

hsa_circ_0004121 and hsa_circ_0030162 Differentially Expressed in Plasma of Patients with Recurrent Implantation Failure

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Abstract

Background: Recurrent or repeated implantation failure (RIF) is a significant challenge that hampers the success rate of assisted reproductive technology (ART) in achieving pregnancy. The underlying mechanisms of RIF remain unclear. Recent studies have identified distinct expression patterns of circular RNAs (circRNAs) in the endometrial tissues of individuals with RIF. The objective of this research is to evaluate the expression of six candidate circRNAs in the plasma of RIF patients.

Methods: The study included a total of sixty participants, comprising 30 RIF patients and 30 age-matched controls. Specific primers were designed for six circRNAs (hsa_circ_0001713, hsa_circ_0004121, hsa_circ_0030162, hsa_circ_0034642, hsa_circ_0034762, and hsa_circ_0092337), and expression analysis was carried out by RT-qPCR. Blood samples were collected from individuals during the implantation window, and cell-free RNA was extracted from plasma. Statistical analysis was performed using Graphpad Prism software.

Results: Control and RIF groups had mean ages of 34.68 ± 6.2 and 36.80 ± 3.8 years, respectively. Plasma from RIF patients showed significant downregulation of hsa_circ_0030162 ($p=0.02$) and upregulation of hsa_circ_0004121 ($p=0.003$) compared to controls. Bioinformatic analysis predicted hsa-miR-125a-3p and hsa-miR-125a-5p as potential targets of hsa_circ_0030162 and hsa_circ_0004121, respectively.

Conclusion: This study demonstrates the differential plasma expression of hsa_circ_0030162 and hsa_circ_0004121 in RIF patients, consistent their expression in endometrial tissue. These circRNAs may contribute to RIF pathogenesis. Further research is needed to validate these findings and explore their clinical utility.

Keywords: Circular RNA, Plasma, Pregnancy, Recurrent implantation failure.

Introduction

Assisted reproductive technology (ART) has a roughly 30% embryo implantation rate, with 10% of in-vitro fertilization (IVF) patients experiencing recurrent implantation failure (RIF) (1, 2) which is a significant challenge due to its extremely low pregnancy rate and poorly understood etiology. It involves complex interactions between the female

partner, male partner, and the embryo itself (3). Endometrial receptivity issues may account for up to two-thirds of implantation failures (4). Current methods for assessing endometrial receptivity are invasive, highlighting the urgent need for non-invasive biomarkers to predict RIF. Discovering and validating such biomarkers would greatly advance diagnostics

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Received: 21 Dec, 2024; Accepted: 3 Feb, 2025

and prediction in this area.

Advances in high-throughput sequencing and molecular biology have revealed the potential roles of non-coding RNAs (ncRNAs) in various biological processes through post-transcriptional regulation (5, 6). Studies show altered expression of ncRNAs, including microRNAs (miRNAs) (7-11), long non-coding RNAs (lncRNAs) (12-15), and circular RNAs (circRNAs) (16-22), in the endometrium of women with RIF. This suggests their involvement in implantation and their potential as diagnostic and therapeutic biomarkers for RIF. CircRNAs, a new category of ncRNAs, demonstrate remarkable stability compared to linear RNAs, even in the bloodstream, and exhibit high abundance and tissue specificity (23, 24). Despite the prevalence of circRNAs in blood, only a limited number of studies have investigated their potential as biomarkers in plasma samples from patients with RIF.

Two studies conducted by Lin Liu et al in 2017 (22) and Tianxiang Ni et al in 2021 (20) investigated circRNA expression in endometrial tissue from RIF patients. The data from the first study (22) are freely available (GEO: GSE147442) and have been frequently reanalyzed along with several other studies (16, 17, 21). After screening the results of these publications, six circRNAs were selected based on their upregulation and downregulation, their expression in the endometrium, and their presence in at least two studies. These circRNAs include hsa_circ_0092337, hsa_circ_0004121, hsa_circ_0034642, hsa_circ_0034762, hsa_circ_0030162, and hsa_circ_0001713. This study will assess the plasma expression of these six circRNAs in RIF patients to validate the endometrial findings and evaluate their potential as predictive biomarkers.

Materials and Methods

Sample collection and study population

This study, approved by the Tehran University of Medical Sciences Ethics Committee, involved 30 RIF patients and 30 age-matched controls, under 40, undergoing IVF treatment

in 2024 at Vali-e-Asr Hospital. All participants provided informed consent. Mid-luteal phase blood samples were collected from Day P+3 to P+6 (implantation window) during a monitored natural cycle.

Unexplained RIF was defined as three or more failed transfers of high-quality embryos (or at least four embryos across multiple cycles) without identifiable causes (3). The control group comprised women undergoing their first IVF/ intracytoplasmic sperm injection (ICSI) cycle due to male factor infertility or by choice, who achieved a live birth after one or two transfer cycles.

Patients were excluded if they had: 1) uterine abnormalities (congenital anomalies, polyps, fibroids, adenomyosis); 2) hydrosalpinx, chromosomal abnormalities, or endocrine disorders; or 3) received hormone therapy or intrauterine procedures in the preceding three menstrual cycles.

Sample Processing

A 10 mL portion of peripheral blood was obtained through phlebotomy using collection tubes containing EDTA as an anticoagulant. Upon arrival at the medical laboratory, the samples were assessed for quality. Samples with low plasma volume, signs of hemolysis, elevated bilirubin levels, or visible particles were excluded from testing, and a repeat blood collection was requested.

Plasma was isolated within two hours of collection via centrifugation (1500 g for 10 minutes at 4 °C, followed by 15,000 g for 10 minutes at 4 °C) and stored at -80 °C.

RNA Isolation

Specifically, 1.5 mL of RNax-Plus® extraction kit (CinnaGen Co., Iran) solution was added to 500 µl of plasma, vortexed for 15 seconds at high speed, and left at room temperature for 10 minutes. Subsequently, 500 µl of chloroform was added to the mixture, vortexed for 15 seconds, and left at room temperature for an additional 10 minutes. The supernatant was transferred to a new tube after centrifugation at 12,000 g for 10 minutes at 4 °C. Following this, 1000 µL of Isopropanol, 2

μL of RNA carrier, and 50 μL of 3 M sodium acetate were added to the supernatant and thoroughly mixed. After approximately 25 min in $-20\text{ }^{\circ}\text{C}$, the mixture was centrifuged at 20,000 g for 20 min at $4\text{ }^{\circ}\text{C}$. Lastly, the resulting white pellet was washed with 1000 μL of 80% pre-cold ethyl alcohol and dissolved in 30 μL of nuclease-free water.

cDNA Library Construction

Purified RNA was converted to cDNA on the same day. cDNA synthesis was carried out in accordance with the producer's instructions of the SMOBIO kit (Hsinchu, Taiwan). In brief, a final volume of 20 μL of cDNA was acquired by reverse transcribing 1500 ng total RNA with random primers and then stored at $-20\text{ }^{\circ}\text{C}$ until further use.

RT-qPCR

Specific primers for circRNAs were designed using CircPrimer (25) and software NCBI primer BLAST tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Table 1). In plasma samples, ACTB served as the endogenous control, and the relative expression of circRNAs was calculated using the $2^{-\Delta\Delta\text{Ct}}$ formula.

The RT-qPCR was conducted using the Amplicon SYBR Green PCR Kit (Amplicon, Denmark) on the Applied Biosystems StepOne Real-Time PCR System (Thermo Fisher Scientific, USA). PCR was performed with an initial denaturation at $95\text{ }^{\circ}\text{C}$ for 15 minutes, followed by 40 cycles of $95\text{ }^{\circ}\text{C}$ for 10 seconds (denaturation) and $59\text{ }^{\circ}\text{C}$ for 30 seconds (annealing/extension).

Table 1. Primer sequences for circRNAs and ACTB gene.

circRNA	Primer Sequence (5'→3')	Length	Product length
hsa_circ_0001713	ACCAATGCACACACCTATAATGA	23	98
	TGCTGCTGATAACGACATACTC	22	
hsa_circ_0004121	CAAGCATTGTGTTCAGTGTG	22	112
	TCTTTGCCTGTTCTTGCTCC	20	
hsa_circ_0030162	ACTCATTGAGAGATGGTCAGTAGG	25	102
	CTTTCGGTACCTTCGCCCTC	20	
hsa_circ_0034642	TGCACTGCAGCTCTCACC	18	128
	GTTCCAACCCACACTCTCCA	20	
hsa_circ_0034762	AGGCTTTTGGACAAGTGGGT	20	128
	CATCAGGGGAGAAGGCAAGG	20	
hsa_circ_0092337	GATAACTGTCTCGTCCGTCCT	21	134
	TTAGCAGTCCCGCTTAGCTC	20	
ACTB	GTGGCCGAGGACTTTGATTG	20	76
ACTB	CCTGTAACAACGCATCTCATATT	23	

Bioinformatic analysis

We initially utilized the CircBank database (<http://www.circbank.cn/>) to predict the miRNA binding sites on differentially expressed circular RNAs (DEcircRNAs).

Subsequently, we employed the mirDIP database (<http://ophid.utoronto.ca/mirDIP/index.jsp>) to forecast the mRNA binding sites for the miRNAs identified from DEcircRNAs. From

this analysis, we constructed a competing endogenous RNA (ceRNA) functional network comprising circRNA-miRNA-mRNA interactions. For visualization, we used Cytoscape version 3.10.3 (<https://cytoscape.org/>) to illustrate the ceRNA network.

Statistical analysis

The study employed the unpaired t-test to assess differences among the RIF and control groups. Statistical significance was considered for P-values < 0.05. Statistical analyses were performed using GraphPad Prism 8.0 software.

Results

Characteristics of the study population

A total of 30 patients with RIF and 30 fertile women who underwent IVF were included in the study. The groups were matched based on age. The average age of the individuals was 34.68±6.2 in controls and 36.80±3.8 in RIF cases. The clinical characteristics of the study samples are detailed in Table 2.

Expression of Selected circRNAs in RIF Samples Compared to Control Samples

In our analysis of selected circRNA expression in RIF samples, we found that hsa_circ_0092337 (hsa_circRPL13_004) and hsa_circ_0001713 (hsa_circWBSCR17_007) did not exhibit significant expression. Consequently, due to their very low expression levels, further evaluation of these circRNAs was not pursued.

On the other hand, hsa_circ_0034642 (hsa_circVPS18_004) and hsa_circ_0034762 (hsa_circMAPKBP1_002) demonstrated a slight increase in expression in RIF plasma

samples compared to control samples. This increase was not statistically significant (p-value: 0.94) (Fig. 1).

Significant downregulation of hsa_circ_0030162 (hsa_circTPT1_003) and significant upregulation of hsa_circ_0004121 (hsa_circRFX8_003) were observed in RIF samples compared to control samples, with p-values of 0.02 and 0.003, respectively (Fig. 2)

CeRNA functional network of hsa_circ_0030162 and hsa_circ_0004121

To investigate the dysregulated circRNAs hsa_circ_0030162 and hsa_circ_0004121, we analyzed two sets of miRNAs. Initially, we utilized the CircBank database to identify predicted miRNAs for these circRNAs and extracted differentially expressed miRNAs from the GSE71332 dataset. We then identified common miRNAs present in both lists. For hsa_circ_0030162, we discovered three shared miRNA targets (hsa-miR-125a-3p, hsa-miR-204-5p, hsa-miR-6512-5p), with hsa-miR-125a-3p exhibiting the highest score in the CircBank database. In the case of hsa_circ_0004121, we identified 11 miRNA targets (hsa-miR-141-5p, hsa-miR-125a-5p, hsa-miR-4713-3p, hsa-miR-3156-5p, hsa-miR-144-5p, hsa-miR-125b-5p, hsa-miR-424-3p, hsa-miR-188-5p, hsa-miR-4507, hsa-miR-145-5p, hsa-miR-4324), with hsa-miR-125b-5p and hsa-miR-141-5p scoring highest in the CircBank database. We focused on hsa-miR-125b-5p for further analysis. Subsequently, we utilized the mirDIP database to predict gene targets for hsa-miR-125a-3p and hsa-miR-125a-5p, identifying eleven shared genes (Fig. 3).

Table 2. Clinical characteristics of patients and controls.

	Control (N=30)	Cases (N=30)	P value
Age (years)	34.68±6.2	36.80±3.8	0.87
BMI (kg/m ²)	25.41±3.2	25.62±3.7	0.80
Infertility duration (years)	5.74±4.1	5.58±4.6	0.88
Basal FSH (IU/L)	5.48±2.1	6.25±1.8	0.126

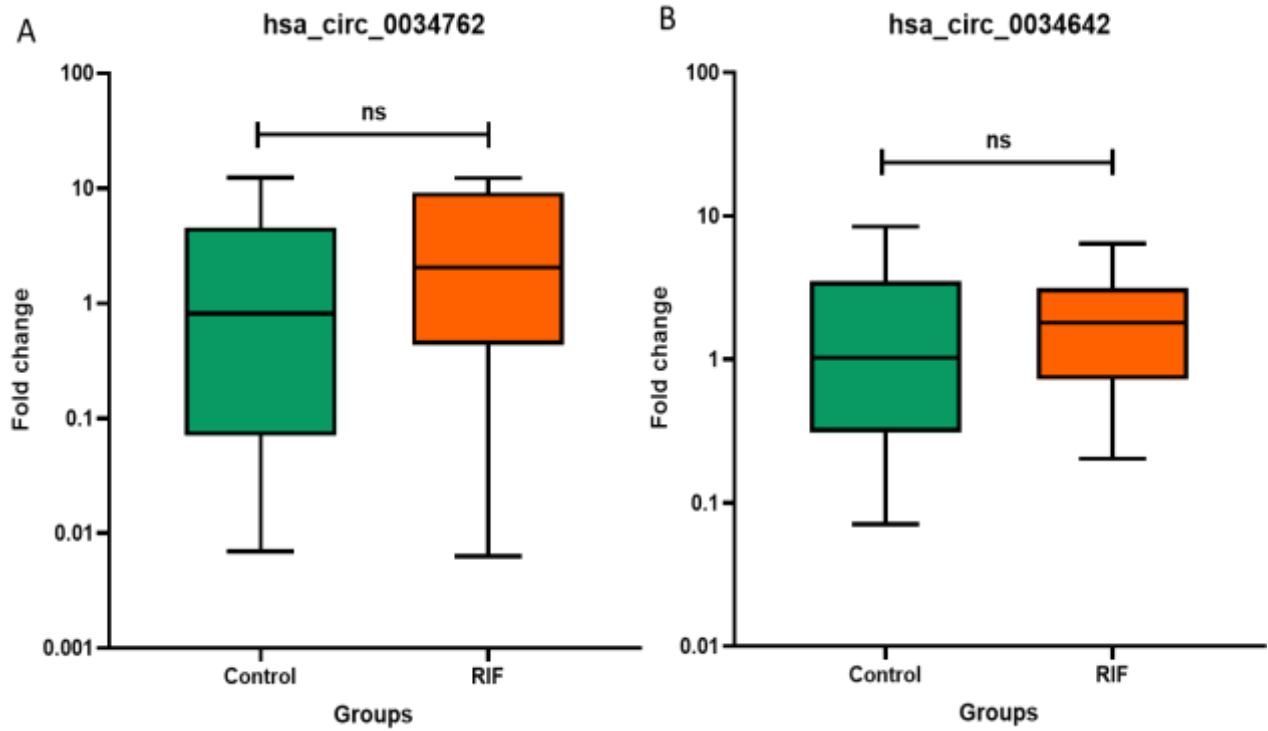


Fig. 1. Expression level of hsa_circ_0034642 and hsa_circ_0034762 in RIF samples. Expression of hsa_circ_0034762 (A) and hsa_circ_0034642(B) in RIF plasma samples in comparison to controls sample showed slightly elevated expression that did not significant. ns: not significant.

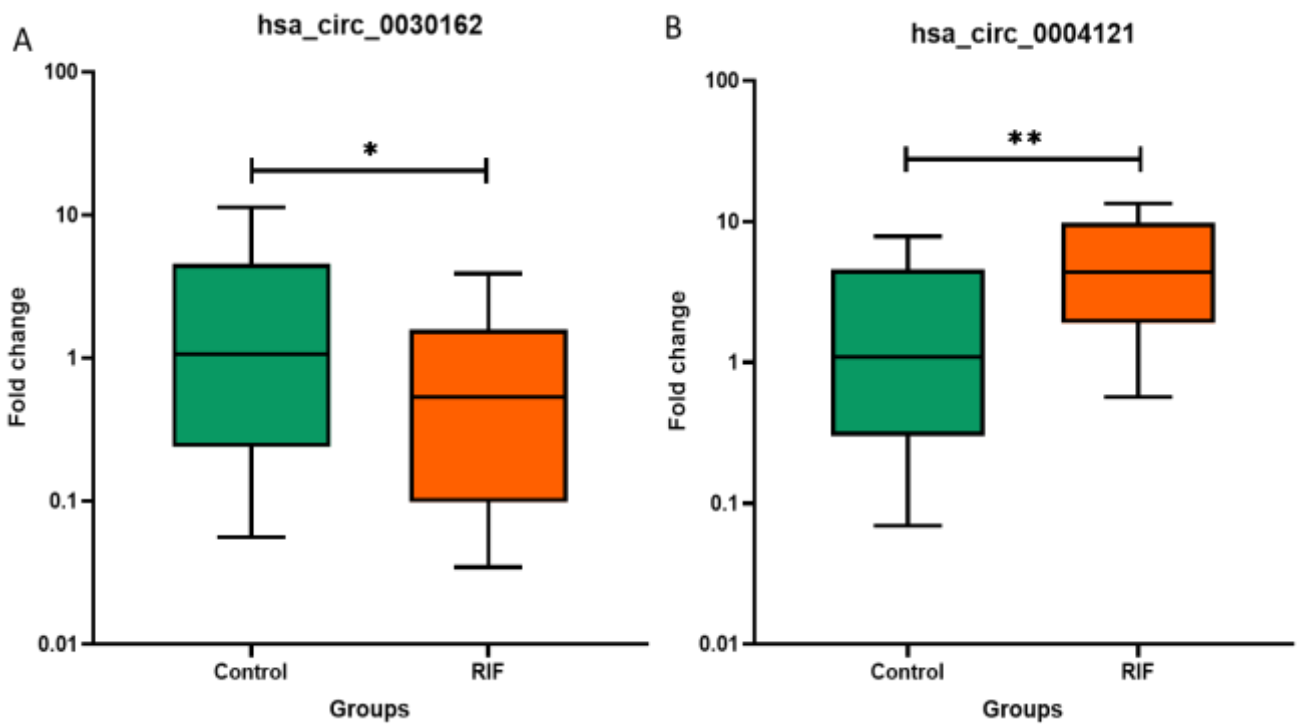


Fig. 1. Expression of hsa_circ_0030162 and hsa_circ_0004121 in RIF samples. Expression of hsa_circ_0030162 (A) and hsa_circ_0004121 (B) in RIF plasma samples in comparison to controls samples significantly dysregulated. *: <0.05, **: <0.005.

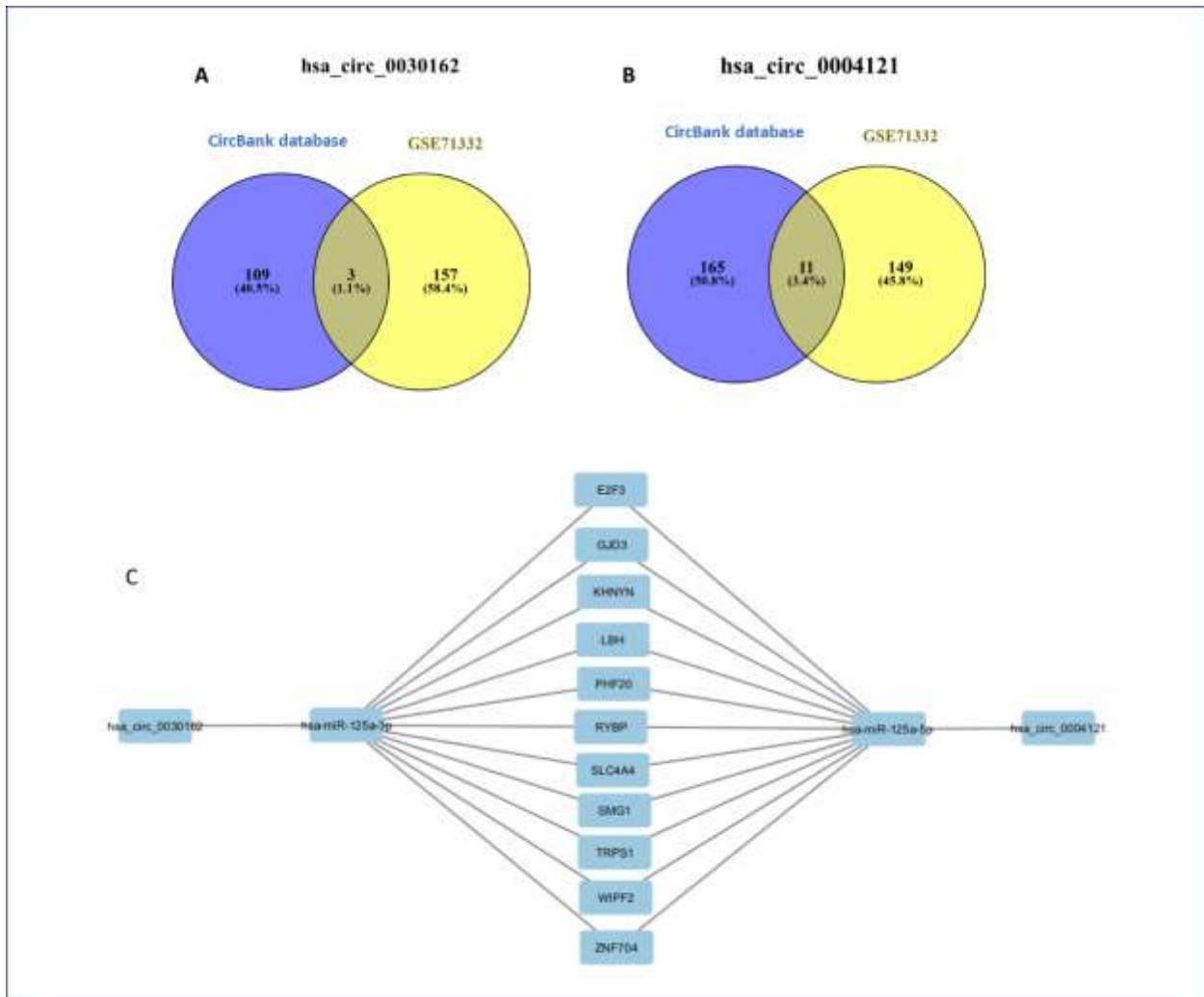


Fig. 3. CircRNA–miRNA–mRNA regulatory network, which consists of hsa_circ_0030162 and hsa_circ_0004121, two predicted target microRNAs, and 11 predicted target genes.

Discussion

In this study, we evaluated the expression levels of six circular RNAs, including hsa_circ_0092337, hsa_circ_0004121, hsa_circ_0034642, hsa_circ_0034762, hsa_circ_0030162, and hsa_circ_0001713, in the plasma of patients with RIF compared to fertile women. Among these circRNAs, hsa_circ_0030162 showed a significant downregulation, while hsa_circ_0004121 exhibited a significant upregulation in the RIF samples. Bioinformatic analysis revealed the importance of hsa-miR-125a-3p and 5p in the regulation of these circRNAs.

hsa_circ_0030162 (also known as hsa_TPT1_0000600 and hsa_circTPT1_003), located on chromosome 13 (hg19: 45911304-45914319), is derived from exons 2-5 of the *TPT1* gene (ENST00000379056.1). Tumor Protein Translationally-Controlled 1 (TPT1), a highly conserved oncogene, plays crucial roles in cell development, proliferation, differentiation, anti-apoptosis, immune defense, cytoskeletal regulation, and tumor suppression (26). It is found in the nucleus, cytoplasm, and extracellular space (27), with extracellular function facilitated by exosome secretion via TSAP6 (28). Previous studies

have reported varying expression of hsa_circ_0030162 in various tissues, including downregulation in esophageal squamous cell carcinoma (29), and involvement in bladder cancer invasion via the hsa_circTPT1_003–hsa-miR-218-5p–CCNE2/SMC4 pathway (30). Furthermore, Luo (21) and Ni (20) observed downregulation in endometrial tissue of RIF patients. This study confirms significant downregulation of hsa_circ_0030162 in the plasma of RIF patients (Fig. 2).

hsa_circ_0004121 (also known as hsa_RFX8_0000600, hsa_circRFX8_003, and hsa-RFX8_0013), located on chromosome 2 (hg19: 102027081-102038934), is derived from exons 6-12 of the *RFX8* gene (ENST00000376826.2). RFX8, a member of the Regulatory Factor X (RFX) family of transcription factors, is a relatively newly characterized protein with largely unknown function (31). Elevated expression has been observed in stomach adenocarcinoma (32) and implicated in AML tumorigenesis and chelerythrine sensitivity (33). Studies on hsa_circ_0004121 have shown downregulation in ischemic stroke patients (34) and its involvement in a circRNA-miRNA pair (hsa_circ_0004121-miR-326) targeting CD8 subunit alpha (CD8A), a protein highly expressed in abdominal aortic aneurysm tissues (35). Luo (21) and Ni (20) reported upregulation of hsa_circ_0004121 in endometrial tissue of RIF patients. This study demonstrates significant upregulation of hsa_circ_0004121 in the plasma of RIF patients (Fig. 2).

Bioinformatic analysis revealed that hsa_circ_0030162 and hsa_circ_0004121 target hsa-miR-125a-3p and hsa-miR-125a-5p, respectively, both members of the hsa-miR-125 family and exhibiting high scores in the CircBank database and differential expression in the endometrium of RIF patients. Upregulation of hsa-miR-125a-3p in placenta is associated with early pregnancy loss (36), while downregulation in the first trimester

maternal plasma is linked to spontaneous preterm delivery (37). Dysregulation of hsa-miR-125a may influence inflammatory responses, tissue remodeling (38), cell migration, and proliferation (39). Furthermore, polymorphisms in pri-miR-125a affect mature miRNA expression and are associated with recurrent pregnancy loss (40). These findings suggest that the hsa-miR-125 family (including hsa-miR-125a-3p and hsa-miR-125a-5p), along with hsa_circ_0030162 and hsa_circ_0004121, play significant roles in pregnancy loss and potentially recurrent implantation failure.

This study identified differential plasma expression of hsa_circ_0030162 and hsa_circ_0004121 in RIF patients compared to fertile controls. These findings warrant further investigation into the roles of these circRNAs, and their miRNA targets (hsa-miR-125a-3p and hsa-miR-125a-5p), in RIF pathogenesis.

Ethics

This study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. Prior to the study, ethical approval was obtained from the research ethics committee of Tehran University of Medical Sciences (code No: IR.TUMS.IKHC.REC.1401.250).

Conflict of interest

There are no competing interests or conflicts of interest present.

Funding

This study was approved and financially supported by research deputy of Tehran University of Medical Sciences (code No: IR.TUMS.IKHC.REC.1401.250)

Acknowledgments

The authors would like to express their sincere gratitude to the patients and staff of Vali-e-Asr Hospital for their invaluable contributions to this study.

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