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Original Article

MicroRNA-22 suppresses metastasis by targeting SNAI1 in the ovarian cancer cell line SKOV-3

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Abstract: MicroRNAs (miRNAs), a class of small non-coding RNAs, regulate gene expression by targeting specific mRNAs to trigger translational repression or mRNA degradation. MiR-22 is aberrantly expressed in human ovarian cancer compared to normal tissues, and it plays an important role in regulating ovarian cancer metastasis. However, the concrete mechanism underlying the regulation of ovarian cancer metastasis by miR-22 remains ambiguous. In this study, the effects of miR-22 on metastasis and metastasis-associated targets were investigated in the human ovarian cancer cell line SKOV-3. Using a transwell migration assay and a cell wound healing assay, we discovered that miR-22 inhibited metastasis. A bioinformatic analysis predicted that snail homolog 1 (SNAI1) was a target of miR-22. The prediction was validated by qRT-PCR, Western blotting, an immunofluorescence assay, and a luciferase reporter assay. Furthermore, the effects of miR-22 on the expression of E-cadherin, a downstream target of Snail, were also investigated. These results suggested that miR-22 might inhibit the epithelial-to-mesenchymal transition (EMT) and metastasis by directly targeting SNAI1 for degradation in the human ovarian cancer cell line SKOV-3.

Keywords: MiR-22, SNAI1, E-cadherin, metastasis, ovarian cancer

Introduction

Ovarian carcinoma, the most lethal gynecological cancer, is the fifth leading cause of cancer death in women, and it is not diagnosed until its late stages in most cases [1, 2]. Patients with ovarian cancer commonly suffer from a poor prognosis, which largely results from aggressive metastasis in the peritoneal cavity [3, 4]. The mechanism involved in ovarian cancer metastasis is still unclear. Thus, further research is of great importance in managing this malignant carcinoma [5].

MicroRNAs (miRNAs), a set of endogenously initiated non-coding RNA molecules that are approximately 22 nucleotides in length, post-transcriptionally regulate the expression of certain genes by cleaving target messenger RNAs or by repressing translation through incomplete base pairing in the 3'un-translated regions (3'UTRs) of target mRNAs [6-8]. miRNAs regulate the expression of a large number of target genes in various pathways and therefore affect basic physiological processes, including proliferation, differentiation, apoptosis, and tumorigenesis [9-12]. miRNAs exhibit different regulatory functions. For example, studies show that miR-193a inhibits cell proliferation by repressing c-kit expression in acute myeloid leukemia [13]. MiR-372 has been shown to significantly inhibit cell proliferation and apoptosis in ovarian carcinoma cell lines [14]. MiR-21 functions as an oncogene and is associated with increased proliferation and decreased apoptosis in human breast cancer cells [15]. miR-9 increases breast cancer metastasis and invasion by targeting CDH1 [16]. To date, miRNA expression profiling is used as a signature to identify different cancer types, and it also acts as an accurate classification system for poorly differentiated tumors [17].

The expression of the miR-22 primary transcript that locates in the 5'-untranslated region of an open reading frame (C17orf91) of chromosome

17p13.3 is driven by its promoter [18]. miR-22 is expressed in most human tissues. It is abundant in heart, smooth muscle, bladder, and adipose tissues. However, it is low in ovary, thymus, intestine, colon, and thyroid tissues [19]. Recent studies indicate that miR-22 plays an important role in many carcinomas. miR-22 induces cell apoptosis in clear ovarian carcinoma; restores the cell senescence in senescent human fibroblasts; acts as an anti-angiogenesis factor in colon cancer cell lines; inhibits cell proliferation in lung carcinoma cell line A549, breast carcinoma cell line MCF-7, and prostate cancer cell line PC-3 [19-22]; promotes cell metastasis in transformed human bronchial epithelial cells [23]; and miR-22 attenuates the cellular proliferation, migration, and invasion of HCC cells [24]. Metastasis is a crucial step in the process of tumorigenesis. One hypothesis for the possible underlying mechanism is concentrating on the epithelial-mesenchymal transition (EMT) [25], which plays a central role in metastasis. In EMT, cells are transformed from a polarized epithelial phenotype to a highly motile mesenchymal or fibroblastoid phenotype [26, 27]. Some mesenchymal-associated genes, including vimentin, N-cadherin, and fibronectin, are up-regulated. Some epithelial-associated markers, such as E-cadherin and cytokeratins, are down-regulated [28]. The transcription factor Snail, a zinc finger protein encoded by the SNAI1 gene and belonging to the Snail superfamily, induces EMT by down-regulating the expression of E-cadherin and up regulating the expression of vimentin, leading to cell migration, invasion, and tumorigenesis [29]. Thus, a detailed characterization of Snail is considered an important breakthrough and provides new insights into the molecular mechanisms of tumor invasion [30, 31].

Aberrant expression of miR-22 might show effects on ovarian cancer metastasis [32]. But the actual mechanism remains unclear. In this study, our results showed that miR-22 played an important role in regulating metastasis in the human ovarian cancer cell line SKOV-3. In addition, we identified that SNAI1 was a direct target of miR-22. Furthermore, the expression of E-cadherin, a specific gene of epithelial cells, was positively correlated with miR-22.

Materials and methods

Cell culture and transfection

The human ovarian cancer cell line SKOV-3 was cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) with 10% calf serum (CS), 100 units/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA), and 100 mg/L streptomycin (Sigma) and grown at 37°C in an incubator containing 5% CO_2.

Transfection was performed with lipofectamine 2000 (Invitrogen) using miR-22 overexpression and suppression reagents (Invitrogen): miR-22 mimics and an miR-22 inhibitor, respectively, at a final concentration of 50 nmol/L, and the transfection efficiency was measured by quantitative RT-PCR.

Wound-healing motility assay

Transfected SKOV-3 cells were seeded into 6-well plates and allowed to grow to a 90% confluence. The confluent monolayers were scratched with a pipette tip, and the plates were washed twice with a fresh medium to remove non-adherent cells. Then, they were maintained and cultured for 12 h. Cells migrated onto the wound surface, and the distances of both surfaces were observed under an inverted microscope (Leica DMI-4000B, Wetzlar, Germany) equipped with a camera (Leica) at a designated time.

Transwell migration assay

The SKOV-3 cells were collected using 5 mM EDTA in PBS after transfection for 48 h. Then, cells at a density of 8×10^4 per well were cultured in a chamber with a serum-free medium, under which 8-μm PET (Millipore, Billerica, MA, USA) was inserted. These chambers were placed into 24-well plates. A complete medium containing 20% CS was added to the lower chamber. The transwells were then attached to the 24-well plate. After incubation for 12 h, the migrated cells were stained using a viola crystalline solution. Six random fields from each of the inserts were counted using phase contrast microscopy (Leica).

MTT assay

The assessment of cell viability was performed using an MTT assay. First, SKOV-3 cells were transfected with miR-22 mimics, the miR-22 inhibitor, and NC, respectively. The cells were seeded into 96-well tissue culture plates at a density of 5×10^3 cells per well in RPMI 1640 containing 10% CS and maintained at 37°C. The cells were continually cultured for 48 h. Then, 0.5 mg/mL of MTT was added to each

Table 1. Sequences of the primers used in this study

Primer name	Primer sequence (5'-3')
Snail-F	TCGGAAGCCTAACTACAGCGA
Snail-R	AGATGAGCATTGGCAGCGAG
E-cadherin-F	CGAGAGCTACACGTTCACGG
E-cadherin-R	GTGTCGAGGGAAAAATAGGCTG
GAPDH-F	AAGGTCGGAGTCATCGGATT
GAPDH-R	CTGGAAGATGGTGATGGGATT

well and incubated for 4 h at 37°C. Afterwards, formazan was dissolved in 150 μL DMSO. Absorbance was measured at 450 nm (λ).

Extraction of total RNA, reverse transcription PCR and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Briefly, for each sample, 2 μg of total RNA was reverse-transcribed using M-MLV (Promega, Madison, WI, USA) to synthesize the first-strand of cDNA following the standard protocols.

To detect the expression of mature miR-22, miR-22 primer and EzOmics SYBR qPCR kits were purchased from Biomics (Jiangsu, China). Amplification procedures were conducted as follows: 94°C for 5 min, followed by 30 cycles at 94°C for 30 s and 60°C for 30 s, and finally at 72°C for 10 min. To detect the expression levels of the Snail and E-cadherin genes, we used their respective primer sequences (listed in **Table 1**), which were all synthesized by Biomics. Amplification procedures were conducted as follows: 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 56°C for 45 s, and 72°C for 45 s, and finally at 72°C for 10 min. PCR product sizes and sequences were identified by a melting curve analysis, and the band sizes were confirmed by electrophoresis in 1.5% (wt/v) agarose gel with glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as the internal control.

Western blotting

SKOV-3 cells transfected with mimics, an inhibitor, and NC, respectively, and were harvested and lysed in a lysis buffer (Promega). Protein concentrations were determined by a Bradford protein assay (Bio-Rad, Berkeley, USA). Samples (120 μg) were subjected to SDS-PAGE, and proteins were transferred to polyvinylidene difluoride membranes (Millipore) and subjected to the standard western blot procedures. Antibodies against Snail and E-cadherin, respectively, were purchased from Cell Signaling Technology. Proteins were detected using the enhanced chemiluminescence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.), and the bands were quantified by density analysis using Quantity One software (BioRad).

Immunofluorescence assay

Cells transfected with miR-22 mimics, the miR-22 inhibitor, and NC, respectively, were seeded into a 6-well plate with glass coverslips. 48 h after transfection, the medium was removed and the cells in the indicated wells were fixed by 4% paraformaldehyde for 20 min and blocked with 3% BSA in PBS for 1 h at room temperature. They were subsequently blotted with Snail monoclonal antibody (1:1000) (Cell Signaling Technology) and then blotted with an FITC-conjugated secondary antibody for 1 h. After that, the fluorescent dye DAPI was used to stain nuclei for 1 h and confocal laser scanning (Carl Zeiss Microscopy, Jena, Germany) was used for observation.

Luciferase reporter assay

The sequences (listed in **Table 2**) that contain portions of the 3'UTR of SNAI1 mRNA with the wild-type and mutant binding sites for miR-22 and HindIII/SpeI were synthesized by Sangon Biotech Co., Ltd (Shanghai). They were cloned to a position, which was the downstream of the luciferase coding region in the pMiR-Report Fluc vectors (Ambion, Foster City, CA, USA). HEK 293T cells were seeded into 24-well plates and grew to approximately 40% confluence. Afterwards, the reporter plasmid was co-transfected using Lipofectamine 2000 (Invitrogen) with miR-22 mimics and NC, respectively. After transfection for 48 h, the cells were collected and assayed for luciferase activity using the Glomax 96 luminometer (Promega). Transfection efficiency was normalized through β-galactosidase activity.

Statistical analysis

All experiments were performed in triplicate. The data are shown as the mean ± SEM for each group and were assessed by analysis of

Table 2. Sequences of SNAI1-WT and SNAI1-Mut

Sequence name	Sequence (5'-3')
SNAI1-WT-F	CTAGTGGCAGACTAGAGTCTGAGATGCCCCGAGCCCAGGCAGCTATTTCAGCCTCCTGA
SNAI1-WT-R	AGCTTCAGGAGGCTGAAATAGCTGCCTGGGCTCGGGGCATCTCAGACTCTAGTCTGCCA
SNAI1-Mut-F	CTAGTGGCAGACTAGAGTCTGAGATGCCCCGAGCCCATAGCTTGCTTTCAGCCTCCTGA
SNAI1-Mut-R	AGCTTCAGGAGGCTGAAAGCAAGCTATGGGCTCGGGGCATCTCAGACTCTAGTCTGCCA

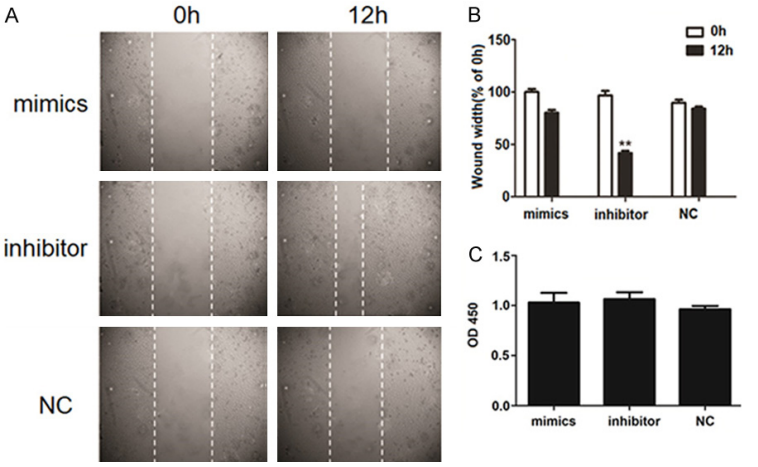

Figure 1. MiR-22 inhibited the migration of SKOV-3. A, B. Scratch wound migration assay. A. Movement of cells into the wound is shown for the miR-22 mimics, the inhibitor, and the negative control transfected cells at 0 and 12 h post scratch. B. The percentage of open space areas covered by the SKOV-3 cells transfected with miR-22 mimics was reduced to 47%, which was significantly lower than the percentage for the negative control (*P < 0.05, **P < 0.01 vs. negative control group). C. MTT assay of SKOV-3 cells after transfection with miR-22 mimics, inhibitor, or NC. Data were presented as the mean ± SEM, n = 3, *P < 0.05, **P < 0.01 vs. NC group.

variance (ANOVA). If this analysis indicated significant differences between the group means, then each group was compared with the normal control group (NC) by using the Dunnett t (2-sided) analysis. P < 0.05 was considered to be statistically significant.

Results

The influence of miR-22 on cell migration

Cell motility, used for determining the metastatic potential of cancer cells, is commonly investigated by a wound healing assay and a transwell migration assay. The relationship between miR-22 and motility of human ovarian cancer cells line SKOV-3 was examined by the above methods after treatment with mimics, an inhibitor, and NC, respectively. **Figure 2C** shows the up- and down-regulation of miR-22 expression. Confluent monolayers of cells were scratched and then cultured for 12 h. As shown in **Figure 1A** and **1B**, the up-regulation of miR-22 led to a significant decrease of wound healing cell migration in the miR-22 mimics-transfected group copared with that in the NC-transfected group and miR-22 inhibitors-transfected group, respectively. Meanwhile, the transwell migration assay displayed a similar result (**Figure 2A**, **2B**). SKOV-3 cells transfected with miR-22 mimics led to significantly decreased metastasis compared with the NC-transfected group and the miR-22 inhibitors-transfected group, respectively. Both the wound healing and transwell migration assays showed that miR-22 had an inhibitory effect on SKOV-3 cell migration. In addition, the MTT assay results showed that there was no significant difference between the miR-22 mimics-transfected group and the inhibitors-transfected group (**Figure 1C**). This confirmed that the inhibitory effect of miR-22 on SKOV-3 cell metastasis observed in the wound healing and transwell migration assays.

Identification of EMT related protein snail as a target of miR-22

It is generally accepted that miRNAs exert their functions by regulating the expression of their target genes. To elucidate the inhibitory mechanism of miR-22 on tumor migration, we used the TargetScan program (version 6.0) to search for any potential direct target genes of miR-22. One of the predicted genes SNAI1 was found to contain a complementary site for the seed region of miR-22 in its 3'-UTR (**Figure 3A**). Snail, a zinc-finger transcription factor encoded by SNAI1, promotes motility and invasion in cancer cells by inducing EMT [33]. Western blotting, an immunofluorescence assay, and a luciferase reporter assay were performed to validate whether SNAI1 was a target of miR-22.

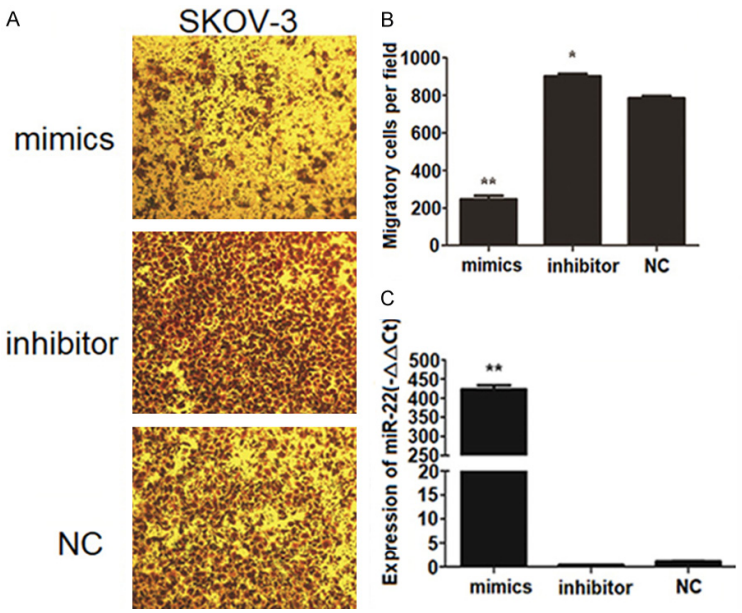

Figure 2. MiR-22 inhibited the migration of SKOV-3. A, B. Transwell migration assays. A. After initial equilibrium, the SKOV-3 cells transfected with the mimic, inhibitor and negative control were suspended in a fresh medium without calf serum, and then added to the inserts. Twelve hours after seeding, the cells on the lower surface of the inserts were fixed and stained. Six visual fields of each insert were randomly counted under a microscope. B. The migratory cell numbers of the SKOV cells transfected with miR-22 mimics were significantly less than the numbers of cells transfected with the negative control and the inhibitor. The percentage reduction was nearly 69%. C. The expression of miR-22 in SKOV-3 cells transfected with miR-22 mimics, inhibitor, or NC. Data were presented as the mean ± SEM, n = 3, *P < 0.05, **P < 0.01 vs. NC group.

First, we utilized western blotting to investigate the effects of miR-22 on Snail expression. As shown in **Figure 3C** and **3D**, the expression of Snail was significantly reduced in the miR-22 mimics-transfected group compared with the NC-transfected group ($P < 0.05$). In contrast, the expression of the Snail protein in the miR-22 inhibitor-transfected group was increased. To further determine whether miR-22 might down-regulate the expression of Snail, an immunofluorescence assay was used. Cells transfected with miR-22 mimics produced a significant reduction of green fluorescence under the confocal laser microscope (Carl Zeiss) (**Figure 4**). Cells transfected with the negative control showed only background staining. These results support the hypothesis that Snail is a target of miR-22.

miR-22 repressed snail expression through direct interaction with a conserved target site in the 3'-UTR of SNAI1 mRNA

In the luciferase reporter assay, a human SNAI1 3'UTR fragment containing a wild-type or mutant-type miR-22 binding site was cloned into the downstream of the firefly luciferase reporter gene. As a result, the relative luciferase activity of the reporter containing wild-type SNAI1 3'-UTR was found to be suppressed to nearly 50%. In contrast, there was no significant difference between the reporter containing mutant SNAI1 3'-UTR and the NC-reporter (**Figure 3B**). These results strengthened the hypothesis that miR-22 down-regulates the expression of SNAI1 by binding to the 3'UTR of SNAI1. Overall, SNAI1 was shown to be a direct target of miR-22.

The effect of miR-22 on E-cadherin expression

The adhesion protein E-cadherin, a downstream target of the transcription factor Snail, plays a central part in the process of EMT. The down-regulated expression of E-cadherin causes cells to develop a metastatic potential [30, 34]. Our results showed that the expression of E-cadherin was up-regulated at the mRNA and protein levels in cells treated with miR-22 mimics (**Figure 5**). The down-regulation of miR-22 revealed an opposite result. These findings implied that miR-22 exhibits a positive correlation with E-cadherin expression and inhibits the EMT process in SKOV-3 cells.

Discussion

It is well known that miRNAs are involved in many steps of tumorigenesis, including proliferation, differentiation, apoptosis, and metastasis [10, 35]. Recent studies on the profiles and the functions of miRNA in cancers provide valuable information on the molecular pathogenesis of several tumor types like those of ovarian, breast, lung, colorectal, and prostate cancers [36-40]; as well as those of hepatocellular carcinoma and glioblastoma [41-43]. MiR-22, locates on the arm of chromosome 17 and is highly conserved across many vertebrate species. However, whether miR-22 is a proto-

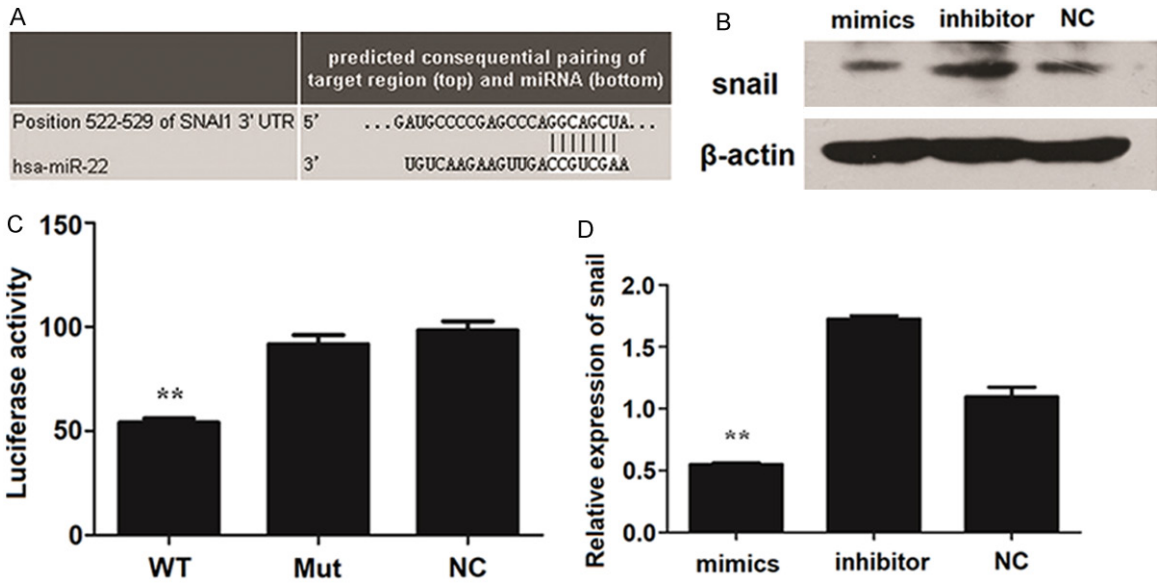

Figure 3. MiR-22 directly targeted SNAI1 mRNA. A. SNAI1 was one of the putative targets of miR-22 predicted by Targetscan 6.0. B. Vectors containing the wild-type or mutant miR-22 binding site from the 3'UTR of SNAI1 mRNA, and miR-22-mimics or NC, were co-transfected into HEK-293T cells. 48 hours later, the fluc activity in the cells was measured and normalized through β-galactosidase activity. Data are presented as the mean ± SEM, n = 3, *P < 0.05, **P < 0.01 vs. NC group. C. SKOV-3 cells were transfected with miR-22-mimics, an inhibitor, and control miRNA. 72 h after transfection, Snail protein and β-actin were detected by western blot. D. Quantitative data of densitometry analyses. The ratio of Snail protein to β-actin was displayed as the mean ± SEM, n = 3, *P < 0.05, **P < 0.01 vs. NC group.

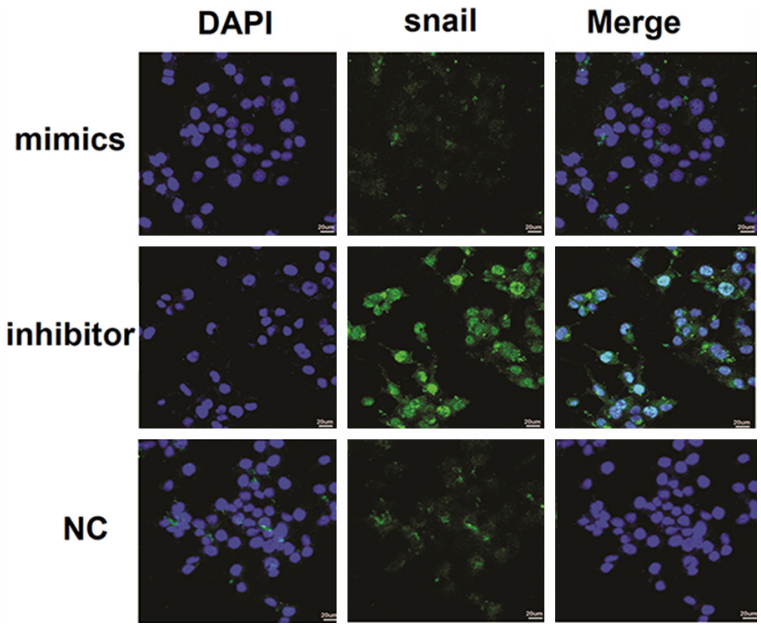

Figure 4. Immunofluorescence analysis of snail expression. MiR-22-mimics, inhibitor, and NC were transfected into SKOV-3. Green, Snail was immunestained with anti-snail; blue, nuclei were stained with DAPI.

oncogene or an anti-oncogene is still being debated. miR-22 has been shown to regulate PTEN expression through translational repression and increase cell motility in transformed the human bronchial epithelial cell line 16HBE-T [23]. In addition, miR-22 also functions as a tumor suppresor. Histone deacetylase 4 (HDAC4), which plays a critical role in cancer development, has been demonstrated to be a target of miR-22 [44]. The c-Myc binding protein (MYCBP) promotes cancer development through the induction of c-Myc transcription. But these effects could be abrogated by miR-22 through its binding to MYCBP [45]. A recent study showed that miR-22 repressed cell mobility by targeting EVI-1 in metastatic breast cancer cells [46]. J. Li *et al*. reported that miR-22 inhibited cell migration and invasion in the ovarian cancer cell lines SKOV-3 and SKOV-3ip [32]. In addition, it has been reported

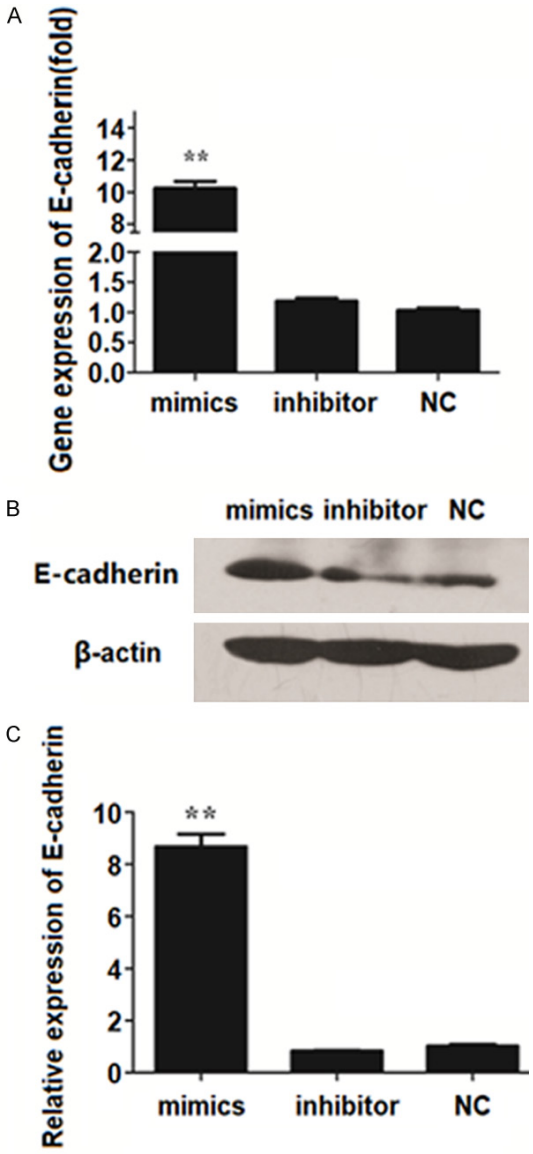

Figure 5. miR-22 regulated the expression of E-cadherin. A. Expression of the E-cadherin gene in the SKOV-3 cells transfected with mimics, inhibitor, and NC was measured by quantitative RT-PCR. Data were presented as the mean ± SEM, n = 3, *P < 0.05, **P < 0.01 vs. NC group. B. The SKOV-3 cells were transfected with miR-22-mimics, an inhibitor, and NC. 72 h after transfection, E-cadherin protein and β-actin were detected by western blot. C. Quantitative data of densitometry analyses. The ratio of E-cadherin protein to β-actin was displayed as the mean ± SEM, n = 3, *P < 0.05, **P < 0.01 vs. NC group.

that miR-22 functions as a tumor suppressor by targeting SIRT1 in renal cell carcinoma and breast cancer [22, 47, 48]. And miR-22 inhibits lung cancer cell and bladder cancer EMT and invasion by targeting Snail [49, 50]. However, the regulatory mechanisms of miR-22 in ovarian cancer is still unclear.

In this study, the results of gain- and loss-of-function experiments indicated that miR-22 was inversely correlated with the migration abilities of the ovarian cancer cell line SKOV-3. When cells were transfected with miR-22 mimics, their migration ability was significantly repressed in comparison to cells transfected with the miR-22 inhibitor or NC (**Figures 1**, **2**). Then we explored how miR-22 inhibited metastasis. We identified SNAI1 to be a direct target of miR-22 (**Figures 3**, **4**). Meanwhile, Snail siRNA suppressed the migration the of ovarian cancer cell line SKOV-3 (data not shown), which further confirmed our results.

Tumor metastasis is the most prominent problem in the clinical treatment of cancer, as most cancer-related deaths are associated with disseminated disease rather than the primary tumor. The processes of metastasis involve the following sequential steps: cell motility, tissue invasion, intravasation, dissemination through the blood or lymph nodes, extravasation, and finally, proliferation at a new site [51, 52]. The two leading theories about the origin of metastasis are the EMT hypothesis and the cancer stem cell hypothesis. Epithelial cancer cells that undergo EMT display properties, which can be all but indistinguishable from those of cancer stem cells. The EMT hypothesis and the cancer stem cell hypothesis are complementary to some extent [53]. Under normal circumstances, EMTs emerge in the phase of embryonic development in many organisms. During this period, cells acquire a morphology that enables them to migrate into the extracellular environment. An increasing number of studies suggest that EMT plays a significant role in metastatic tumor formation [26, 54, 55]. EMT is induced by several oncogenic pathways (peptide growth factors, Src, Ras, Ets, integrin, Wnt/beta-catenin, and Notch) [56]. One important marker for identifying EMT is the loss of E-cadherin. When the expression of E-cadherin is down-regulated, cell motility is induced through either the activation of the Wnt/β-catenin signaling pathway or cooperation with the TGFβ/Smad signaling pathway [57, 58]. Our results showed that the expression of E-cadherin was up-regulated by miR-22 (**Figure 5**), indicating that miR-22 plays an important role in EMT regulation.

Studies have shown that the zinc-finger protein snail promotes EMT development, metastasis, and organ fibrosis in many cancers, including

ovarian carcinoma, lung carcinoma, breast carcinoma, and colon carcinoma [59]. Huber and his colleagues reported that Snail repressed the transcription activity of E-cadherin by binding to the E-box of the E-cadherin promoter [57]. According to our results, miR-22 inhibited EMT development and up-regulated E-cadherin expression by targeting SNAI1 in the ovarian cell line SKOV-3.

In conclusion, we found that miR-22 plays a vital role in ovarian cancer cell metastasis. The aberrant expression of miR-22 increases the metastatic activities of ovarian cancer cells, demonstrating that miR-22 is a negative regulator in ovarian cancer metastasis. Moreover, we confirmed the negative correlation between miR-22 and SNAI1 and identified SNAI1 as a direct target of miR-22. Additionally, our results suggest that miR-22 regulates EMT activity by up-regulating E-cadherin expression. Our studies provide new insights into the mechanisms underlying the effects of miR-22 on the inhibition of ovarian cancer metastasis.

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Disclosure of conflict of interest

None.

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