# Original Article miR-221-3p involves in the invasion of trophoblast and angiogenesis through AMPK pathway and leads to unexplained recurrent spontaneous abortion

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**Abstract:** This study was aimed to find the mechanism of miR-221-3p in response to unexplained recurrent spontaneous abortion (URSA). We selected the women who had suffered curettage from January 2013 to January 2016 and were diagnosed with URSA. The qRT-PCR was performed to detect the mRNA expression of miR-221-3p, hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), vascular endothelial growth factor (VEGF) and AMP-activated protein kinase (AMPK). The western blot was conducted to evaluate the post-translational expressions of the proteins mentioned above. A total of 36 URSA patients and 32 controls were included in this study. Suppression of miR-221-3p significantly reduced the mRNA and protein expression of HIF-1 $\alpha$ , VEGF and p-AMPK. p-AMPK activator obviously promoted the expression of HIF-1 $\alpha$ , VEGF and p-AMPK in miR-221-3p inhibitor transfected JAR cells. miR-221-3p plays important roles in URSA and does its function in the invasion of trophoblast and angiogenesis by regulating VEGF and HIF-1 $\alpha$  through AMPK signal pathway.

Keywords: Unexplained RSA, miR-221-3p, HIF-1α, VEGF, AMPK

#### Introduction

Recurrent spontaneous abortion (RSA), which is defined as three or more consecutive pregnancy losses, is a common complication of women during gestation period. About 1% of couples who what to have children are troubled with RSA [1]. There are many causes for RSA, including hyperhomocysteinemia, chromosomal abnormity, anatomical disorders, endocrine disorders, infection, immune factors, chronic endometritis and lifestyle factors [2-4]. If the cause of RSA is diagnosed, the treatment can be taken accordingly. However, the cause of approximately 50% RSA cases cannot be determined, which is defined as unexplained RSA (URSA) [5].

Recently, some factors have been reported to be associated with the URSA. For examples, Perfetto et al. [6] found that decrease of IL-22 levels in the uterine decidua in URSA patients may lead to a disruption of decidual homeostasis and eventually cause early pregnancy loss. Another study [7] showed that compared to the normal pregnant women, the URSA patients would display significantly higher ratio of decidual Th1/Th2 cytokines. Except for cytokines, some genes play important roles in the development of URSA. C677T mutation of methylenetetrahydrofolate reductase gene (MTHFR) had been reported to be significantly associated with URSA in the East Asian population [8. 9]. Besides, some URSA related polymorphisms in other genes, such as Human leukocyte antigen (HLA)-G gene [10], Protease-activatedreceptor 1 [11] and folic acid metabolism-related genes [12] were also found. However, the mechanism of URSA is still unclear until now.

Micro RNA (miRNA) is a class of small non-coding RNA. It can target to the 3'-UTR of certain

Table 1. The primers of genes used in this study

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Target	Forward (5'-3')	Reverse (5'-3')
VEGF	GAAACCATGAACTTTCTGCTG	TCCTTCCTCCTGCCCGGCTA
HIF-1α	CAAGAACCTACTGCTAATGCC	GTTAGGGCTTCTTGGATGAG
AMPK	CAGGCATATGGTGGTCCATAGAG	TCATGGGATCCACCTGCAGC
GAPDH	CCACTCCTCCACCTTTGAC	ACCCTGTTGCTGTAGCCA

genes and involve in the development of many diseases. So far, several researches have reported the association between miRNAs and URSA [13, 14], and a circulating miRNA miR-221-3p has been found to be significantly upregulated in villus tissues of URSA patients [13]. Nevertheless, the mechanism of the miR-221-3p on URSA development remains unknown.

In this study, we detected the expression levels of miR-221-3p in URSA patients. In order to explore the role of miR-221-3p in invasion of trophoblast and angiogenesis in URSA patients, we evaluated the expression of two biomarkers for invasion of trophoblast and angiogenesis: hypoxia inducible factor- $1\alpha$  (HIF- $1\alpha$ ) and vascular endothelial growth factor (VEGF). Our results will provide a reference for discovering the molecular mechanism of URSA development.

#### Materials and methods

# Patients and samples

This study was warranted by ethics committee of Affiliated Hospital of Binzhou Medical University. The informed consents were acquired from all the patients. There were 36 URSA patients who had suffered curettage in the Affiliated Hospital of Binzhou Medical University from January 2013 to January 2016. and 32 healthy controls were included in this study. All the included women aged from 24 to 32 years. The gestation ages of the included URSA patients and controls were 54-65 d and 45-60 d, respectively. In URSA group, 18 patients had endured spontaneous abortion twice, 6 patients suffered 3 times and 12 experienced 4 or more times. The patients with confirmed unavoidable abortion in clinic (abdominal pain or vaginal bleeding occurred and B ultrasonic showed no original heart beat) and spontaneous abortion for two or more times before were grouped into URSA patients. The patients with normal pregnancy (blood human chorionic gonadotropin level and B ultrasonic test showed normal embryo development) asked for abortion at the same period were considered as controls. The patients who had the following diseases were excluded from this study: chromosome abnormality, anatomical deformity, reproductive tract infections, endocrine and immune diseases. Villus samples

from the URSA patients and controls after curettage were selected and washed repeatedly by normal saline. The samples were frozen in liquid nitrogen and saved at -80°C for further experiments.

# Cell line and cell transfection

Human cytokine-7-positive trophoblast cells (HTR-8/SVneo) (CRL-3271<sup>TM</sup>) and human choriocarcinoma JAR cell line (HTB-144<sup>TM</sup>) were purchased from American Type Culture Collection (ATCC, Rochcille, MD, USA). JAR cells and HTR-8/SVneo cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS, Gibco, USA), 100 U/mL penicillin, and 100 U/mL streptomycin in an atmosphere of 5% CO<sub>2</sub> at 37°C. miR-221-3p inhibitor/mimic/ scramble and AMPK activator A769662 was purchased from Sangon Biotech (Shanghai, China). Cell transfection was performed using the Lipofectamine 2000 according to the manufacture's protocol.

# Cell proliferation assay

Cell proliferation ability was assessed using MTT assay. Cells transfected with silenced vectors at logarithmic stage were cultured in DMEM/F12 medium including 10% FBS. Cells were adjusted to  $5 \times 10^3$  cells and injected into the 96-well plates. After being cultured for 24 h, cells were centrifuged at 12,000 rpm, and then supernatant was removed. A total of 20 µL MTT was added in each well and cultured for 4 h. Finally, 150 µL dimethylsulfoxide (DMSO) was added and mixed with the cells for 10 min. Absorbance at 570 nm of cells in each well was observed under an absorption spectrophotometer (Olympus, Japan).

# qRT-PCR analysis

Total RNA and miRNA extraction from tissues and cells was conducted using TRIzol Reagent (Invitrogen, CA, USA) following the instruction. Then the isolated RNA was treated with RNse-free Dnase I (Promega Biotech, USA).



**Figure 1.** The mRNA expression of miR-221-3p in different tissues and cells. A: The relative mRNA expression of miR-221-3p in villous tissues from URSA patients and controls. B: The relative mRNA expression of miR-221-3p in HTR-8/SVneo and JAR cell lines.

Consequently, concentration and purity of isolated RNA were measured using SMA 400 UV-VIS (Merinton, Shanghai, China). Purified RNA was used for cDNA synthesis with the PrimerScript 1st Strand cDNA Synthesis Kit (Invitrogen, USA). Expressions of targets were detected in an Eppendorf Mastercycler (Brinkman Instruments, Westbury, NY) using the SYBR ExScript RT-gPCR Kit (Takara, China) according to the manual. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified and detected. Phosphoglyceraldehyde dehydrogenase (GAPDH) was chosen as the internal controls. Primers used for targets (HIF-1 $\alpha$  and VEGF, AMPK and GA-PDH) amplification were shown in Table 1. The miR-221-3p RT primers and forward primers were "GTCGTATCCAGTGCAGGGTCCGAGGTATT-CGCACTGGATACGACGAAACC" and "GAGCTAC-ATTGTCTGCTGGGT", respectively. U6 was used as an internal reference and the primers were follows: U6-RT-primer: AACGCTTCACGAATTTGC-GT, U6-Forward primer: CTCGCTTCGGCAGCA-CA and Reverse primer: AACGCTTCACGAATT-TGCGT.

#### Western blotting

Cells were lapped with radioimmunoprecipitation assay (RIPA, Sangon Biotech) lysate containing phenylmethanesufonyl fluoride (PMSF, Sigma, USA), and then were centrifuged at 12,000 rpm for 10 min at 4°C. Supertanant was collected for the measurement of protein concentrations using BCA protein assay kit (Pierce, Rochford, IL). Protein samples (50 µg per lane) were separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and blotted onto polyvinylidene difluoride membranes, blocked in PBST (0.1% triton in 19 PBS) and probed with primary antibodies (1:1,000 for HIF-1 $\alpha$ , VEGF and p-AMPK, 1:5,000 for GAPDH) overnight at 4°C. The membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. The immunoreactive protein bands were developed by enhanced chemilumines-cence. The immunoreactive bands were analyzed by a densitometer.

#### Statistical analysis

All data were displayed as mean  $\pm$  standard error of mean (SEM) in this study. Independent sample t-test was used to calculate the difference between two groups using the GraphPad Prism 5.0 software (San Diego, CA). Analysis of variance (ANOVA) was used to calculate the difference for more than 3 groups. The P < 0.05 was defined as statistically significant.

#### Results

# miR-221-3p expression in URSA tissue and cell line

As shown in **Figure 1A**, the expression level of miR-221-3p in URSA group was significantly higher than in controls. Then, we detected the miR-221-3p expression in HTR-8/SVneo and JAR cells and the result showed that the mRNA level of miR-221-3p in JAR cells were significantly higher than in HTR-8/SVneo cells (**Figure 1B**).

# VEGF and HIF-1 $\alpha$ expression in URSA tissue and cell line

In URSA patients, VEGF and HIF-1 $\alpha$  showed significant lower mRNA and protein levels than in controls (**Figure 2A**). The same result was found in the cell lines (**Figure 2B**). Both VEGF and HIF-1 $\alpha$  showed significantly higher expression levels in HTR-8/SVneo cells (**Figure 2**).

# miR-221-3p suppression promoted cell proliferation

After transfecting four miR-221-3p vectors into JAR cells, we found that the decrease of the miR-221-3p expression could improve the via-



**Figure 2.** The mRNA and protein expression of VEGF and HIF-1 $\alpha$  in different tissues and cells. (A, B) The relative mRNA (A) and protein (B) expression of VEGF and HIF-1 $\alpha$  in villous tissues from URSA patients and controls. (C, D) The relative mRNA (C) and protein (D) expression of VEGF and HIF-1 $\alpha$  in HTR-8/SVneo and JAR cell lines.



**Figure 3.** Transfection of miR-221-3p vectors into JAR cells. A: The mRNA expression of miR-221-3p in different transfected cells. B: Cell viability of miR-221-3p in different transfected cells after transfection.



Figure 4. The mRNA and protein expression of VEGF and HIF-1 $\alpha$  in different miR-221-3p transfected cells. A: The mRNA expression of VEGF and HIF-1 $\alpha$  in miR-221-3p inhibitor/mimic/scramble transfected cells. B: The protein expression of VEGF and HIF-1 $\alpha$  in miR-221-3p inhibitor/mimic/scramble transfected JAR cells.

bility of the JAR cells (Figure 3). The miR-221-3p scramble transfected cells showed similar proliferation trend with the control group (Figure 3A). In the miR-221-3p-inhibitor transfected cells, the cell viability was significantly increased after transfecting for 96 h (Figure 3B). However, miR-221-3p mimic transfected cells showed a significant lower cell viability than control and miR-221-3p scramble transfected cells.

miR-221-3p suppression promotes the expression of VEGF and HIF-1 $\alpha$ 

As shown in **Figure 4A**, the suppression of the miR-221-3p significantly increases the mRNA expression of VEGF and HIF-1 $\alpha$ . However, in miR-221-3p mimic transfected cells, the VEGF and HIF-1 $\alpha$ expression levels were significantly reduced. In addition, miR-221-3p scramble transfected cells displayed a similar VEGF and HIF-1 $\alpha$  expression levels with the control.

The protein expressions showed similar trend with mRNA (Figure 4B).

miR-221-3p suppression promotes the activation of AMPK signal pathway

In villus tissues from URSA patients and JAR cell line, p-AMPK mRNA and protein expressions were downregulated compared to the controls and HTR-8/SVneo cells (**Figure 5A-D**). After transfection, the inhibition of miR-221-3p expression significantly induced the expression of p-AMPK. However, miR-221-3p mimic transfected cells showed obviously lower p-AMPK expression than control. In addition, no significant difference was found in p-AMPK expression between miR-221-3p scramble transfected cells and control (**Figure 5E, 5F**).

In order to verify the role of miR-221-3p in AMPK signal pathway, the AMPK activator A769662 was used. The result showed that

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**Figure 5.** The mRNA and protein expression of p-AMPK and AMPK in different tissue and cells. (A, B) The mRNA (A) and protein (B) expression of p-AMPK and AMPK in villous tissues from URSA patients and controls. (C, D) The mRNA (C) and protein (D) expression of p-AMPK and AMPK in HTR-8/SVneo and JAR cell lines. (E, F) The mRNA (E) and protein (F) expression of p-AMPK and AMPK in miR-221-3p inhibitor/mimic/scramble transfected cells. GAPDH was considered as reference.



**Figure 6.** The effect of AMPK activator A769662 on miR-221-3p inhibitor/mimic/scramble transfected cells. (A, B) the mRNA (A) and protein (B) expression of p-AMPK and AMPK in miR-221-3p inhibitor/scramble transfected cells with or without A769662. (C, D) the mRNA (C) and protein (D) expression of VEGF and HIF-1 $\alpha$  in miR-221-3p inhibitor/scramble transfected cells with or without A769662.

A769662 had no effect on VEGF, HIF-1 $\alpha$  and p-AMPK expression (**Figure 6**). However, when miR-221-3p inhibitor and A769662 were co-transfected into JAR cells, the mRNA

levels of VEGF, HIF-1 $\alpha$  (Figure 6C, 6D) and p-AMPK (Figure 6A, 6B) were significantly reduced. AMPK had no changes in any treatment.

# Discussion

In the present study, we found the expression of miR-221-3p increased in the embryo villus tissues from URSA women. In further experiment, miR-221-3p could regulate HIF-1 $\alpha$  and VEGF which are two biomarkers involved in invasion of trophoblast and angiogenesis. Moreover, through the interaction between miR-221-3p and the AMPK activator A769662, we suggest that miR-221-3p probably acts on trophoblast invasion and angiogenesis by activating the p-AMPK signal pathway.

VEGF is an important angiogenesis promoter and plays key role in development and maintenance of the vasculature. VEGF and its receptors (VEGFR-1 and VEGFR-2) are necessary for primary vascular network formation and secondary angiogenesis. VEGF system would be activated under hypoxic conditions [15]. HIF-1a is a transcription factor that responds to hypoxia by inducing expression of genes involved in angiogenesis including VEGF [16]. HIF-1α has been also found to regulate matrigel-induced endovascular differentiation in human extravillous trophoblast cell under normoxia condition [17]. In our study, VEGF and HIF-1α were downregulated in villous tissues from URSA patients and JAR cell line. However, both two genes were upregulated in miR-221-3p inhibitor transfected JAR cells. Therefore, the URSA may inhibit the expression of miR-221-3p which can activate VEGF and HIF-1 $\alpha$  expression.

AMPK is a stress responsive protein kinase that is inhibited by hypoxia environment and other cellular stresses that leads to decrease of cellular ATP levels. Previous studies have reported that AMPK signal play vital roles in many human diseases and involve in cell proliferation, apoptosis, migration, and invasion. Phosphorylation of AMPK could be activated by hypoxia in human umbilical vein endothelial cells, and inhibition of AMPK signaling could suppress both cell migration to VEGF and in vitro differentiation in hypoxia [18]. In addition, AMPK signal could also stimulate the VEGF expression and angiogenesis in human glioblastoma and skeletal muscle [19, 20]. Another previous study reported that the AMPK also worked in placenta tissue of pregnant rats and humans and AMPK signal would be activated in hypoxia to increase arterial blood flow and protect the fetus [21]. In our results, we found that the AMPK was suppressed in villous tissues from URSA patients and JAR cells. This response of AMPK may be modulated by miR-221-3p. Because the AMPK expression was inhibited in miR-221-3p over-expressed JAR cells and upregulated in miR-221-3p inhibited JAR cells. Moreover, our results showed that the AMPK activator A769662 could significantly promote the activation of AMPK signal in miR-221-3p inhibited JAR cells. However, the activator A769662 could not work in miR-221-3p scramble transfected cells. These results identify that the miR-221-3p can involve in invasion of trophoblast and angiogenesis by regulating VEGF and HIF-1 $\alpha$ , and these responses may be processed by activation of AMPK signal pathway.

In conclusion, we firstly identified the role of miR-221-3p and explored its primary molecular mechanism on URSA. Our results confirm that miR-221-3p play important roles in URSA and do its function in invasion of trophoblast and angiogenesis by regulating VEGF and HIF-1 $\alpha$  through AMPK signal pathway.

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#### Disclosure of conflict of interest

None.

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#### References

- [1] Elnashar A. Recurrent miscarriage: Does any treatment help? Comment. Middle East Fertility Society Journal 2015; 9: 27-28.
- [2] Wouters MG, Boers GH, Blom HJ, Trijbels FJ, Thomas CM, Borm GF, Steegers-Theunissen RP, Eskes TK. Hyperhomocysteinemia: a risk factor in women with unexplained recurrent early pregnancy loss. Fertil Steril 1993; 60: 820-825.

- [3] Stephenson MD. Recurrent early pregnancy loss. Semin Reprod Med 2011; 29: 461-462.
- [4] Rai R and Regan L. Recurrent miscarriage. Lancet 2006; 368: 601-611.
- [5] Practice Committee of the American Society for Reproductive Medicine. Evaluation and treatment of recurrent pregnancy loss: a committee opinion. Fertil Steril 2012; 98: 1103-1111.
- [6] O'Hern Perfetto C, Fan X, Dahl S, Krieg S, Westphal LM, Bunker Lathi R, Nayak NR. Expression of interleukin-22 in decidua of patients with early pregnancy and unexplained recurrent pregnancy loss. J Assist Reprod Genet 2015; 32: 977-984.
- [7] Yuan J, Li J, Huang SY and Sun X. Characterization of the subsets of human NKTlike cells and the expression of Th1/Th2 cytokines in patients with unexplained recurrent spontaneous abortion. J Reprod Immunol 2015; 110: 81-88.
- [8] Cao Y, Xu J, Zhang Z, Huang X, Zhang A, Wang J, Zheng Q, Fu L, Du J. Association study between methylenetetrahydrofolate reductase polymorphisms and unexplained recurrent pregnancy loss: a meta-analysis. Gene 2013; 514: 105-111.
- [9] Nair RR, Khanna A and Singh K. MTHFR C677T polymorphism and recurrent early pregnancy loss risk in north Indian population. Reprod Sci 2012; 19: 210-215.
- [10] Wang X, Jiang W and Zhang D. Association of 14-bp insertion/deletion polymorphism of HLA-G gene with unexplained recurrent spontaneous abortion: a meta-analysis. Tissue Antigens 2013; 81: 108-115.
- [11] Grisaru-Granovsky S, Salah Z, Maoz M, Tevet A, Margalioth E, Samueloff A, Altarescu G, Bar-Shavit R. Protease-activated-receptor 1 polymorphisms correlate with risk for unexplained recurrent pregnancy loss: a pilot study querying an association beyond coagulation. Eur J Obstet Gynecol Reprod Biol 2015; 185: 13-18.
- [12] Cao Y, Zhang Z, Zheng Y, Yuan W, Wang J, Liang H, Chen J, Du J, Shen Y. The association of idiopathic recurrent early pregnancy loss with polymorphisms in folic acid metabolism-related genes. Genes Nutr 2014; 9: 1-8.

- [13] Qin W, Tang Y, Yang N, Wei X and Wu J. Potential role of circulating microRNAs as a biomarker for unexplained recurrent spontaneous abortion. Fertil Steril 2016; 105: 1247-1254, e3.
- [14] Li D and Li J. Association of miR-34a-3p/5p, miR-141-3p/5p, and miR-24 in Decidual Natural Killer Cells with Unexplained Recurrent Spontaneous Abortion. Med Sci Monit 2016; 22: 922.
- [15] Gaengel K and Betsholtz C. Endocytosis regulates VEGF signalling during angiogenesis. Nat Cell Biol 2013; 15: 233-235.
- [16] Ema M, Taya S, Yokotani N, Sogawa K, Matsuda Y and Fujii-Kuriyama Y. A novel bHLH-PAS factor with close sequence similarity to hypoxiainducible factor 1α regulates the VEGF expression and is potentially involved in lung and vascular development. Proc Natl Acad Sci U S A 1997; 94: 4273-4278.
- [17] Fukushima K, Murata M, Hachisuga M, Tsukimori K, Seki H, Takeda S, Asanoma K, Wake N. Hypoxia inducible factor 1 alpha regulates matrigel-induced endovascular differentiation under normoxia in a human extravillous trophoblast cell line. Placenta 2008; 29: 324-331.
- [18] Nagata D, Mogi M and Walsh K. AMP-activated Protein Kinase (AMPK) Signaling in Endothelial Cells Is Essential for Angiogenesis in Response to Hypoxic Stress. J Biol Chem 2003; 278: 31000-31006.
- [19] Ouchi N, Shibata R and Walsh K. AMP-activated protein kinase signaling stimulates VEGF expression and angiogenesis in skeletal muscle. Circ Res 2005; 96: 838-846.
- [20] Neurath KM, Keough MP, Mikkelsen T and Claffey KP. AMP-dependent protein kinase alpha 2 isoform promotes hypoxia-induced VEGF expression in human glioblastoma. Glia 2006; 53: 733-743.
- [21] Skeffington K, Higgins J, Mahmoud A, Evans A, Sferruzzi-Perri A, Fowden A, Yung HW, Burton GJ, Giussani DA, Moore LG. Hypoxia, AMPK activation and uterine artery vasoreactivity. J Physiol 2015; 594: 1357-69.