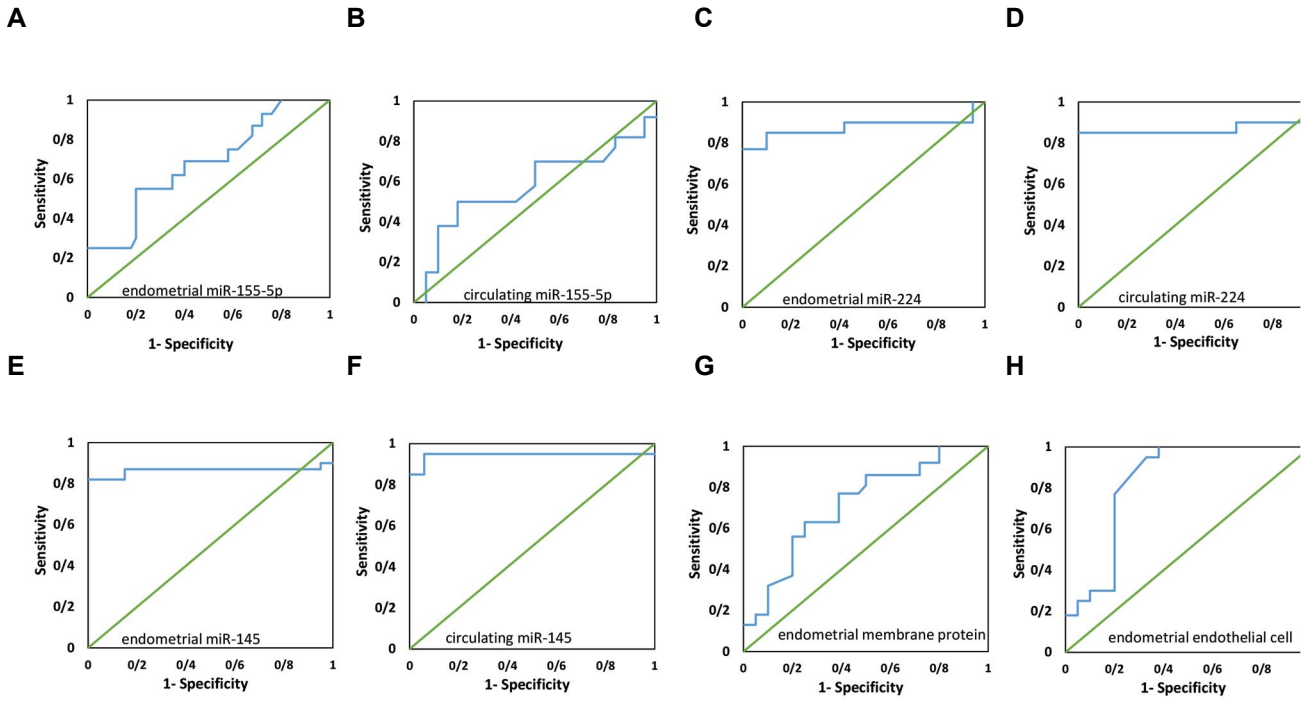

Fig.4: Receiver operator characteristic (ROC) curve analysis for examining the probability of *MPP-5*, *PECAM-1*, miR-224, miR-145, and miR-155-5p for RIF. **A.** Endometrial miR-155-5p, **B.** Circulating miR-155-5p, **C.** Endometrial miR-224, **D.** Circulating miR-224, **E.** Endometrial miR-145, **F.** Circulating miR-145, **G.** Endometrial membrane protein palmitoylated-5 (*MMP-5)*, and **H.** Endometrial endothelial cell adhesion molecule-1 (*PECAM-1*) in patients with repeated implantation failure (RIF).

Table 2: Prediction of outcome according to ROC curve analysis

miRs, *PECAM-1*, and *MPP-5* expression	TN	TP	FN	FP
Endometrial miR-224 expression	14	18	3	3
Circulating miR-224 levels	16	18	0	3
Endometrial miR-145 expression	16	18	0	3
Circulating miR-145 levels	16	18	0	3
Endometrial *PECAM-1* expression	15	16	1	5
Endometrial *MPP5* expression	10	13	6	8

ROC; Receiver operator characteristic, TP; True positive, TN; True negative, FN; False negative, and FP; False positive.

Discussion

Accumulating evidences show that cell adhesion molecules (CAMs), including *PECAM-1* and *MPP-5,* and an array of endometrial miRNAs have the capability to predict RIF (2, 12); however, exploring the correlation between *PECAM-1* and *MPP-5* with miRNAs presents a complex and challenging matter. Our findings showed decreased expression levels of *PECAM-1* and *MMP-5* in women with RIF compared to normal pregnant females. We observed decreased expression of endometrial miR-155-5p, and increased expressions of endometrial and circulating miR-145 and miR-224 in women with RIF. Circulating miR-224, endometrial miR-145, and *PECAM-1* could be potential biomarkers for detection of RIF.

The function of PECAM-1 in regulation of embryo implantation is poorly understood despite reports that a number of CAMs are expressed in the decidual endothelial cell and in the trophoblast, and are necessary for correct implantation (6, 7). However, consistent with our findings, the expression level of *PECAM-1* was reported to decrease in the early proliferation and late proliferative phases. The expression level of *PECAM-1* reduces in women with RIF, which indicates that this molecule can be a good predictor for RIF (6). There is a significant positive correlation between expression levels of *PECAM-1* and vascular damage, which suggests that reduced expression levels of *PECAM-1* can justify a reduction in trophoblast invasion (9, 23), and lead to the embryo's inability to implant. *PECAM-1* has an important role in *TGF-β1* expression and regulation. Reduced expression levels of *PECAM-1* and *TGF-β1* have been found in women with RIF (6), which indicates that *PECAM-1* may have a key role in regulation of *TGF-β1* expression followed by proper implantation.

Few studies have assessed the association between *MPP-5* and implantation in humans. In line with our finding, a mutation in *MPP-5* has been reported to delay epithelial polarization and decrease transepithelial electrical resistance (24), which may be followed by implantation failure. A reduction in *MPP-5* expression may lead to a decreased interaction of MMP-5 with other CAMs and result in failure of the embryo to migrate (25, 26) followed by implantation failure. The results of a study in mice indicated that an interaction between MPP-5 with other CAMs is essential for the maintenance of polarity during different stages of embryo and implantation development (27); therefore, a reduction in *MPP-5* levels can upset polarity of the embryo and result in implantation failure. On the contrary, the results of a cross-sectional study on preeclampsia showed that *MPP-5* had higher expression in the severe early-onset preeclampsia group compared to control subjects. This discrepancy can be justified by the stage of pregnancy and vascular damage that happens in preeclampsia.

Endometrial and circulating miRNAs can predict ER and are recognized as potential reliable biomarkers for implantation and RIF (25, 28). Consistent with our findings, it has been reported that miR-145, miR-155-5p, and miR-224 play critical roles in infertility and implantation (29). An increased expression level of miR-145 has been detected in infertile women with endometriosis (30). miR-145 has been reported to over-express in women with recurrent pregnancy loss relative to normal pregnant women (31). MiR-145 has been shown to regulate the expression of a number of genes associated with adhesion molecules, and make a substantial contribution to endometrial receptivity, placentation and implantation (20, 32). Abnormal miR-145 expression can be correlated to abnormal expression of tyrosine kinase receptor (*RTKN)*, oestrogen receptor (*ER-α),* insulin-like growth factor 1 receptor (IGF-1R) and other signalling factors that can damage ER and result in implantation failure (33, 34). Research has shown a link between miR-224 overexpression and implantation failure, which supported the results of the current study (33). MiR-224 overexpression negatively affects embryo development (35) and resultant implantation failure. MiR-224 upregulation leads to implantation defects in women with recurrent miscarriage (36).

In the present study we have shown that the expression level of circulating miR-155-5p did not significantly change in RIF patients; however, the expression levels of endometrial miR-155-5p significantly decreased in RIF patients compared to control subjects. MiR-155-5p is a crucial regulator of a number of genes, including *TGFβ* and *MMP,* by which it modulates the inflammatory and immune responses and proper implantation (34). In contrast to our finding, miR-155-5p has been reported to upregulate in the RIF patients (22). Decreased expression levels of miR-155-5p were found to increase extracellular matrix degradation and the decidualization process of proper implantation (37). Overexpression of miR-155-5p could inhibit the smad2/3 signalling pathway and repress cell proliferation, migration, apoptosis, and invasion in the endometrium, which leads to suppression of implantation (38). More experimental and clinical research are required to elucidate the exact association of miR-155-5p with implantation failure in RIF patients.

We observed a positive correlation between expression levels of endometrial miR-155-5p and circulating miR-155-5p and miR-224 in patients with RIF. This correlation might be the result of the common signalling pathways and regulatory mechanisms behind endometrial miR-155-5p and circulating miR-155-5p and miR-224 action on their target tissues and cells (28). Our findings did not show any significant correlation between the expression levels of endometrial and circulating miR-244 in RIF patients, which indicates that the factors associated with expression profiles of endometrial and circulating miR-244 are likely different; however, more molecular research is required to determine the factors associated with miR-244 expression in RIF patients. Unlike the RIF group, we observed a negative correlation between circulating and endometrial miR-224, and a positive correlation between circulating and endometrial miR-145 and miR-155-5p in the control subjects, which suggests that the expression profiles of miR-224, miR-145, and miR-155-5p follow different pathways in RIF patients. It has been shown that adipocytes release numerous miRNAs in plasma, and this indicates that plasma miRNAs can be suitable biomarkers for evaluation of certain diseases (39). ROC and Pearson analysis in the current study demonstrated a significant correlation between circulating and endometrial miR-155-5p and miR-224 in RIF patients, which suggests that miR-155-5p and miR-224 are released from endometrial tissue into circulation; therefore, they can be proper biomarkers for evaluation of RIF. The discrepancy between miRNAs signature in control subjects and RIF patients highlights the importance of the physiological and pathophysiological state of ER and susceptibility to implantation failure. It is suggested that an endogenous miRNA regulatory and control mechanism to normalize circulating miRNA levels might be absent (8).

Conclusion

Our findings show that downregulation of *PECAM-1, MPP-5*, miR-145, and miR-224 along with upregulation of miR-145 and miR-224 most likely result in decreased ER and implantation defects

in RIF patients. In addition, circulating miR-224, endometrial miR-145, and *PECAM-1* appear to be potential biomarkers for prediction of RIF. Although we observed a positive correlation between circulating miR-155-5p and endometrial *PECAM-1* expression, the correlation between miR-145 and miR-224, and endometrial *PECAM-1*, and association of miR-145, miR-224, miR-155-5p with endometrial *PECAM-1* and *MPP-5* expression remains to be elucidated.

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Authors' Contributions

S.F., M.F.M.; Proposed the idea, concept and design of the study. J.J., N.M., L.K., A.M., R.F., S.A.-F., S.B.T., M.Kh. M.F.M., R.R., A.T.; Contributed to the overall research project and performed the experiments and data analysis. R.R., S.F., J.J.; Wrote the manuscript draft. S.F., R.R.; Reviewed and revised the draft. All authors approved the final version of this manuscript.

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