Integrated miRNA-seq analysis reveals the molecular mechanism underlying the effect of acupuncture on endometrial receptivity in patients undergoing fertilization: embryo transplantation

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Received: 2 July 2019 / Accepted: 12 November 2019 / Published online: 27 November 2019
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Abstract

Endometrial receptivity (ER) is the main factor affecting in vitro fertilization (IVF) and embryo transplantation. Previous studies have shown that acupuncture might be useful for increasing ER. However, the underlying microRNA (miRNA) molecular mechanisms deserve to be further elucidated. In this study, we performed small RNA sequencing of endometrial samples from infertile women who either underwent acupuncture therapy or did not. Differentially expressed microRNAs (DEmiRNAs) were identified and used to predict target genes. Then, the functional and pathway were analyzed for the target genes. Moreover, quantitative reverse transcription PCR (qRT-PCR) was performed to validate the RNA-seq results. Finally, the miRNA-gene network was conducted by Cytoscape. A total of 39 DEmiRNAs were identified between the acupuncture group and the control group. The functional enrichment analysis suggested that the target genes of the DEmiRNAs were significantly enriched in GO biological process (BP) terms associated with transcription, such as regulation of DNA-templated transcription and positive regulation of DNA-templated transcription. The pathway analysis showed that DEmiRNAs might be involved in acupuncture therapy via Endocytosis, Axon guidance, Oxytocin signaling, the Hippo pathway, and Estrogen signaling pathways. Significant downregulation of hsa-miR-449a and hsa-miR-449b-3p, and significant upregulation of hsa-miR-3135b and hsa-miR-345-3p in the RNA-seq results were validated by qRT-PCR. Besides, these four DEmiRNAs and their 34 target genes conducted a miRNA-gene network. Our results predict that hsa-miR-449a, hsa-miR-3135b and hsa-miR-345-3p may underly mechanisms by which acupuncture therapy help increase ER and promote endometrium receptivity in preparation for in vitro fertilization and embryo transplantation.

Keywords In vitro fertilization-embryo transplantation · Endometrium receptivity · Acupuncture · miRNA-seq

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Introduction

During the past decades, researchers have made great advances in the field of in vitro fertilization (IVF) and embryo transfer for assisted reproductive technology (ART); however, implantation failure is still a major cause of low pregnancy rate in ART (Toth et al. 2011). The most important factors affecting the success of IVF include age, endosperm quality, and endometrial receptivity (Wu et al. 2014). Several studies have shown a significant correlation between endometrial receptivity (ER), pregnancy rate, and endometrial thickness (EMT), which is considered to be an indicator of ER. Patients with EMT > 7 mm on the day of human chorionic gonadotropin (HCG) administration had higher pregnancy rates than patients with a thin endometrium (EMT < 7 mm) (Kunicki et al. 2014; Kasius et al. 2014). Medications such as aspirin, vitamin E, and pentoxifylline have been used to increase EMT (Tesarik et al. 2004; Tourgeman et al. 2001; Weckstein et al. 1997; Acharya et al. 2009; Gleicher et al. 2013; Letur-Konirsch and Delanian 2003), but their effect is limited.

Acupuncture is a traditional Chinese medicine approach with more than 2000 years of history, recorded in The Medical Classic of the Yellow Emperor, an ancient treatise on this topic (Cochrane et al. 2014; Zhou and Benharash 2014). Many studies have shown that acupuncture is effective in the treatment of diseases (Linde et al. 2016b; Manheimer et al. 2012; Linde et al. 2016a; Johansson and Stener-Victorin 2013; Kuang et al. 2013). The use of acupuncture in reproductive endocrinology and infertility has become popular worldwide (Kuang et al. 2013). In addition, acupuncture applied on the lower limbs and in the lower abdomen can increase blood flow to the ovaries and uterus, and can thus increase the thickness of the endometrial lining (Stener-Victorin et al. 1996; Napadow et al. 2008). However, clinicians trained in Western medicine often refuse to recommend acupuncture to their patients, mainly because the specific mechanism of acupuncture treatment is unclear.

The relationship between acupuncture and endometrial receptivity has not been studied. To further elucidate the molecular mechanisms of acupuncture, in this study, we performed microRNA (miRNAs) sequencing of endometrial samples from infertile women who either underwent acupuncture therapy or did not. Then, identified the differentially expressed miRNAs (DEmiRNAs), and their functions were predicted. Moreover, a competitive miRNA–mRNA network was constructed to further interpret the molecular mechanism of acupuncture therapy on low endometrium receptivity. We anticipate our results could help understand the role of acupuncture in treating women's health.

Materials and methods

Patients

The endometrial samples were collected from infertile women with thin endometrium at childbearing age undergoing embryo transplantation at our hospital from January 2016 to June 2017. All women experienced infertility for 1.5–9 years caused by thin endometrium combined with salpingitis, polycystic ovarian syndrome, or diminished ovarian reserve. A total of 12 women who planned to receive IVF embryo transplantation (ET) were recruited in the study (Table 1). The women were assigned to either the case group ($n = 6$) or the control group ($n = 6$) according to their characteristics or response during previous cycles. IVF was performed in women of both groups as previously described (Jeong et al. 2018). Additionally, women in the case group underwent acupuncture at six points, i.e., Guanyuan (CV4), Zigong (EX-CA1), Zusanli (ST36), Sanyinjiao (SP6), Shenshu (BL23), and Ciliao (BL32). Acupoint selection is based on traditional Chinese medicine theory and classical acupoint consensus and reference literature (Tanaka 2002; Villahermosa et al. 2013). EMT was measured at the maximal distance between each myometrial–endometrial interface by vaginal ultrasonography in the mid-luteal phase. This study was approved by the ethics committee of Nanjing Medical University (No. 2014, 204). All women provided written informed consent.

Table 1 Primers sequences for qRT-PCR

Gene	Direction	Sequence
U6	Forward	5' CGATACAGAGAAGATTAGCAT GGC 3'
hsa-miR-449a	Forward	5' GCGCAGTGGCAGTGTATTGTT 3'
hsa-miR-449b-3p	Forward	5' GTGATGCAGCCACAACCTACCC 3'
hsa-miR-449c-5p	Forward	5' CGCAGTAGGCAGTGTATT GCTAG 3'
hsa-miR-4463	Forward	5' GCTCAGCAGGAGACTGGGGT 3'
hsa-miR-4446-3p	Forward	5' ACTACATCAGGGCTGGCA GTGA 3'
hsa-miR-3135b	Forward	5' ATTCGGCTGGAGCGAGTGC 3'
hsa-miR-345-3p	Forward	5' TGGTTATTAGCCCTGAACGAG 3'
Reverse		5' AGTGCGTGTCTGGAGTTCG 3'

Forward is the specific primer for miRNA, and Reverse is the primer matching RT

Acupuncture therapy procedure

Women in the case group received acupuncture from the first day of two menstrual cycles before entering IVF-ET to the day of IVF-ET, with a frequency of once every other day. The procedure was as follows: women rested in a supine position; needles with a length ranging from 25 to 40 mm were inserted, and mild reinforcing and attenuating techniques were applied. The needle is retained for 30 s upon arrival of qi, then twirled 90°, and then lifted and thrust within a range of 2 mm with a frequency of 60–100 times/min. The needles were retained for 30 min at each acupuncture point. During needle retention, Han's acupoint neurostimulator (HANS, LH202) was connected to the needles at Ciliao (BL32) and Zigong (EX-CA1). The frequency of HANS was 2–15 Hz and the intensity of stimulation was adjusted depending on the patients' condition and comfort. Acupuncture was applied at Guanyuan (CV4) and Zusanli (ST36) as follows: the needle was inserted 2 cm and held vertically at each point for 10 min. The acupuncture session was stopped if the patient could not tolerate the discomfort.

RNA extraction

Endometrial samples were obtained during the mid-luteal phase of the menstrual cycle. Total RNA from the endometrial samples of the two groups was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). A NanoDrop 2000 spectrophotometer (Wilmington, DE, USA) was used to measure the quantity and quality of the extracted RNA. The qualified RNA samples with an A260/280 ratio > 1.9 were used for cDNA library construction as described previously [21].

Small RNA library construction and sequencing

NEBNext Multiplex Small RNA Library Prep Set for Illumina (NEB, Ipswich, MA, USA) was used to construct small RNA libraries. First, the RNA (1 µg total RNA) was ligated to the adapter; afterward, reverse transcription and PCR amplification were performed. Second, purification of amplified PCR products was performed using a QIAquick PCR Purification Kit (NEB, Ipswich, MA, USA), and fractions were size-separated in 8% tris-borate-EDTA and 8% polyacrylamide gel electrophoresis (TBE-PAGE). Third, we evaluated the library quality using the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Last, we performed RNA sequencing at Shanghai Yingbio Biotechnology Co., Ltd., using the Illumina HiSeq 2500 Genome Analyzer platform in pair-end mode.

Sequencing data processing

The raw reads were qualified using Fast-QC (v0.11.7) (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) by filtering empty reads, adaptor sequences and low-quality sequences (> 50% of bases whose Q scores were ≤ 10%). We used the internationally recognized Burrows-Wheeler Aligner algorithm for miRNA mapping. We compared the filtered clean reads with the human miRNA, genome and Rfam databases, and obtained the expression of miRNA. Gene expression abundance was calculated in fragments per kilobase of exon per million fragments mapped (FPKM). miRNAs that were differentially expressed between the two groups (DEmiRNAs) were screened using the DESeq software (Wang et al. 2010), meeting the criteria of log₂ fold change (FC) > 1 and false discovery rate (FDR) < 0.05.

Table 2 General characteristic of women in this study

Terms	Case (n = 6)	Control (n = 6)	P
Age, years	31 ± 2.37	27.5 ± 2.43	0.03
Infertility years	5.75 ± 2.40	2.83 ± 0.68	0.02
BMI, kg/m ²	21.52 ± 1.35	20.77 ± 1.01	0.3
Age of menarche, years	14.33 ± 1.51	13 ± 0.89	0.09
Menstrual cycle, days	58.75 ± 60.04	32.08 ± 10.10	0.3
Menstrual duration	6.58 ± 0.86	5.58 ± 1.16	0.12
EMT before acupuncture and moxibustion	9.3 ± 1.15	10.26 ± 1.34	0.21
EMT after acupuncture and moxibustion	8.65 ± 0.73	10.26 ± 1.34	0.02

EMT was measured on the day of transplant

BMI body mass index, EMT endometrium thickness

Difference between the case and control groups was compared by Student's test. *P* < 0.05 indicated statistical significance

miRNA target genes prediction

The target genes of the DE miRNAs were predicted using miRanda and RNAhybrid (Kruger and Rehmsmeier 2006; Rehmsmeier et al. 2004). The energy threshold was set at < -20 and the alignment score was set at > 150 for miRanda prediction, and we used a free energy threshold of < -25 for RNAhybrid prediction. Only the target genes predicted by the two methods were retained for further analysis. Cytoscape V3.6.0 was used to construct the miRNA–gene network.

Functional enrichment analysis

The function of the gene was predicted by Gene Ontology (GO) analysis (<http://www.geneontology.org>), which covers three categories: cellular component (CC), molecular function (MF), and biological process (BP) (Ashburner et al. 2000). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (<https://www.kegg.jp/>) was performed to identify the cellular signal pathways in which DE miRNAs target genes are involved. The threshold of significance for GO and KEGG analyses was defined as $FDR < 0.05$.

Validation of the DE miRNAs

The expression levels of seven key DE miRNAs were validated by quantitative reverse-transcription PCR (qRT-PCR). First, total RNA from endometrial samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Afterward, complementary DNA (cDNA) was reverse transcribed and synthesized using the PrimeScript RT kit (Takara, Dalian, China). Then, qRT-PCR was performed using a SYBR-Green PCR kit (Roche Diagnostics, Indianapolis, IN, USA) and a qRT-PCR system of StepOnePlus (Applied Biosystems, Foster City, CA, USA). The primer sequences used for the PCR are shown in Table 1. The qRT-PCR cycling conditions were as follows: 95 °C for 10 min, 45 cycles of 95 °C for 15 s, 60 °C for 60 s, dissociation at 95 °C for 10 s, 60 °C for 1 min, and 95 °C for 15 s. Relative gene expression was determined by the $2^{-\Delta\Delta C_t}$ method using the *U6* gene as internal control. The qRT-PCR were all repeated three times.

Statistical analysis

RNA sequence data were analyzed by bioinformatics methods as indicated above. Other data were analyzed using SPSS 21.0 (IBM, Chicago, USA). Differences between the two groups were compared by two-tailed Student's *t* test, and $P < 0.05$ was accepted as statistically significant.

Results

General characteristics of the women included in the study

The characteristics of the women in the two groups are shown in Table 2. The age and duration of infertility (in years) were

Fig. 1 Differentially expressed (DE) microRNAs (miRNA) between women who either received acupuncture (case group, Case 1–6) or did not (control group, NC 1–6). Hierarchical cluster analysis of DE miRNAs in the control and case groups by small RNA-seq. The red and green colors denote miRNA mean upregulation and downregulation, respectively

significantly higher in the case group than in the control group ($P=0.03$ and $P=0.02$, respectively). The other characteristics, including BMI, age of menarche, menstrual cycle, menstrual duration, and EMT before acupuncture, were comparable between the two groups ($P>0.05$). However, EMT was significantly increased upon acupuncture therapy ($P<0.05$).

Identification of DE miRNAs

Considering $P<0.05$ to indicate statistically significant differences, a total of 39 DE miRNAs (including 14 upregulated miRNAs and 25 downregulated miRNAs) were screened in the case group and compared with those in the control group. Hierarchical cluster analysis indicated that these DE miRNAs allow separating the samples into case and control groups (Fig. 1), suggesting the reliability of our analysis.

Functional analysis of the DE miRNAs

To further interpret the DE miRNAs, we performed GO and pathway enrichment analysis for the target genes. A total of 23 GO BP terms were significantly enriched ($FDR<0.05$). Most enriched GO BP terms were related to transcription (e.g., DNA-templated transcription, regulation of DNA-templated transcription, positive regulation of DNA-templated transcription, and positive regulation of transcription from

RNA polymerase II promoter), transport (e.g., synaptic transmission), development (e.g., nervous system development and negative regulation of cell proliferation) (Fig. 2a). The pathway analysis revealed that 78 terms were enriched for the target genes of the DE miRNAs, based on the cutoff value of $FDR<0.05$, including Endocytosis, Axon guidance, Oxytocin signaling pathway, Hippo signaling pathway, Glioma, Ras signaling pathway, Proteoglycans in cancer, Rap1 signaling pathway, Phosphatidylinositol signaling system, Wnt signaling pathway, Insulin signaling pathway, Pathways in cancer, Neurotrophin signaling pathway, Morphine addiction, PI3K-Akt signaling pathway, Inflammatory mediator regulation of TRP channels, FoxO signaling pathway, Prostate cancer, Focal adhesion, cGMP-PKG signaling pathway, Chronic myeloid leukemia, Melanoma, Glutamatergic synapse, Cholinergic synapse, Dopaminergic synapse, mTOR signaling pathway, Estrogen signaling pathway, Vascular smooth muscle contraction, Gastric acid secretion, ErbB signaling pathway (Fig. 2b).

Validation of DE miRNAs

To validate our results, we selected seven DE miRNAs and performed qRT-PCR for further confirmation. As shown in Fig. 3, compared with control group, significantly downregulated expression of hsa-miR-449a and hsa-miR-449b-3p, and significantly upregulated expression of hsa-miR-3135b and hsa-miR-345-3p revealed by RNA-seq results were validated by qRT-PCR; however, the difference between the case and the control groups in the downregulation of hsa-miR-449c-5p, and in the upregulation of hsa-miR-4446-3p and hsa-miR-4463, did not reach statistical significance ($P>0.05$) (Fig. 3). The raw data were uploaded to Sequence Read Archive (SRA) (<http://www.ncbi.nlm.nih.gov/>) with accession PRJNA587020.

Fig. 2 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis results. The top 20 enrichment terms of the GO BP analysis (a), and the top 30 enrichment terms of

the pathway analysis (b) of DE miRNAs. Significant terms are shown in red and non-significant terms are shown in green (P value <0.05)

Fig. 3 Validation of expression of the selected upregulated (a) and downregulated DEMiRNAs (b) in endometrial tissues of the case and control groups by qRT-PCR. The asterisk indicates statistically significant differences (t test, $P < 0.05$), $n = 6$

Construction of miRNA–gene network map

To better understand the interaction between miRNA and target genes in the estrogen pathway, a miRNA–gene network map was constructed using Cytoscape (Fig. 4). This network was constructed with upregulated and downregulated miRNAs along with their target genes. In this network, several miRNA–gene relationships were predicted. For example, the upregulation of both hsa-miR-3135b and hsa-miR-345-3p included adenylate cyclase 2 (ADCY2) and growth factor receptor-bound protein 2 (GRB2) as common target genes. Upregulated miRNAs included hsa-miR-3135b and hsa-miR-345-3p, with 21 and 10 predicted target genes, respectively. Downregulated mRNAs include hsa-miR-449a and hsa-miR-449b-3p, having 5 and 4 predicted target genes, respectively.

Fig. 4 The miRNA–gene network for DEMiRNAs was verified by qRT-PCR. The red and blue colors represent upregulation miRNA, and downregulation miRNA, respectively. The target gene predicted by TargetScan is shown in green

Discussion

The endometrium is a hormone-dependent tissue and the primary target organ of estrogen, which is essential for endometrial proliferation (Tourgeman et al. 2001; Hapangama et al. 2015). Several studies on ER and embryo implantation have reported the effects exerted by estrogen and estrogen receptors (Lessey and Young 2014; Young 2013; Dorostghoal et al. 2018; Kobayashi et al. 2017). The differential RNA was significantly enriched in this pathway. Hence, one of our objectives was to target the estrogen pathway. Based on the miRNA–gene network, we found that upregulated hsa-miR-3135b and hsa-miR-345-3p can be targeted to ADCY2 (Fig. 4). The ADCY2 gene is insensitive to Ca^{2+} , and codes for a membrane-associated enzyme that catalyzes the formation of the secondary messenger cyclic adenosine monophosphate (cAMP). The inhibition of cAMP-mediated signaling can activate the production of blood platelets, which increases the incidence of pregnancy in cows (Lange-Consiglio et al. 2015; Raslan and Naseem 2015; Cheng et al. 2002). Taken together, we hypothesized that acupuncture treatment may affect the activity of ADCY2 by affecting the expression of hsa-miR-3135b and hsa-miR-345-3p, thereby affecting the activity of the downstream cAMP signaling cascade and ultimately increasing ER.

Hsa-miR-449a was verified by qRT-PCR that significantly downregulates in the case group, and targeted the calmodulin-like protein 3 (CALML3), which is involved in the estrogen metabolic pathway. CALML3 is an estrogen

receptor alpha coregulator that functions as a sensor and regulates gene expression (Qin et al. 2017). Previous studies have shown that CALML3 regulates myosin-10 (Arase et al. 2009; Rogers and Strehler 2001), which may play an important role in cell adhesion and motility, which influence ER (Yonezawa et al. 2003; Tokuo and Ikebe 2004; Bohil et al. 2006; Gnainsky et al. 2015; Evans-Hoeker and Young 2014). Our results indicate that acupuncture might increase ER by downregulating hsa-miR-449a and increasing the expression of CALML3 and myosin-10.

According to the pathway analysis, the Hippo pathway showed obvious changes. The Hippo pathway is an evolutionarily conserved signaling pathway that plays essential roles in growth control (Zhao et al. 2011). The activation of the Hippo/Yes-Associated Protein Pathway (YAP) promotes cell proliferation and Anti-apoptosis gene expression in the endometrium (Zhu et al. 2017). YAP, one of the core components of the Hippo pathway, can mediate the decidualization of uterine endometrial stromal cells in humans (Song et al. 2018). Previous research has found that the degradation of phosphorylated YAP downregulates interferon tau gene (*IFNT*) transcription after conceptus attachment to the uterine endometrium (Kusama et al. 2016). *IFNT* acts on the endometrium, regulating genes that play an important role in ER and conceptus growth, as well as in development and implantation (Spencer et al. 2004; Kim et al. 2003; Gray et al. 2004; Nagaoka et al. 2003; Gray et al. 2006; Kunii et al. 2018). Based on the above research results, we speculate that acupuncture exerts its effects through the Hippo pathway, and downregulates the *IFNT* gene, thus increasing ER.

Our results predict that hsa-miR-449a, hsa-miR-3135b and hsa-miR-345-3p may be underlying molecules by which acupuncture therapy may help increase ER and promote endometrium receptivity in preparation for in vitro fertilization-embryo transplantation.

Acknowledgements This work was supported by the National Natural Science Foundation of China [Grant numbers 81473767, 81873371, 81403477, 81603674, 81403481, 81804179] and by the Jiangsu Provincial Science and Technology Project of the Traditional Chinese Medicine Bureau [Grant number YB2017005].

Author contribution Conceptualization: Youbing Xia; Methodology: Yanyun Mu, Qian Li, Jie Cheng; Formal analysis and investigation: Yanyun Mu, Qian Li, Jie Shen, Zhengyun Xie, Zhao Gao, Wenjing Zhang, Qixin Hua; Writing—original draft preparation: Yanyun Mu, Jie Shen, Youling Gao; Writing—review and editing: Qian Li, Xun Jin, Liangjun Xia; Funding acquisition: Youbing Xia; Resources: Jie Cheng, ...; Supervision: Youbing Xia. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability The raw data were uploaded to Sequence Read Archive (SRA) (<http://www.ncbi.nlm.nih.gov/>) with accession PRJNA587020.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this paper.

Ethical approval This study was approved by the ethics committee of Nanjing Medical University (No. 2014, 204).

Informed consent All women provided written informed consent.

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