Effects of miR-492 on migration, invasion, EMT and prognosis in ovarian cancer by targeting SOX7

Zhong Wang¹, Yihua Liu², Min Wang¹, Jinsheng Zhao³

¹Department of Reproductive Genetics, Liaocheng People’s Hospital, Liaocheng 252000, China; ²Central Laboratory, Liaocheng People’s Hospital, Liaocheng 252000, China; ³Department of Chemistry, Liaocheng People’s Hospital, Liaocheng 252000, China.

Summary

Purpose: Ovarian cancer (OC) is one of the most common malignancies in females with high mortality rate. MicroRNAs (miRNAs or miRs) serve as oncogenes or tumor suppressors in various human cancer types, including OC. The aim of this study was to explore the roles of miR-492 in OC.

Methods: Two human ovarian cancer cell lines, SKOV3 and CAOV3, and a normal ovarian cell line IOSE80 were used in this study. Real-time quantitative polymerase chain reaction (RT-qPCR) was conducted to measure the mRNA levels of miRNAs and genes. The protein levels of epithelial-mesenchymal transition (EMT) associated genes were calculated using Western blot. Transwell assay was utilized to evaluate the migratory and invasive capacities.

Results: MiR-492 was overexpressed while SRY-box 7 (SOX7) was lowly expressed in OC tissues and cells. Upregulation of miR-492 or downregulation of SOX7 predicted poor prognosis of OC patients. MiR-492 regulated the expression of SOX7 via directly binding to the 3’-untranslated region (3’-UTR) of SOX7 mRNA in SKOV3 OC cells. The expression of miR-492 had a negative relationship with SOX7 in OC tissues. MiR-492 promoted the migration, invasion and EMT through SOX7 in SKOV3 cells. SOX7 could partially reverse the role of miR-492 on the migratory, invasive and EMT abilities in SKOV3 cells.

Conclusions: MiR-492 promoted the migratory, invasive and EMT abilities through SOX7 in OC. This suggested that miR-492/SOX7 axis may be an effective candidate therapeutic target for the treatment of OC.

Key words: miR-492, SOX7, ovarian cancer, EMT

Introduction

Ovarian cancer (OC) has the highest mortality rate among all gynecological malignancies [1,2]. Despite the huge progress in the therapy of OC, the 5-year overall survival for OC patients is 35% [3,4]. Therefore, it is urgent to explore the underlying mechanisms of OC and find new therapeutic targets to improve the clinical outcome of OC patients.

MicroRNAs (miRs), an evolutionarily conserved classical short non-coding RNAs 18-25 nucleotides in length, mediate the expression of target gene through directly targeting the 3'-untranslated region (3'-UTR) of their mRNA, thereby contributing to the modulation of multiple malignancies and thus offering promising treatment options [5,6]. Increasing evidence revealed that miRs acted as key regulators in modulating multiple biological processes including cell growth, fat metabolism, cell cycle, metastasis and cell apoptosis [7]. MiR-492 has been reported to function as an oncogene and is overexpressed in several diseases including cervical cancer, psoriasis, atherosclerosis and hepatoblastoma [8-11]. A few studies have indicated that miR-492 promoted tumor progression being involved in oxaliplatin resistance in colon cancer and improved the proliferation of hepatic cancer [12,13] However, Wu et al [14] revealed that miR-492 in-
hibited the growth and invasion of renal clear cell carcinoma cells, while improving their apoptosis and adhesion. The roles of miR-492 transcription regulation and the regulatory mechanisms remain unclear. Therefore, the aim of this study was to investigate the important roles of miR-492 in OC.

SRY-box 7 (SOX7) belongs to the SOX (SRY-related HMG-box) family and is reported to be a transcriptional factor involved in regulating embryonic development [15]. Qin et al [16] have revealed that SOX7 was involved in cell activation and mediated acute allograft kidney injury in mice. Recently, SOX7 has been validated to be a tumor suppressor that regulated a variety of cancer-related pathways [17,18]. SOX7 has been indicated to act as a tumor suppressor in renal cell carcinoma to suppress cell proliferation and arrest cell cycle at G0/G1 [19]. The aim of this study was to explore the roles of miR-492 in OC.

Methods

Tissue samples

Between January 2016 to June 2018, 49 OC patients were selected from Liaocheng People’s Hospital and 49 pairs of cancer tissues and paracancer tissues were obtained. The fresh tissues were immediately frozen in liquid nitrogen after surgical resection and stored at −80°C. All patients signed informed consent and the study was approved by the Ethics Committee of Liaocheng People’s Hospital. This research was conducted in accordance with the Declaration of Helsinki.

Cell culture

Two human ovarian cancer cell lines SKOV3 and CAOV3 and a normal ovarian cell line IOSE80 were obtained from Cobioer (Shanghai, China). All the cells were cultured using the Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). All of the cell lines were maintained in incubator at 37°C in a humidified atmosphere of 5% CO2.

Cell transfection

The miR-492 mimic, the miR-492 inhibitor and their negative controls (NC) were purchased from RiboBio (Guangzhou, China). The pc-DNA 3.1-SOX7 vector (Hanbio Biotechnology, Shanghai, China) vector was employed to overexpress the SOX7 in OC cells. SKOV3 cells seeded in 6-well plates were used to perform the transfection by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

Real-time quantitative polymerase chain reaction (RT-qPCR) assay

The total RNAs were extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and were quantified on a NanoDrop ND-1000 (NanoDrop Wilmington, DE, USA). The DNA was removed from RNA samples using DNase I (Thermo Fisher Scientific, Waltham, MA, USA). The first complementary deoxyribose nucleic acid (cDNA) strand was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Finally, quantitative analysis was carried out using a SYBR Premix Ex Taq Kit (TaKaRa, Dalian, China). Quantitative PCR was conducted at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The primers were as follows: miR-492: forward 5′-TTAGGCCTGGGGAACG-3′, reverse 5′-TTTGGGACTAGCACTT-3′; U6: forward 5′-CTCGTTCGGACGACA-3′, reverse 5′-AACGCTTCAGAATTTCGG-3′; SOX7: forward 5′-GGGGCCGCGCCGCGAGCTGATA-3′, reverse 5′-CGGGATCACATCAAGGCAC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward 5′-GACGCCATCTTCCTGTT-3′, reverse 5′-CACCGACCTTATCGATCAT-3′. The relative expression levels of miR-492 and SOX7 were normalized to U6 and GAPDH levels, respectively, utilizing the 2−ΔΔCt method.

Luciferase reporter assay

To generate psiCHECK-SOX7 reporter, the SOX7 3′-UTR sequences containing the potential binding sites of miR-423 were cloned into psiCHECK-2 vector (Promega, Madison, WI, USA) at Hanbio Biotechnology. Meanwhile, KOD-plus mutagenesis Kit (Toyobo, Osaka, Japan) was employed to perform the mutation. The wild type or the mutated SOX7 were designated as psiCHECK-SOX7-WT (WT) and psiCHECK-SOX7-MUT reporter (MUT), respectively. The miR-492 mimic or the miR-492 inhibitor along with the wild type or the mutant SOX7 were co-transfected into SKOV3 cells. Following this, the luciferase activity was evaluated using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) in accordance with the manufacturer’s instructions after transfection of 48 h.

Western blot assay

The total proteins were extracted from tissues and cell lines by the radioimmunoprecipitation assay (RIPA) Lysis and Extraction Buffer (Thermo Fisher Scientific, Waltham, MA, USA) incorporated with protease inhibitor complex (cocktail, Roche Diagnostics, Basel, Switzerland). Subsequently, the concentration of the protein was calculated by using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal proteins were fractionated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electro-transferred onto a nitrocellulose membrane (Millipore, Bedford, MA, USA). After blocking for 1 h in 5% skim milk at room temperature, the membrane was immunoblotted with primary antibodies at 4°C overnight. The primary antibodies were against SOX7, E-cadherin, N-cadherin, Vimentin and GAPDH. Next, the membrane was incubated with the HRP-conjugated secondary antibody for 1 h at room temperature. Finally, the immunoreactive signals on Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA) were assessed by Pierce Enhanced Chemiluminescence Detection Kit (ECL) Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA).
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Transwell assay

The migratory and invasive properties were assessed using the transwell chamber assay (pore size, 8 µm) covered with or without Matrigel (BD Bioscience, Franklin Lakes, NJ, USA). Then, SKOV3 cells were resuspended in serum-free medium and seeded into the upper chamber of a 24-well transwell insert. Meanwhile, DMEM medium with 20% FBS was added to the lower chamber as chemoattractant. After incubation of 48 h, the non-migrated or non-invasive cells were wiped with cotton swabs, while the migrated or invaded cells were fixed with 100% methanol and stained with 0.1% crystal violet solution (Sigma-Aldrich, Louis, MO, USA). Cells were photographed with an inverted light microscope (Zeiss, Germany) at 200x magnification and the number of cells was counted in five random fields.

Statistics

The data shown are presented as the mean ± standard deviation (SD) from at least three independent experiments. Statistical analyses were performed using SPSS 20.0 software, Chicago, IL, USA. Differences between experimental groups were analyzed by Student’s t-test when only two groups. Comparison between multiple groups was carried out using one-way analysis of variance (ANOVA) test followed by post hoc test (least significant difference). The relationship between miR-492 and SOX7 expression was assessed by Spearman’s correlation analysis. P<0.05 was considered to indicate a statistically significant difference compared with the respective control.

Results

Overexpression of miR-492 predicted poor prognosis of ovarian cancer patients

The mRNA level of miR-492 was evaluated in 49 pairs of OC and peritumor normal tissues by RT-qPCR. OC tissues showed a higher miR-492 mRNA level than the corresponding peritumor normal tissues (p<0.01) (Figure 1A). The association between the expression of miR-492 and the overall survival was assessed using the Kaplan-Meier method which showed that the expression of miR-492 was connected with overall survival (p<0.05) (Figure 1B).

Moreover, the expression of miR-492 was calculated in OC cell lines SKOV3 and HO-8910 and normal ovarian epithelial cell line IOSE386. Similar with the tissues, the expression of miR-492 was upregulated in OC cell lines SKOV3 and HO-8910 than the normal ovarian cell line IOSE386 (p<0.05) (Figure 1C). To explore the function of miR-492 in ovarian cancer cells, the miR-492 expression was...
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Figure 2. MiR-492 promoted cell metastasis and EMT of SKOV3 cells (A and B). The migratory and invasive abilities were enhanced by the miR-492 mimic, while they were inhibited by the miR-492 inhibitor. C: Western blot results revealed that miR-492 promoted the EMT ability in SKOV3 cells. *compared with the NC mimic group or NC inhibitor group, p<0.05.

Figure 3. MiR-492 regulated the expression of SOX7 through directly binding to its 3'-UTR mRNA. A: SOX7 was predicted to be a target of miR-492 by TargetScan. B: Luciferase reporter assay was performed to verify that miR-492 directly binding to SOX7. C: MiR-429 regulated the expression of SOX7 in SKOV3 cells. *compared with the NC group or the NC mimic group, p<0.05. #compared with the NC group or NC mimic group, p<0.05.
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MiR-492 was up- or down-regulated using miR-492 mimic or miR-492 inhibitor in SKOV3 cells, and calculated by RT-qPCR (p<0.05) (Figure 1D).

MiR-492 improved the metastatic potential and EMT of SKOV3 cells

Transwell assay was applied to evaluate the migratory and invasive abilities in SKOV3 cells. The results illuminated that the miR-492 mimic enhanced the migratory (p<0.05) and invasive (p<0.05) properties, while the migration and invasion were inhibited by miR-492 inhibitor (p<0.05) (Figure 2A and 2B). In addition, the western blot results revealed that the miR-492 mimic promoted the expression of N-cadherin whereas inhibited the expression of E-cadherin. On the contrary, the miR-492 inhibitor suppressed the expression of N-cadherin, whereas improved the expression of E-cadherin (Figure 2C). All the results revealed that miR-492 enhanced the migration, invasion and EMT abilities in SKOV3 cells.

MiR-492 regulated the expression of SOX7 by directly binding to the 3'-UTR of mRNA

The online software TargetScan was used to predict the target genes of miR-492, and SOX7 was found to be a target gene with a possible binding site at 1417 to 1423 bases. As shown in Figure 3A, the binding sequences located at the 3'-UTR of the mRNA were mutated from CAGGUCC to GUCCAGG. The luciferase reporter assay was carried out to prove that miR-492 directly targeted the 3'-UTR of SOX7 mRNA. MiR-492 overexpressing SKOV3 cells transfected with the WT-SOX7-3'UTR construct showed significantly decreased luciferase activity (p<0.05). On the contrary, the miR-492 mimic failed to alter the luciferase activity of the mutated 3'-UTR (p>0.05) (Figure 3B). In addition, SKOV3 cells were transfected with the miR-429 mimic or the miR-429 inhibitor, and the mRNA levels of SOX7 were assessed. Not unexpectedly, miR-429 inhibited the expression of SOX7 (p<0.05), while the miR-429 inhibitor promoted the expression of SOX7.

**Figure 4.** Downregulation of SOX7 predicted worse prognosis of ovarian cancer patients. A: The expression of SOX7 was lower in ovarian cancer tissues than the matched paracancer tissues. B: The expression of miR-492 had negative connection with SOX7 expression in SKOV3 cells (p<0.05). C: Overexpression of RBM5 predicted worse outcome of ovarian cancer patients. D: The expression of SOX7 was lower in ovarian cancer cell lines SKOV3 and HO-8910 than in normal ovarian epithelial cell line IOSE386. *compared with the normal control group or the IOSE386 group, p<0.05. **compared with the normal control group and IOSE386 group, p<0.01.
SOX7 in SKOV3 cells (p<0.05) (Figure 3C). All the results revealed that miR-492 regulated the expression of SOX7 in SKOV3 cells.

Downregulation of SOX7 predicted poor prognosis of OC patients

To explore the important roles of SOX7 in OC, the expression of SOX7 was assessed by RT-qPCR assay. Compared with the normal tissues, SOX7 was found to be downregulated in OC tissues (p<0.05) (Figure 4A). Because the expression of SOX7 was lowly expressed in OC, we were not sure over the connection between the expression of SOX7 and miR-492. As expected, we discovered that the expression of miR-492 had a negative connection with SOX7 expression in SKOV3 cells (p<0.05) (Figure 4B). Moreover, Kaplan-Meier method indicated that overexpression of SOX7 predicted worse outcome of OC patients (p<0.05) (Figure 4C). Meanwhile, the expression of SOX7 was significantly lower in SKOV3 (p<0.01) and HO-8910 (p<0.05) than normal ovarian epithelial cell line IOSE386 (Figure 4D).

SOX7 partially reversed the functions of miR-492 on cell metastasis

To further confirm the important roles of SOX7 in miR-492 overexpressing cells, pcDNA3.1-SOX7 plasmid was co-transfected into miR-492 overexpressing SKOV3 cells (p<0.05) (Figure 5A). Subsequently, cell migration and invasion of SKOV3 cells were calculated by transwell assay. The results showed that migration and invasion increased significantly when co-transfected with the miR mimic and SOX7 compared with control (p<0.05) (Figure 5B). In addition, overexpression of SOX7 suppressed the EMT ability by inhibiting the expression of N-cadherin while enhancing the expression of E-cadherin in SKOV3 cells (Figure 5C). These results demonstrated that SOX7 could partially reverse the functions of miR-492 on OC cells migratory, invasive and EMT abilities.

Discussion

MiRs specifically interact with the sequence of the 3’-UTR of the homologous mRNA target to inhibit post-translational gene expression [20,21]. Frowein et al [22] have indicated that overexpression of miR-492 enhanced the metastatic potential and was associated with worse outcome of hepatoblastoma. Consistent with Frowein et al [22], miR-492 was found to be upregulated in OC tissues and also in cell lines. What’s more, overexpression of miR-492 predicted worse outcome of ovarian cancer patients. Even in cervical squamous
cell carcinomas, miR-492 promoted the viability and invasion, and it was associated with radiotherapy response [23]. However, the status and roles of miR-492 in OC progression is still not known. Our findings were consistent with the entire study, and we found that aberrant expression of miR-492 promoted the migration, invasion and EMT, while the migration, invasion and EMT were impaired by reduction of miR-492 in OC. MiR-492 promoted cell proliferation and cell cycle through directly binding to SOX7 mRNA in breast cancer [24]. We proposed that miR-492 regulated the expression of SOX7 by directly binding to the 3'-UTR of SOX7 mRNA in OC cells. The expression of miR-492 had a negative connection with the expression of SOX7 in OC tissues.

Previous studies indicated that SOX7 was a target gene of multiple miRs that included miRNA-24, miRNA-452, miR-595 and miR-616 [25-28]. It has been reported that SOX7 was associated with cell proliferation through Wnt/β-catenin signaling in endometrial cancer [29]. In lung adenocarcinoma, low expression of SOX7 was related to poor prognosis [30]. Consistent with these findings, we found that SOX7 was downregulated in OC tissues and cell lines compared to the normal tissues and normal cells. Low expression of SOX7 induced cell apoptosis and suppressed cell proliferation in colorectal cancer [31]. SOX7 has been demonstrated to act as a tumor suppressor in breast cancer cell growth and metastasis [32]. It’s the first time for us to propose that re-expression SOX7 reduced the abilities of metastasis and the EMT in miR-492 mimic-transfected cells, compared with the cells that were transfected only with the miR-492 mimic.

**Conclusions**

MiR-492 acts as an oncogene to promote the metastasis and EMT by directly binding to the 3'-UTR of SOX7 mRNA in OC cells. These findings provide new insights into the molecular mechanisms of miR-492 in OC metastasis, suggesting that miR-492 may be a therapeutic target for OC. However, the effect of miR-492 on OC metastasis has not been verified in vivo, which is a limitation of this article, and we will study that in our next article.

**Conflict of interests**

The authors declare no conflict of interests.

**References**

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