MiR-15a-5p Inhibits Migration and Invasion of Ovarian Cancer Cells by Targeting PELP1

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Research

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Abstract

Background: Ovarian cancer is one of the most common malignancy of the female reproductive system. Hsa-miR-15a-5p (miR-15a-5p) has been reported with tumor-suppressing roles in various cancers. This study aims to determine the role of miR-15a-5p during the progression of ovarian cancer.

Methods: We used bioinformatics, luciferase reporter assays, wound-healing, transwell invasion assays, quantitative Real-time polymerase chain reaction (qRT-PCR) and Western blot to dissect the molecular mechanism of how miR-15a-5p may cause metastasis in ovarian cancer.

Results: The upregulation of miR-15a-5p inhibited growth, migration and invasion in ovarian cancer cells. Furthermore, miR-15a-5p suppressed epithelial mesenchymal transition (EMT) of ovarian cancer cell in vitro, evidenced by expression alteration of E-cadherin and vimentin. Proline-, glutamic acid- and leucine-rich protein 1 (PELP1) was identified as the direct target of miR-15a-5p and downregulated by miR-15a-5p. The inhibitory effect of miR-15a-5p on migration, invasion and EMT was rescued by PELP1. Additionally, downregulation of PELP1 mimicked the suppressive impact of miR-15a-5p on ovarian carcinoma cells.

Conclusions: Our data indicated that miR-15a-5p inhibited migration, invasion and EMT of ovarian cancer cells by targeting PELP1, which might relate to the progression and metastasis of ovarian cancer.

Background

Ovarian cancer is one of the most common malignancy of the female reproductive system, with five-year survival rates below 45% [1, 2]. Most ovarian cancer patients will suffer a recurrence of the tumor after standard treatment due to the metastatic potential of ovarian cancer [3]. Because the ovaries are located deep in the pelvic cavity, most ovarian cancer patients are asymptomatic at beginning [4]. It is often difficult to diagnose ovarian cancer at an early stage because of its insidious pathogenesis and lack of early detection methods [5]. The majority ovarian cancer patients are diagnosed at an advanced stage with peritoneal spread and massive ascites, and only 19% are diagnosed at its early stage [6]. Although traditional therapeutic advantage for advanced-stage disease has been proven, the 5-year cause-specific survival for most ovarian carcinoma that are diagnosed at stage III or IV is 43% [7]. Metastasis is the leading cause of death in patients with ovarian cancer, but the molecular mechanisms still remain obscure.

Epithelial-mesenchymal transition (EMT) is a process in which polar epithelial cells are capable of moving freely between cells, thus enhancing tumor metastasis [8–10]. EMT cells lose their epithelial characteristics and acquire mesenchymal properties [11]. EMT is the crucial physiological phenomenon that helps cancer cells escape from intercellular junctions and become more metastatic [12, 13].

MicroRNAs (miRNA) are 22 nucleotides long, highly conserved noncoding RNAs that negatively regulate gene expression post-transcriptionally through binding to the 3' untranslated region [14]. Previous studies

reported that miRNA dysregulation is causal in lots of diseases, including tumor [15]. MiRNA have also been found to regulate EMT in many tumor cells. For example, the downregulation of miR-214 induces EMT by directly targeting FGF9 in gastric cancer-associated cells [16]. MiR-630 is upregulated in epithelial ovarian cancer and associated with proliferation and migration of tumor cells [17]. Besides, the forced expression of miR-101 suppresses migration and invasion by targeting ZEB1 in ovarian cancer cells [18].

MiR-15a is a part of miR-15a/miR-16 cluster, which is located in human genome 13q14.2. MiR-15a has been reported to inhibit invasion and metastasis of cancer cells in hepatocarcinoma, gastric cancer and chronic lymphocytic leukemia [19–21]. Furthermore, it plays a potential role in the prediction of recurrent colorectal adenocarcinoma [22]. The role of miR-15a-5p in ovarian cancer is poorly understood.

In this study, we explored the role of miR-15a-5p in the invasion and EMT of ovarian cancer cells, and dissected the underlying mechanism. In addition, we demonstrate that miR-15a-5p expression inhibits migration and invasion by targeting Proline-, glutamic acid- and leucine-rich protein 1 (PELP1) in ovarian cancer cells.

Methods

Cell culture and transfection

Ovarian cancer cell lines SKOV3 and OVCAR3 were obtained from Guangzhou Jennio Biotech Co.,Ltd. (Guangzhou, Guangdong, China) and cultured in RPMI1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (HyClone, Logan, UT, USA) at 37 °C, 5%CO₂. Cells were seeded in 12-well plates (5 × 10⁴ per well) and transfected with miR-15a-5p mimic or the negative control (miR-NC) (GenePharma, Shanghai, China). The pcDNA3.1-PELP1 expression plasmid was constructed by GenePharma (Shanghai, China). Small interfering RNA (siRNA) was used for the knockdown of PELP1. Pooled PELP1-specific siRNAs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine™ 3000 was used for transfection according to the manufacture's instruction.

Western blot

Cells in each group were harvested and washed twice with cold PBS, then lysed in RIPA buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail) on ice for 30 min. Total protein concentrations were evaluated using a BCA protein assay kit (Pierce, Thermo Scientific). Total protein (40 µg) was separated using 10% SDS-PAGE and transferred to nitrocellulose membranes. Subsequently, the membranes were blocked by 5% (w/v) nonfat milk for 1 h at room temperature and overnight incubation at 4 °C with primary antibodies against β-actin (Sigma 1:5000), PELP1 (Cell Signaling Technology 1:1000), E-cadherin (Cell Signaling Technology 1:1000) and vimentin (Cell Signaling Technology 1:1000). Finally, the membranes were incubated with secondary antibodies for 1 h and scanned by an Odyssey two-colour infrared laser imaging system (LI-

COR Biosciences, Lincoln, NE, USA). Protein relative quantification were analyzed by using ImageJ software and normalized to β -actin.

Total RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from cells with an RNA isolation kit (Ultrapure RNA Kit, CWBiotech) and mRNA was reverse transcribed into cDNA by a Takara Reverse Transcriptase M-MLV kit according to the manufacturer’s instructions. qRT-PCR was performed on a Roche Light Cycler System with SYBR Green PCR Master Mix kit (Roche, Switzerland). The cycling conditions were as follows: incubation at 95 °C for 5 min, 40 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 20 s and extension at 72 °C for 20 s. GAPDH was used as the internal control. Gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

Table 1
Primer sequences used in quantitative real-time reverse transcription polymerase chain reactions

Gene	Sequence
PELP1	Forward: 5'-TCAGTAATGCACGTCTCAGTTC-3'
	Reverse: 5'-CTCCGAAGCCAAGACACACAG-3'
miR-15a-5p	Forward: 5'-TAGCAGCACATAATGGTTTGTG-3'
	Reverse: 5'-CTCAACTGGTGTCTGTGGA-3'
Snail	Forward: 5'-TCGGAAGCCTAACTACAGCGA-3'
	Reverse: 5'-AGATGAGCATTGGCAGCGAG-3'
U6	Forward: 5'-CTCGCTTCGGCAGCACA-3'
	Reverse: 5'-ACGCTTCACGAATTTGCGTGTC-3'
GAPDH	Forward: 5'-AGGTCGGAGTCAACGGATTTG-3'
	Reverse: 5'-AGGCTGTTGTCATACTTCTCAT-3'

Luciferase Reporter Assay

Luciferase activity was detected using a luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer’s instruction.

Wound-healing Assay

Cells were seeded into a 6-well plate (4×10^5 cells per dish) and cultured for 24 h. After the wound was made using a 200 μ L pipette, cells were washed twice with PBS to remove non-adherent cells. The wound sizes were observed by a microscope (ZEISS, Axio Image Z2) at 0 h and 24 h.

Transwell Invasion Assay

Cells were seeded in the upper chamber with an 8 µm pore polycarbonate membrane (Corning, NY, USA) that were pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The chamber was in 24-well plates, and 600 µL medium containing 20% FBS was added to the lower chambers. After incubation for 36 h, the cells were stained with 1% cresol violet (Amresco, Solon, OH, USA). Invaded cells were observed with a microscope (ZEISS, Axio Image Z2).

Cell Viability Assay

Cell viability were assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (Promega Corporation, USA). SKOV3 or OVCAR3 cells were seeded into 96-well culture plates (3×10^3 cells per well) and cultured for 24 h, 48 h and 72 h. Then, cells were incubated with 5 mg/mL MTS solution for 4 h. Optical density (OD) values were recorded at 490 nm using a microplate reader (Bio-Tek, Winooski, VT, USA).

Statistical analysis

Data were presented as the mean $\pm$ SD error and analyzed with one-way ANOVA. *P* values less than 0.05 was considered as significance. Each experiment was performed at least three times.

Results

MiR-15a-5p targets PELP1 and inhibits PELP1 expression

PELP1 oncogenic signaling are involved in the progression of some cancers. To investigate whether the expression of miR-15a-5p genes regulates PELP1, online bioinformatics website Targetscan (http://www.targetscan.org/vert_71/) was used to find out the potential binding sites of PELP1 3'UTR that are targeted by miR-15a-5p (Fig. 1A). To test the targeting relationship between miR-15a-5p and 3'UTR of PELP1, the luciferase reporter assay was performed. SKOV3 and OVCAR3 cells were co-transfected with miR-15a-5p mimics and pMIR-PELP1-UTR. The results showed that miR-15a-5p reduced the luciferase activity by 54% by co-transfection with pMIR-PELP1-3'UTR, while that of PELP1 3'UTR-mut did not change significantly (Fig. 1B). SKOV3 and OVCAR3 cells transfected with miR-15a-5p mimic construct significantly decreased PELP1 gene and protein expression compared with mimics NC groups, while cells transfected with miR-15a-5p inhibitor showed significantly higher expression of PELP1 (Fig. 1C-E). These results confirmed that miR-15a-5p inhibited the expression of PELP1 by binding to its mRNA 3'UTR.

MiR-15a-5p down-regulates the migration and invasion of SKOV3 and OVCAR3

To test the effect of miR-15a-5p on the invasion ability of ovarian cancer, cells were transfected with miR-15a-5p mimics or inhibitor. Real-time PCR confirmed that transfection of miR-15a-5p mimics increased the miR-15a-5p gene expression significantly compared to NC group, while that of miR-15a-5p inhibitor

decreased the expression level (Fig. 2A). For the MTS assay, SKOV3 and OVCAR3 cells transfected with miR-15a-5p mimics induced a decrease in cell viability compared to NC group. While the viability of cells was increased in miR-15a-5p inhibitor group (Fig. 1B, C). Cell wound scratch assay and transwell assay were performed to measure the metastasis and invasion ability. In cell wound scratch assay, the cell migration rate was decreased in miR-15a-5p mimics group and increased in inhibitor group, which were consisted with transwell assay results (Fig. 1D, E). As results showed, the number of penetrating cells was significantly decreased in miR-15a-5p mimics group than in mimics NC group. Inhibition of miR-15a-5p remarkably increased the number of penetrating cells (Fig. 2F, G). Taken together, these results indicated that miR-15a-5p might regulate the migration and invasion of SKOV3 and OVCAR3 cells in vitro.

MiR-15a-5p suppresses EMT in ovarian cancer cells

During tumor metastasis, vimentin and E-cadherin molecules plays notable roles. EMT-related protein vimentin and E-cadherin was detected to measure the EMT transition of ovarian cancer cells. The protein expression level of E-cadherin was increased 2.65-fold in miR-15a-5p mimics transfected group and decreased in in miR-15a-5p inhibitor group compared to NC group. While that of vimentin were decreased by 68% after miR-15a-5p mimics transfection and increased 2.19-fold after transfection of inhibitor (Fig. 3A-D). To examine the effect of miR-15a-5p transfection on EMT process, the gene expression of EMT-related transcription factors Snail was detected. The gene expression of Snail was decreased in miR-15a-5p mimics transfected group and increased in miR-15a-5p inhibitor group when compared with that in NC group (Fig. 3E). The results indicated that the invasion inhibition of ovarian cancer cells by transfection of miR-15a-5p might be associated with EMT transition mediated by Snail.

The inhibitory effects of miR-15a-5p is rescued by PELP1

To analyze the role of PELP1 in ovarian cancer cells, SKOV3 and OVCAR3 cells were co-transfected with PELP1 and miR-15a-5p. The real-time PCR results indicated that the expression level of PELP1 was increased and not suppressed by miR-15a-5p, which confirmed the effectiveness of the plasmid (Fig. 4A). After that, MTS assay showed that overexpression of PELP1 increased the viability of SKOV3 and OVCAR3 cells (Fig. 4B). Additionally, wound scratch and transwell assays revealed that the migratory and invade ability of ovarian cancer cells were elevated by PELP1 overexpression (Fig. 4C-F). The protein expression levels of E-cadherin and vimentin were decreased and increased respectively after PELP1 forced expression, indicating that PELP1 accelerated EMT in SKOV3 cells (Fig. 4G-J). Taken together, miR-15a-5p inhibited viability, migration, invasion and EMT of ovarian cancer cells by downregulating PELP1 and the inhibitory effects were neutralized by the overexpression of PELP1.

Knockdown of PELP1 mimics the inhibitory effects of miR-15a-5p on migration and invasion of ovarian cancer cells

Finally, to investigate whether knockdown of PELP1 mimicked the inhibitory effects of miR-15a-5p, the effect of PELP1 knockdown on the migration and invasion of SKOV3 and OVCAR3 cells was examined. The Western blot results revealed that the expression level of PELP1 was decreased in SKOV3 and OVCAR3 cells through application of siRNA targeting PELP1 (Fig. 5A). The MTS assay showed that the viability of SKOV3 and OVCAR3 was decreased by downregulation of PELP1, indicating an oncogenic effect of PELP1 on ovarian cancer growth (Fig. 5B, C). In addition, wound scratch and transwell assays revealed that knockdown of PELP1 in SKOV3 cells suppressed cell migration and invasion, which were consistent with cell viability assay (Fig. 5D-G). Therefore, these results demonstrate that PELP1 knockdown mimics the inhibitory effects of miR-15a-5p on migration and invasion of ovarian cancer cells.

Discussion

Ovarian cancer is a kind of lethal malignancy due to metastatic progression [23]. Most patients have no specific clinical symptoms at an early stage and present with disseminated intra-abdominal metastasis at diagnosis [24]. Recurrence occurs in most ovarian cancer patients with standard therapeutic approaches [23]. Therefore, the molecular mechanisms of cancer cell metastasis are crucial for the diagnosis and targeted therapies of ovarian cancer.

Metastasis of tumor cells is a complex process by which cancer cells spread to distant locations through infiltration and proliferation [25]. Emerging evidence suggests that miRNA regulate cell proliferation, survival and are involved in cancer metastasis [26, 27]. Besides, miRNA have been reported to regulate tumor EMT process through targeting tumor suppressors and oncogenes [28]. The current study first demonstrated that miR-15a-5p inhibits metastasis of ovarian cancer cells by regulating PELP1.

Previous studies reported that miR-15a as tumor suppressor genes on cell proliferation and apoptosis [29]. Since loss of miR-15a displaces the expression balance of anti-apoptotic and pro-apoptotic proteins, miR-15a was considered as prognostic biomarkers in chronic lymphocytic leukemia [21]. It was also reported that overexpression of miR-15a regulated Twist and Snail-mediated EMT through activin/smad signaling pathway in prostate cancer cells [30]. Nevertheless, the upregulation of miR-15a downregulate BCL-2 and cyclin D1 and inhibit ERK signaling pathway to reduce survival of tumor cells in neuroblastoma [31]. Consistently, this current study found that upregulation of miR-15a-5p oppressed migration, invasion and EMT of ovarian cancer cells, indicated oppressed tumor metastasis.

Bioinformatics prediction provides important clues for the study of miRNA function. The bioinformatics online databases targetscan was used to predicted that the miR-15a-5p could potentially target PELP1 (Fig. 1). Recent study revealed that PELP1 was upregulated and associated with clinicopathologic features in gastric cancer samples [32]. Furthermore, it was reported that the PELP1-regulated genes associated with nuclear factor- κ B (NF- κ B) signaling pathway. PELP1 knockdown remarkably reduced medulloblastoma progression in vivo through regulating NF- κ B target genes expression and nuclear

translocation of p65 [33]. Interestingly, PELP1 was also found to drive inflammatory gene expression and active macrophage to promote migration and proliferation of breast epithelial cells [34]. Our study showed that the overexpression of PELP1 enhanced migration and invasion in ovarian carcinoma, which were reversed by miR-15a-5p.

Overall, we demonstrated the suppressive effect of miR-15a-5p on the invasion of ovarian cancer cells. Combined with the luciferase reporter assay, all these results identified that PELP1 is a target gene of miR-15a-5p. In addition, the knockdown of PELP1 mimicked the inhibitory effect of miR-15a-5p on metastasis of ovarian cancer cells. In future research, we will focus on the downstream pathway regulated by PELP1 and explore the special role of PELP1 in ovarian carcinoma.

Conclusion

This study provided new experimental evidence that miR-15a-5p expression inhibits migration, invasion and EMT of ovarian cancer cells. Additionally, PELP1 was identified as a direct target of miR-15a-5p and play a crucial role in the suppressive effect of miR-15a-5p on ovarian carcinoma, which suggested a molecular linkage between PELP1 and miR-15a-5p that determine invasion of ovarian cancer.

List Of Abbreviations

qRT-PCR: Quantitative Real-time polymerase chain reaction

EMT: Epithelial mesenchymal transition

PELP1: Proline, glutamic acid- and leucine-rich protein 1

MiRNA: MicroRNAs

FBS: Fetal bovine serum

siRNA: Small interfering RNA

OD: Optical density

Declarations

Ethics approval and consent to participate

All the procedures described in this report were approved by the Ethics Committee of Zunyi Medical University Affiliated Hospital.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

No competing interests was declared.

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Author contributions

Bing Yang was responsible for the conception, study design, and administrative support. Yujia Yang and Li Yuan were responsible for the study design, data collection and analysis, and manuscript writing and revision.

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Figures

Figure 1

MiR-15a-5p targets PELP1 and inhibits PELP1 expression. (A) Bioinformatic analysis showed that the binding site of miR-15a-5p is present in the PELP1 3'UTR; (B) 3'UTR luciferase reporter assay; (C) qRT-PCR was performed to detect the endogenous mRNA level; (D, E) Western blotting was conducted to detected the protein expression of PELP1 in cells transfected with miR-15a-5p. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figure 2

MiR-15a-5p down-regulates the migration and invasion of SKOV3 and OVCAR3. The SKOV3 and OVCAR3 cells were transfected with miR-15a-5p mimics or inhibitor. (A) qRT-PCR was performed to detect the miR-15a-5p levels; (B, C) MTS assay was used for detection of viability; (D, E) The migratory ability was determined with wound healing assay; (F, G) The invade ability was measured with transwell assay. (the scale bars represent 100 μ m) (* P < 0.05, ** P < 0.01, *** P < 0.001, ns, no significance).

Figure 3

MiR-15a-5p suppresses EMT in SKOV3 and OVCAR3. (A, C) Western blotting was conducted to detect E-cadherin and vimentin in SKOV3 (A) or OVCAR3 (C) cells transfected with miR-15a-5p mimics or inhibitor; (B, D) The gray value of western blot bands in (A) and (C) was quantified. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figure 4

The inhibitory effects of miR-15a-5p is rescued by PELP1. (A) The mRNA level of PELP1 was detected by qRT-PCR in SKOV3 and OVCAR3 cells after PELP1 overexpression; (B) MTS assay was performed to detect the viability of SKOV3 and OVCAR3 cells; (C, D) Wound healing assay was used to detect the migratory ability; (E, F) The invade ability of SKOV3 and OVCAR3 cells was detected by transwell assay; (G, I) Western blotting was conducted to detect E-cadherin and vimentin in SKOV3 (G) or OVCAR3 (I) cells

transfected with miR-15a-5p mimics or inhibitor; (H, J) The gray value of western blot bands in (G) and (I) was quantified. (the scale bars represent 100 μm). (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

Figure 5

Knockdown of PELP1 mimics the inhibitory effects of miR-15a-5p on migration and invasion of ovarian cancer cells. (A) siPELP1 and siCon was transfected into SKOV3 and OVCAR3 cells. The level of PELP1 was detected by western blot; (B, C) Cell proliferation activity was measured by MTS assay; (D, E) SKOV3

and OVCAR3 cells were transfected with siCon or siPELP1. Wound healing assay was conducted; (F, G) Cell invasion ability was measured by the transwell invasion assay.