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Long noncoding RNA DARS-AS1 acts as an oncogene by targeting miR-532-3p in ovarian cancer

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Abstract. – OBJECTIVE: Ovarian cancer is one of the most ordinary malignant tumors. Recently, the role of long noncoding RNAs (lncRNAs) in tumor progression has caught attention of numerous researchers. In this research, lncRNA DARS-AS1 was studied to identify how it functions in the development of ovarian cancer.

PATIENTS AND METHODS: DARS-AS1 expression was detected by Real-time quantitative polymerase chain reaction (RT-qPCR) in ovarian cancer tissue samples. Moreover, functional experiments were conducted to detect the effect of DARS-AS1 on the proliferation and metastasis of ovarian cancer. In addition, the underlying mechanism was explored through luciferase assay and RNA immunoprecipitation (RIP) assay.

RESULTS: In this study, DARS-AS1 expression was remarkably higher in ovarian cancer tissues compared with that in adjacent ones. Cell proliferation was inhibited after DARS-AS1 silenced in ovarian cancer cells. Moreover, cell migration and invasion were also inhibited after DARS-AS1 silenced in ovarian cancer cells. Furthermore, results of luciferase assay and RIP assay showed that microRNA-532-3p (miR-532-3p) was a direct target of DARS-AS1 in ovarian cancer. The expression of miR-532-3p was upregulated after DARS-AS1 was knocked down.

CONCLUSIONS: Our study suggests that DARS-AS1 enhances cell proliferation and metastasis via sponging miR-532-3p in ovarian cancer.

Key Words:
Long noncoding RNA, DARS-AS1, Ovarian cancer, miR-532-3p.

Introduction

Ovarian cancer is one of the most fatal gynecologic malignancies and is also the fifth leading cause of death among malignant cancers[1]. The high mortality of ovarian cancer is associated with the high occurrence of drug resistance and metastasis[2,3]. Therefore, it is urgent to find out new strategies in early detection of these patients and establish new therapeutic techniques for successful intervention.

Recent studies have revealed that long non-coding RNAs (lncRNAs) act as a vital role in a variety of cellular activities of malignant tumors. For example, lncRNA SNHG7 enhances tumor growth ability in osteosarcoma by sponging miR-34[illegible] LncRNA SNHG16 inhibits cell apoptosis in papillary thyroid cancer[5]. LncRNA FENDRR acts as a tumor suppressor[6] in non-small cell lung cancer by targeting miR-761. Silence of lncRNA MEG3 promotes glioma proliferation and metastasis *via* regulating phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway[7].

In this study, we found out that the expression of DARS-AS1 was remarkably higher in ovarian cancer tissues. Moreover, DARS-AS1 promoted the proliferation, migration and invasion of ovarian cancer *in vitro*. In addition, we further found that the function of DARS-AS1 in ovarian cancer was also associated with microRNA-532-3p (miR-532-3p).

Patients and Methods

Tissue Specimens

52 cases of ovarian cancer tissues and their adjacent tissues were collected from patients who received surgery at Chinese PLA General Hospital. No chemotherapy or radiotherapy was received before surgical resection. All cases were diagnosed with ovarian cancer by two independent pathologists without any controversial. Writ-

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ten consent form was obtained from each patient. This study was approved by the Ethics Committee of Chinese PLA General Hospital.

Cell Culture

3 cancer cell lines (A2780, SKOV3 and OVCAR-3), and normal ovarian cell (ISOE80) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were maintained in 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) as well as 100 U/mL penicillin 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Besides, cells were cultured in an incubator containing 5% CO_2 at 37°C. Cell culture medium was refreshed every two days.

Cell Transfection

Specific shRNA against DARS-AS1 was designed and synthesized by Invitrogen (Carlsbad, CA, USA). Negative control shRNA was also synthesized. To establish cell lines with stable knockdown of DARS-AS1, SKOV3 cells were transfected with DARS-AS1 or negative c shRNA through Lipofectamine 3000 reag vitrogen, Carlsbad, CA, USA) according manufacturer's protocol.

RNA Extraction and Real-Tim Quantitative Polymerase Ch tion (RT-qPCR)

Total RNA was extracted fr ultur cancer cells or patients' tu y using TRIzol reagent (TaKaRa B Inc., iga, Japan) and then reverse-tra bed to cD u-gh reverse Transcripti it (TaKaRa B nc., Otsu, Shiga, Japan). performed three times in the follow equ denaturation at 95°C for 1 min followed by at 95°C for 15 s, 60°C f 0 s, and 72°C f 0 s. $2^{-\Delta\Delta Ct}$ method was ut d for lculating relative expression. The p r se ces used for RT-qPCR were as fol AS1 fo rd: 5′- AGCCA-AGGACTGC TT-3′ reverse: 5′-CT-GTACTGGTGGC 3′; glyceraldehyde 3-pho ehydrog (GAPDH), forward: 5′- C CAAGGCT GAGAAC-3′ and reverse TGG CCAGTGGA -3′.

Ce ration Assay

Cell was monitored with Cell Counting Kit-8 ( ) assays (Dojindo, Kumamoto, Japan). Briefly, 2×10³ SKOV3 cells were seeded and incubated in 96-well pla The-se cells were incubated wit RS-A NA for 24 h and then incuba with 10 μ K-8 for 3 h. The absorban measured 450 nm with an ELISA rea m (M kan Ascent, LabSystems Helsinki

Colony Format Ass

To detect the g-ter ffect f DARS-AS1 on ovarian ca iferat colony formation assay were ed. 2 SKOV3 cells/well we aced in ate and culture medium eplaced every 7 day later, colonies we ith 75% ethanol for 30 min and staine th ystal violet. Colonies were phot graphed an

ynyl Deoxyuridine (EdU) Incorporation Assay

dU Kit (Ri o, Guangzhou, China) was for dete g cell proliferation of tran- 10³ transfected cells were cultur well plates. Next, each well was added with 50 μM EdU labeling medium incu- ted for 2 h at 37°C. Finally, cells were stained EdU working solution. Hoechst 33342 s used to label cell nuclei. Representative otograph was taken by a fluorescent microscope (Olympus, Tokyo, Japan).

ranswell Assay

Transwell chambers with 8 μm pores were provided by Corning (Corning, NY, USA). Cells were then seeded into the upper chambers of a 24-well plate. 20 % FBS-DMEM was added to the lower chamber of the culture inserts. After cultured for 24 h, these inserts were fixed with methanol for 30 min and stained by hematoxylin for 20 min. The number of migrated cells was counted by a light microscope (Olympus, Tokyo, Japan). For the transwell invasion assays, the membrane was precoated with 50 μL Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Next, the experiments were the same as described above.

Scratch Wound Assay

1.0×10⁴ cells were seed into a 6-well plate in each well. Three parallel lines were made on the back of each well. After growing to about confluent of 90%, cells were scratched with a pipette tip and cultured in a medium. Cells were photographed under a light microscope after 0 and 48 h. Each assay was independently repeated in triplicate.

Luciferase Reporter Gene Assay

DIANA LncBASE Predicted v.2 was used to predict the potential target gene and fragment sequences containing DARS-AS1 reaction sites. The DARS-AS1 3′-UTR wild-type (WT) sequence named DARS-AS1-WT was 5′-UCUCACUCAAAAGUGGGAGU-3′ and the mutant sequence of DARS-AS1 3′-UTR missing the binding site with miR-532-3p named DARS-AS1-MUT was 5′-UCUCACUCAAAACACCCUCU-3′. Luciferase reporter gene assay kits (Promega, Madison, WI, USA.) were used to detect the luciferase activity of ovarian cancer cells. The luciferase reporter gene vector was constructed, and SKOV3 cells were transfected.

RNA Immunoprecipitation (RIP) Assay

To confirm the endogenous relationship between DARS-AS1 and miR-532-3p, RIP assay was carried out using the EZMagna RIP RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA). Treated SKOV3 cells were collected and lysed using RIP lysis buffer containing protease inhibitor and RNase inhibitor. Cells were incubated with the RIP buffer c… ning magnetic beads coated with Ago2 an… (Millipore, Billerica, MA, USA). IgG acte… negative control (input group). After incuba… for 2 h at 4°C, co-precipitated RNAs were isolat… and measured by RT-qPCR analysis…

Statistical Analysis

All statistical analyses w… perfo… Statistical Product and Serv… (SPSS) 21.0 (IBM Corp., Armonk, N… dependent-sample *t*-test was used … mpar… ffe-rence between the two gr… Moreover, …05 was considered to indic… tatistically …ifi-cant difference.

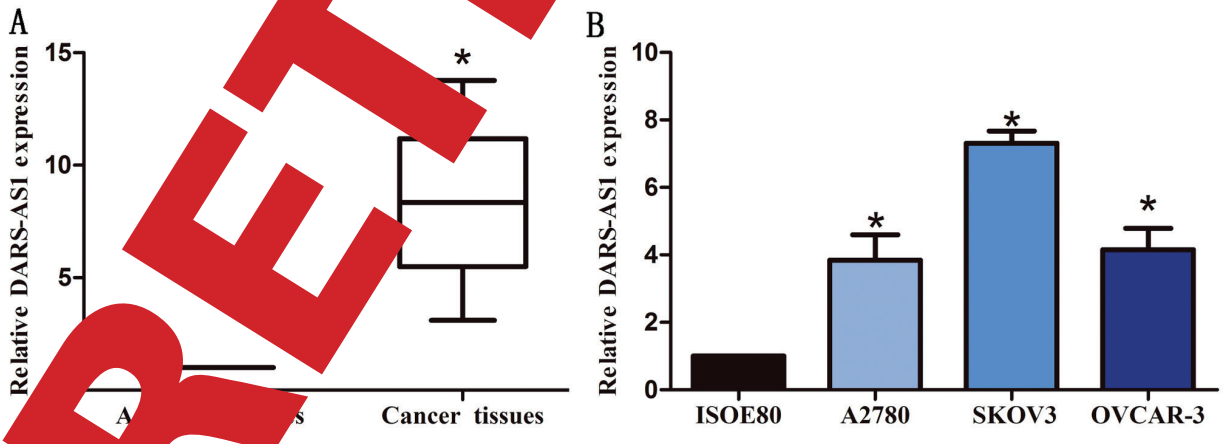
Result…

DARS-AS1 E… …evel … Ovarian Cancer Tissues …lls

First, RT… CR wa… … for detecting DARS-AS… xpression in … ients' tissues and 3 ovaria… ell lines. As a result, DARS-AS1 was si… ca… ulated in tumor tissue samples (Figure 1A). … S1 level of ovarian cancer cel… s higher than t… f ISOE80 (Figure 1B).

…ockdown of DARS-AS1 Inhibited Cell …liferation i… KOV3 Ovarian …cer Cells

… e chose SKOV3 cell line for the kn… DARS-AS1. Next, RT-qPCR was utilized for detecting the DARS-AS1 expression (Figure 2A). Moreover, CCK-8 assay showed … ll growth ability of SKOV3 cells was … ntly repressed after DARS-AS1 was …ocked down (Figure 2B). Colony formation …ssay showed that the colonies were significantly …educed after DARS-AS1 was knocked down (Fi…re 2C). Furthermore, EdU incorporation assay …lso showed that EdU positive cells were reduced after knockdown of DARS-AS1 in SKOV3 cells (Figure 2D).



Figu… …ssion levels of DARS-AS1 were increased in ovarian cancer tissues and cell lines. ***A,*** DARS-AS1 expression was signifi… …eased in the ovarian cancer tissues compared with adjacent tissues. ***B,*** Expression levels of DARS-AS1 relative to GA… …re determined in the human ovarian cancer cell lines and ISOE80 by RT-qPCR. Data are presented as the mean ± standar… error of the mean. *$p<0.05$.

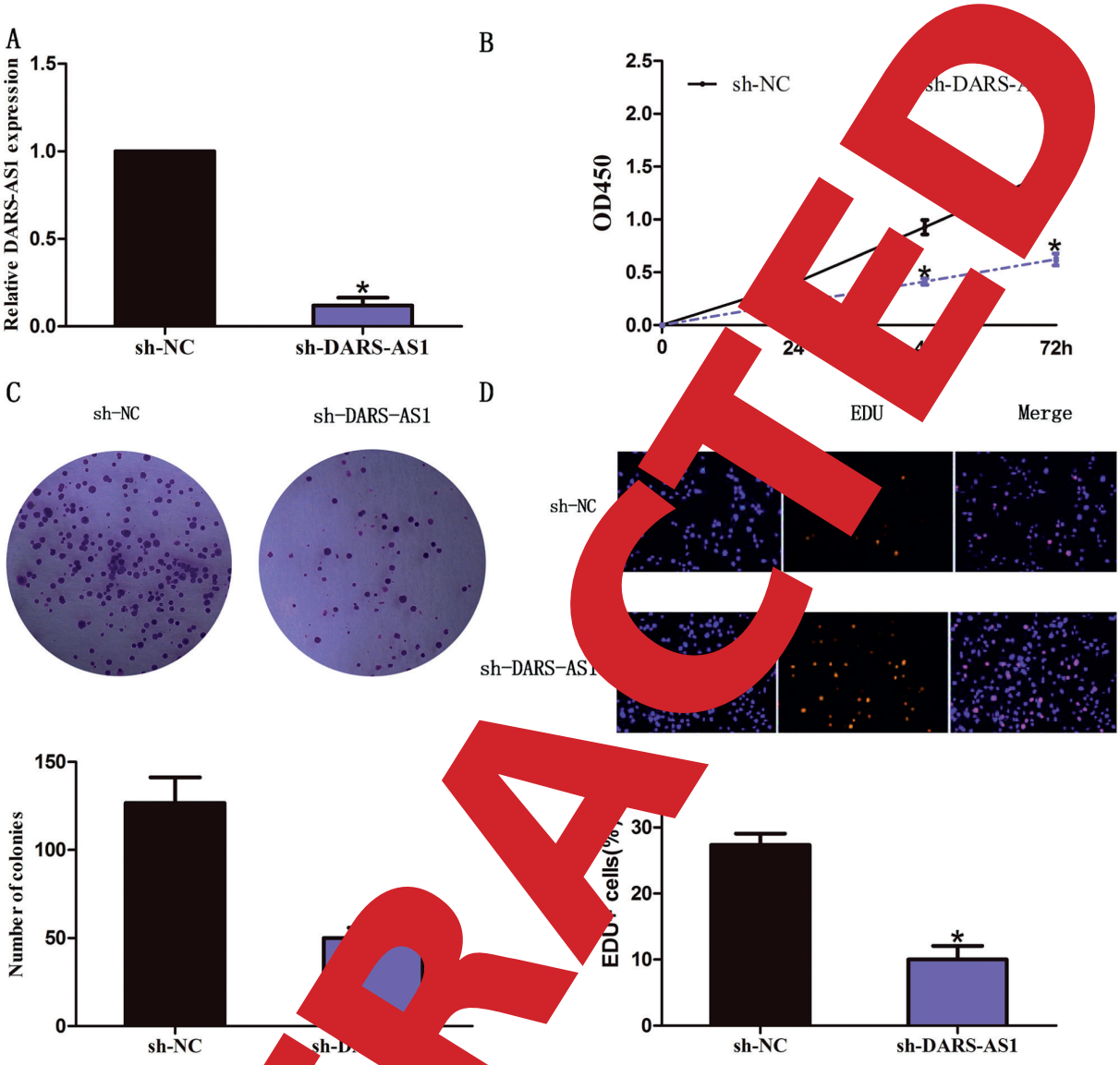


Figure 2. Knockdown of D -AS1 inhibited S ovarian cancer cell proliferation. ***A,*** DARS-AS1 expression in SKOV3 ovarian cancer cells trans DARS-AS1 shRNA (sh-DARS-AS1) and negative control shRNA (sh-NC) was detected by RT-qPCR. GAPDH w ternal control. ***B,*** CCK-8 assay showed that knockdown of DARS-AS1 significantly inhibited cell growth i OV3 cer cells. ***C,*** Colony formation assay showed that the number of colonies was reduced after knockdown of DARS-A V3 cells (magnification: 10×). ***D,*** EdU incorporation assay showed that EdU positive cells were ed after knockdo DARS-AS1 in SKOV3 cells. The results represent the average of three independent experime mean ± standard error of the mean). *$p<0.05$, as compared with the control cells.

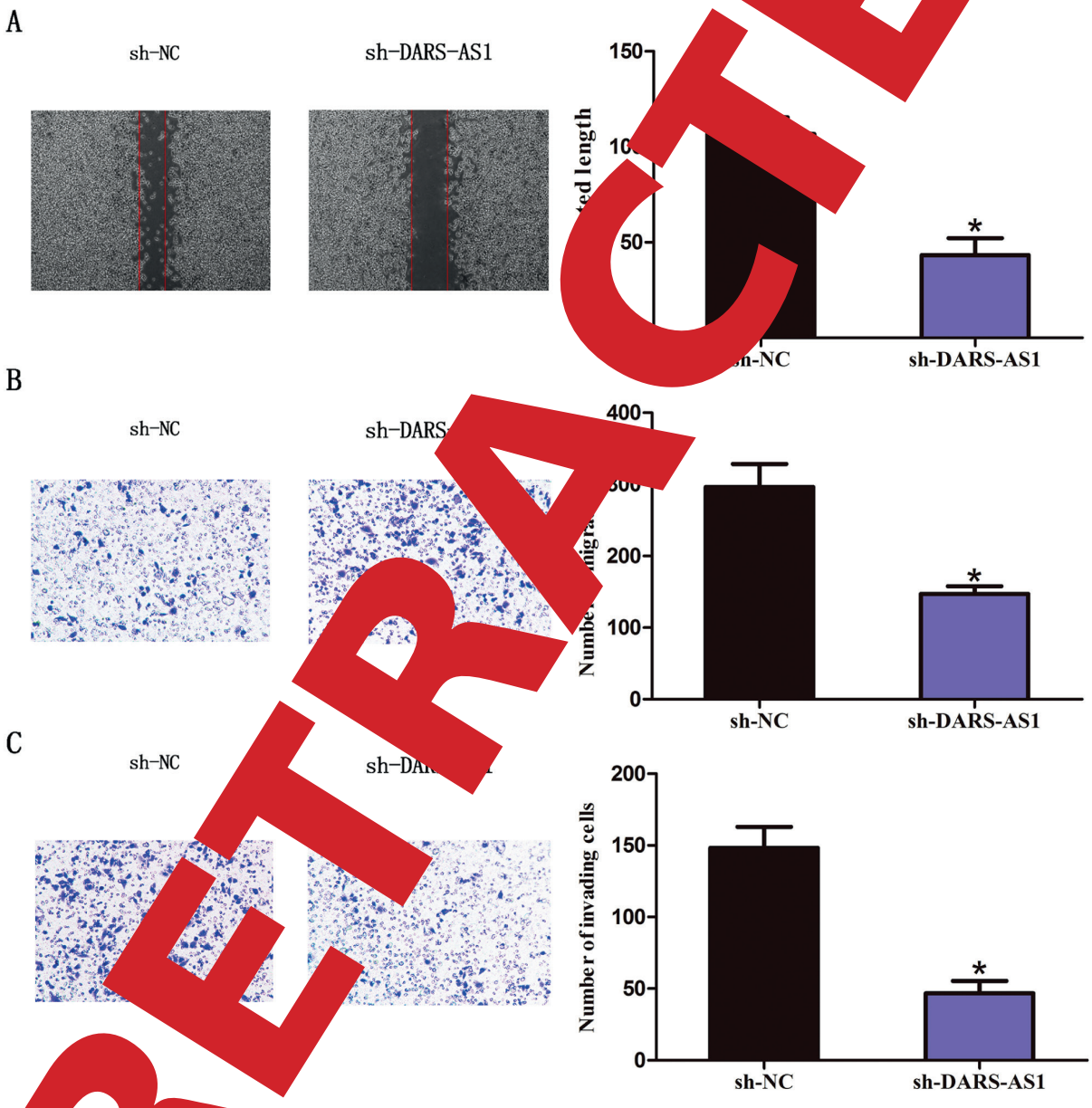
Knockdown of Inhibited Cell Migr nd Inva n SKOV3 Ov cer Cells

atch w howed that the migrated l f S was significantly decreased RS-AS1 was knocked down (Figure 3A). ll assay showed that the number of migrated was significantly reduced after DARS-AS1 was knocked down (Figure 3B). Transwell invasion assay showed that the number of invaded cells was significantly reduced after DARS-AS1 was knocked down (Figure 3C).

The Interaction Between miR-532-3p and DARS-AS1 in Ovarian Cancer

Starbase v2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php) was used to find the miRNAs that contained complementary base with DARS-AS1.

MiR-532-3p was selected from these miRNAs, which were interacted with DARS-AS1 (Figure 4A). RT-qPCR assay showed that the expression of miR-532-3p was higher in DARS-AS1/shRNA cells than that in control cells (Figure 4B). Furthermore, the luciferase assay revealed that co-transfection of DARS-AS1-WT and miR-532-3p largely decreased the lucife while co-transfection of DARS-A iR-532-3p had no effect on luciferase ity either (Figure 4C). Mea le, RIP assa lso identified that miR-532-5 be rem bly enriched in the DARS AS1 g with control group (Figu D).



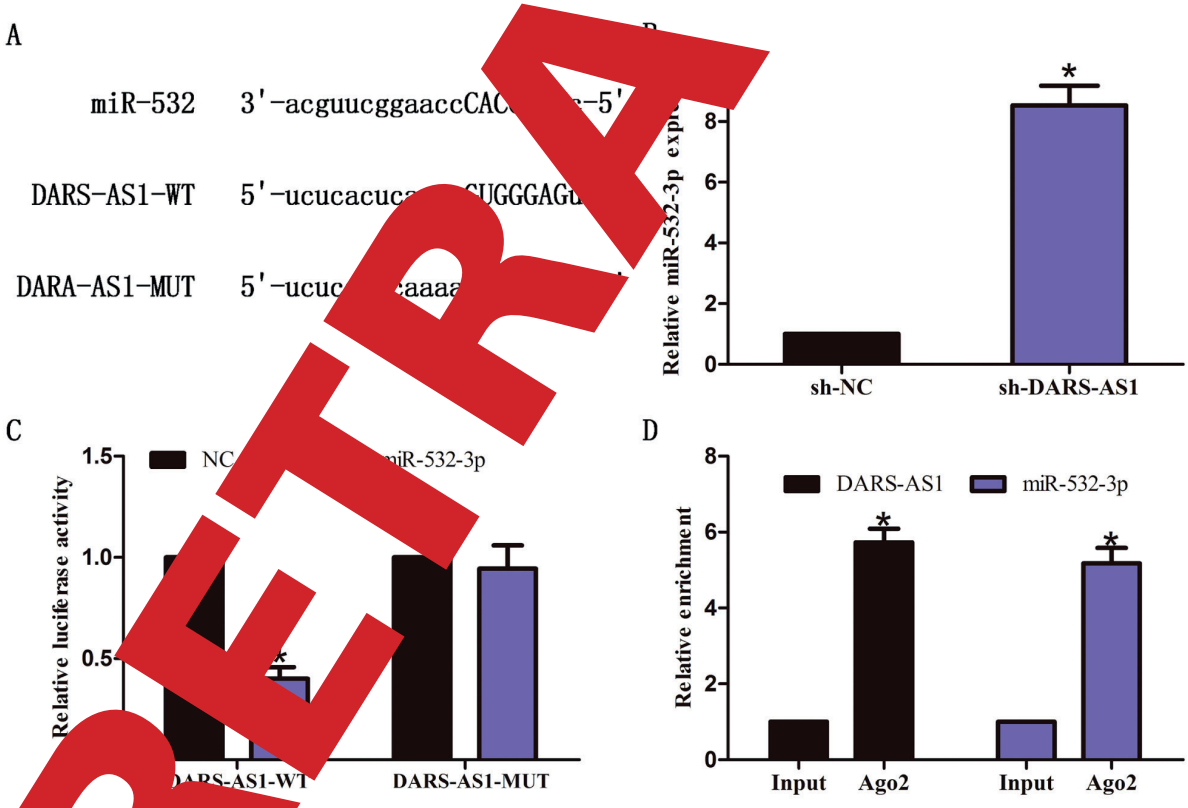
F . K RS-AS1 inhibited SKOV3 ovarian cancer cell migration and invasion. ***A,*** Scratch wound assay sh migrated length of SKOV3 cells was significantly decreased after DARS-AS1 was knocked down (magnification: 4 swell assay showed that number of migrated cells was reduced after knockdown of DARS-AS1 in SKOV3 cells (magn 0×). ***C,*** Transwell invasion assay showed that number of invaded cells was reduced after knockdown of DARS-AS1 in cells (magnification: 40×). The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with the control cells.

Discussion

Ovarian cancer is one of the most fatal gynecologic malignancies and the fifth leading cause of death in cancer. Most ovarian cancer cases are diagnosed at a late stage due to atypical or absent symptoms at early stage. Plenty of lncRNAs have been revealed to play important roles in oncogenesis and progression of ovarian cancer. For example, silence of lncRNA MNX1-AS1 suppresses cell proliferation and migration of ovarian cancer, which may be a potential target for ovarian cancer[8]. LncRNA TUG1 promotes epithelial ovarian cancer cell proliferation and invasion via the WNT/beta-catenin pathway[9]. LncRNA BACE1-AS inhibits the proliferation and invasion of ovarian cancer stem cell and functions as a novel target for treating ovarian cancer[10]. Besides, lncRNA ElncRNA1 functions as an oncogene in the proliferation of epithelial ovarian cancer cells which is upregulated by estrogen[11].

LncRNA DARS antisense R… AS1), located in 2q21.3, is a nove… RNA … ered recently. Its role in cance… velopment … not been studied. Our study … uncovered … association between DARS-… ovaria… ncer progression. The resu… demon… ARS-AS1 was upregulat… n ovarian … amples and cells. Besides… er DA… -AS1 wa… knocked down, ovarian … r cel… olifera…n was inhibited. Meanwh… RS-A… was knocked down, ovari… cane… migra… and invasion were found … be inhib… e data indicated that DAR… S1 promoted … rigenesis of ovarian can… ight act as an oncogene.

Bio…rma… ware predicted miR-532-3p as a possible tar… DARS-AS1. MiR-532-3p fu… ns as a tumor … ppressor in a diverse of …ors by regulating various biological processes[12,13]. For examp…, miR-532-3p is downregulated …bladder cance… d inhibits tumor development …gh targeti… Wnt signaling pathway[14]. MiR-



F… 4. R… sion between DARS-AS1 and miR-532-3p. ***A,*** The binding sites of miR-532-3p on DARS-AS1. ***B,*** …3p expression was increased in sh-DARS-AS1 group compared with negative control shRNA (sh-NC) group. ***C,*** Co… of miR-532-3p and DARS-AS1-WT strongly decreased the luciferase activity, while co-transfection of negative co… and DARS-AS1-WT did not change the luciferase activity. ***D,*** RIP assay identified that DARS-AS1 and miR-532-3p we… ficantly enriched in Ago2-containing beads compared to input group. The results represent the average of three independe… experiments. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

532-3p could induce cell apoptosis of lung adenocarcinoma cancer[15]. Recently, high expression of miR-532-5p is associated with better prognosis in ovarian cancer[16]. Besides, miR-532-3p was reported to repress cell growth and invaded ability in ovarian cancer[17].

In the present study, miR-532-3p could be directly targeted by DARS-AS1 through a luciferase assay. Moreover, miR-532-3p expression could be upregulated through knockdown of DARS-AS1. Furthermore, miR-532-3p was significantly enriched by DARS-AS1 through RIP assay. All these results showed that DARS-AS1 could work as a miR-532-3p sponge in ovarian cancer.

Conclusions

We demonstrated that DARS-AS1 could enhance ovarian cancer cell proliferation and metastasis through sponging miR-532-3p. These findings implied that lncRNA DARS-AS1 could act as a prospective therapeutic target for ovarian cancer.

Conflict of Interest

The Authors declare that they have no conflict of inter

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