Original Article
Mechanism underlying the regulation of lncRNA ACTA2-AS1 on CXCL2 by absorbing miRNA-532-5p as ceRNA in the development of ovarian cancer

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Abstract: Objective: To explore the mechanism underlying the regulation of long non-coding RNA (LncRNA) ACTA2-AS1 on CXCL2 as a ceRNA of miR-532-5p in the progression of ovarian cancer (OC). Methods: A qRT-PCR assay was carried out for analyzing the expression changes of ACTA2-AS1, miR-532-5p, as well as CXCL2 in OC tissues and corresponding healthy paracancerous tissues HOSEpiC (human ovarian epithelial cells), and OC cells. OC cells were grouped and transfected, and the fluorescent in situ hybridization was adopted for evaluating ACTA2-AS1 in the cells. Additionally, a dual luciferase reporter (DLR) assay was carried out for verifying the correlation of ACTA2-AS1 with miR-532-5p and of miR-532-5p with CXCL2. Cells were transfected with si-ACTA2-AS1, miR-532-5p, or CXCL2 overexpression plasmids, and then the cell proliferation, invasion, and apoptosis were determined using MTT, Transwell, and flow cytometry assays, respectively. Results: Compared with paracancerous tissues and HOSEpiC cells, OC tissues and cells showed increased ACTA2-AS1 and CXCL2 expression and decreased miR-532-5p expression (all P<0.05). ACTA2-AS1 acted as ceRNA in OC by negatively regulating miR-532-5p. Additionally, upregulating ACTA2-AS1 intensified the proliferation and invasion of cancer cells and suppressed their apoptosis (all P<0.05), and inhibition of it resulted in opposite results. In contrast, overexpressing miR-532-5p suppressed the proliferation, invasion, and clone formation of the cells and promoted their apoptosis (all P<0.05). The effect of ACTA2-AS1 on OC cells can be partially reversed by overexpressing miR-532-5p. Moreover, CXCL2, positively correlated with ACTA2-AS1 in expression (P<0.0001, r=0.7385), was the target of miR-532-5p, and its overexpression could partially offset the influence of miR-532-5p on OC cells. Conclusion: LncRNA ACTA2-AS1 can act as a tumor promoter in OC by absorbing miR-532-5p as ceRNA and regulating CXCL2, and ACTA2-AS1 inhibitor is expected to play a role in targeted therapy of OC.

Keywords: ACTA2-AS1, miR-532-5p, ovarian cancer, invasion, proliferation, apoptosis

Introduction

Ovarian cancer (OC) is a prevalent malignancy in gynecology and the fifth major cause of female death [1]. There are over 230,000 people diagnosed with the cancer each year. A majority of patients with OC are already in a later stage at the time of diagnosis due to the atypical or asymptomatic feature of early OC. Moreover, OC is highly heterogeneous and metastatic, so patients may respond well to chemotherapy at the beginning, but they are likely to show chemotherapy resistance and recurrence at the late stage and poor prognosis [2]. Therefore, it is of great significance to explore the pathogenesis of OC and find an effective treatment for it.

There is evidence that the dysregulated IncRNAs is related to the progression of tumors including OC [3-5]. For example, long non-coding RNA (IncRNA)-NORAD is up-regulated in cases with epithelial OC, and its knockout can effectively reduce the malignant activity of OC cells [6]. LncRNA UCA1 can promote the propagation of oncolytic vaccinia viruses, thus benefiting the treatment of OC [7]. Additionally, the abnormal overexpression of IncRNA-MALAT1 is related to a high OC stage, OC recurrence, and poor prognosis [8]. These findings suggest that
the pathologic process of OC is linked to the abnormal regulation of several lncRNAs. According to reports, ACTA2-AS1 with high expression in malignant cervical cancer tissues, promotes the invasion and proliferation of the cancer cells and inhibits the cell apoptosis, and knockout of it can strongly suppress the progression of the cancer [9]. One study has found by screening the bioinformatics database, that ACTA2-AS1 is in dysregulation in cases with OC, but the study has not explored how it affects the pathogenesis of OC and its specific mechanism [10]. Therefore, the specific role and regulatory mechanism of ACTA2-AS1 in OC require deeper clarification.

MicroRNAs (miRNAs), 18-22 nucleotides long, are a group of ncRNAs [11]. In general, miRNAs fulfill their functions through guiding miRNA-induced silencing complex and targeting mRNA, which is helpful to inhibit gene expression at the post-transcriptional level [12]. miRNAs have been verified to regulate cancer processes including invasion, metastasis, inflammation, as well as tumor angiogenesis [13-15]. Intriguingly, miR-532-5p is involved in the inhibition of OC progression. Its overexpression inhibits the proliferation, colony formation, as well as invasion of OC cells, and its expression in malignant tissues is significantly lower than that in benign tissues [16-18]. However, the function of miR-532-5p in OC still requires full elucidation, and the interaction of miR-532-5p with ACTA2-AS1 is still unclear.

Chemokine C-X-C motif ligand 2 (CXCL2) belongs to the CXC family. As a protein encoded by a proto-oncogene, it can promote angiogenesis and plays a crucial part in the development and metastasis of tumors [19]. In recent years, the role of CXCL2 in tumor activity has been mentioned often. One study has demonstrated that CXCL2 is expressed at a high level in the serum of patients with OC, and blocking it can effectively inhibit the development of OC [20]. We found CXCL2 was the target of miR-532-5p through online website-based prediction, so we inferred miR-532-5p may affect disease progression by regulating CXCL2.

This study aimed to determine the role and mechanism of ACTA2-AS1 in OC, and found a new mechanism of ACTA2-AS1/miR-532-5p/CXCL2 axis in OC, which provides a theoretical basis for cancer therapy.

Materials and methods

Collection of OC tissues

Biopsy was carried out of OC tissues and corresponding healthy paracancerous tissues of 50 patients undergoing operation in Shanghai Jiao Tong University Affiliated Sixth People's Hospital. All tissues were directly frozen in liquid nitrogen after operation and stored until use. All patients with OC had received no preoperative treatment. Additionally, written informed consent was acquired from each participant before operation, and the study was carried out with permission from the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital.

Cell culture and transfection

Four OC cell strains (SKOV3, A2780, OVCAR, as well as HO-8910) and HOSEpiC provided by the American Type Culture Collection (ATCC) were incubated in RPMI 1640 (31870082, Thermo Fisher Scientific, Inc. USA) supplemented with penicillin and streptomycin, and stored in an incubator with 5% CO₂ at 37°C.

Recombinant lentivirus expressing sh-ACTA2-AS1 (shRNA targets ACTA2-AS1 directly) and the relevant negative control (sh-NC) were established. Sh-ACTA2-AS1 and sh-NC lentiviral particles for transduction (MOI=20) were transfected into SKOV3 cells using 5 μg/mL polybrene (H8761, Beijing Solarbio Science & Technology Co., Ltd., China), and cells stably transfected were screened out using 1 μg/mL puromycin (P8230, Beijing Solarbio Science & Technology Co., Ltd., China) within 2w.

MiR-532-5p mimics, negative control mimics (miR-NC), as well as miR532-5p inhibitor provided by Shanghai Gene Pharma Co., Ltd., or CXCL2 overexpression plasmid (pCDNA3.1-CXCL2) constructed and saved in the laboratory were transfected into SKOV3 cells with lipofectamine 2000 (11668019, Invitrogen, USA) under corresponding instructions, separately.

Dual luciferase reporter (DLR) assay

Wild-type (wt) sequence of ACTA2-AS1 containing miR-532-5p-binding locus or mutant (mut) sequence designed based on the wt sequence was amplified and then introduced into pGL3-
Mechanism of long non-coding RNA (lncRNA) ACTA2-AS1

Table 1. qRT-PCR sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTA2-AS1</td>
<td>Forward: GTTCTGGAGGCTTGATATGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCCTTCATCGGTAGGCAACAAAC</td>
</tr>
<tr>
<td>miR-532-5p</td>
<td>Forward: CTTCCATGCTTGAGTGTA</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTGTTGGAGGTTAATAGATG</td>
</tr>
<tr>
<td>CXCL2</td>
<td>Forward: ACCAGACGT CATGCAACAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCCACACCCCT GTT GCTGTA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: GCUCGGCGACACAUUAUCUAUU</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGCUUCACGAAU UUGCGUGUCAU</td>
</tr>
<tr>
<td>U6</td>
<td>Forward: GCUUCCGGACGACACAUUA</td>
</tr>
<tr>
<td></td>
<td>Reverse: UUGCGUGUCAU</td>
</tr>
</tbody>
</table>

Flow cytometry assay

Suspended cells (300 g) centrifuged at 4°C for 5 min and adherent cells centrifuged after digestion with ethylenediaminetetraacetic acid (EDTA)-free trypsin were collected, separately, and washed twice with pre-cooled phosphate buffer saline (PBS), 300 g cells each time. Then, the cells were centrifuged at 4°C for 5 min. Cells (1-5*10^5) were collected, and the PBS was absorbed out. Subsequently, the cells were suspended with 100 μL 1× Binding Buffer, followed by addition of 5 μL Annexin V-FITC and 10 μL PI Staining Solution and gentle mixing. Afterwards, the cells were let to react for 10-15 min at room temperature in the dark, followed by addition of 400 μL 1× Binding Buffer. Finally, the cells were placed on ice, and evaluated with a flow cytometer or fluorescence microscope within 1 h.

Statistical analyses

Samples in our study were all tested three times. The data were presented as the mean ± standard deviation (X ± sd), and analyzed by SPSS 20.0. Inter-group differences of data were analyzed by the independent-samples t test, and multi-group differences of data were analyzed by the univariate analysis combined with the least-significant difference t (LSD-t) test for pairwise comparison. Differences were considered significant if P<0.05.

Results

**ACTA2-AS1 in OC tissues and cell lines is increased, but miR-532-5p in them is opposite**

A qRT-PCR assay was used to quantify ACTA2-AS1 and miR-532-5p in OC tissues and cell
Mechanism of long non-coding RNA (lncRNA) ACTA2-AS1

To understand their expression in cases with OC. Notably, compared with healthy para-
cancerous tissues (n=50), OC tissues (n=50) showed high ACTA2-AS1 expression (P<0.05; Figure 1A). In addition, in contrast to HOSEpiC, ACTA2-AS1 was greatly up-regulated in OC cell strains (SKOV3, A2780, OVCAR, and HO-8910) (P<0.05; Figure 1B). ACTA2-AS1 expression in the si-ACTA2-AS1 group was significantly decreased compared with the si-NC group (P<0.05; Figure 3A). To understand the role of ACTA2-AS1 in OC cells, we carried out a MTT assay, finding that knock-out of ACTA2-AS1 significantly lowered the proliferation activity of OC cells (Figure 3B). The flow cytometry assay revealed that apoptotic cells in SKOV3 cells increased rapidly once ACTA2-AS1 was down-regulated (Figure 3C). In addition, the Transwell assay showed that invasive cells in OC cells transfected with si-ACTA2-

The siRNA knockout efficiency of ACTA2-AS1 was observed, and the results showed that the expression of ACTA2-AS1 in the si-ACTA2-AS1 group was significantly decreased compared with the si-NC group (P<0.05; Figure 3A). To understand the role of ACTA2-AS1 in OC cells, we carried out a MTT assay, finding that knock-out of ACTA2-AS1 significantly lowered the proliferation activity of OC cells (Figure 3B). The flow cytometry assay revealed that apoptotic cells in SKOV3 cells increased rapidly once ACTA2-AS1 was down-regulated (Figure 3C). In addition, the Transwell assay showed that invasive cells in OC cells transfected with si-ACTA2-AS1 decreased (P<0.05; Figure 3D).
indicate knockout of ACTA2-AS1 can reduce the malignant behavior of OC cells.

**Upregulating miR-532-5p can inhibit the proliferation and invasion of OC cells and promote their apoptosis**

Si-ACTA2-AS1, si-ACTA2-AS1 + anti-miR-532-5p, anti-NC, or si-ACTA2-AS1 + anti-NC was transfected into SKOV3 cells. In SKOV3 cells, transfection of si-ACTA2-AS1 + anti-miR-532-5p inhibited the enhancement of miR-532-5p expression induced by transfection of si-ACTA2-AS1 (P<0.05; Figure 4A). The MTT assay revealed that inhibiting miR-532-5p promoted the proliferation of cells whose ACTA2-AS1 was knocked out (Figure 4B). The flow cytometry assay revealed that inhibiting miR-532-5p suppressed the apoptosis of SKOV3 cells induced by knockout of ACTA2-AS1 (Figure 4C). In addition, the Transwell assay demonstrated that transfecting si-ACTA2-AS1 + anti-miR-532-5p could increase invasive cells in SKOV3 cells inhibited by transfection of si-ACTA2-AS1 (Figure 4D). The above results suggest that knockout of ACTA2-AS1 alleviates the malignant behavior of OC cells through increasing miR-532-5p.

**CXCL2 is the target of miR-532-5p and at a high expression in OC tissues and cells**

To further understand the mechanism of ACTA2-AS1 in OC, we screened and identified the target gene of miR-532-5p. Figure 5A and 5B showed that CXCL2 was greatly upregulated in OC tissues compared with in healthy para-cancerous tissues (P<0.05). The Spearman’s correlation analysis demonstrated that mRNA expression was negatively related to miR-532-5p expression in OC tissues and positively correlated with ACTA2-AS1 expression in them.
Mechanism of long non-coding RNA (lncRNA) ACTA2-AS1

It was verified through Targetscan, a bioinformatics tool, that there were multiple binding loci between CXCL2 and miR-532-5p (Figure 5E). With the aim of verifying their relationship, we carried out a DLR assay, finding that the luciferase activity of 293T cells declined greatly after transfection with CXCL2-wt and miR-532-5p (P<0.05), while that of 293T cells showed no notable change after transfection of CXCL2-mut and miR-532-5p (P>0.05; Figure 5F). Oppositely, the luciferase activity of 293T cells increased greatly after transfection of CXCL2-wt and anti-miR-532-5p (P<0.05; Figure 5G). Additionally, we found the protein level of CXCL2 in SKOV3 cells transfected with miR-532a-5p was inhibited, while that in SKOV3 cells transfected with miR-532-5p + pcDNA-ACTA2-AS1 was up-regulated (both P<0.05; Figure 5H). The above data indicate that CXCL2 is the direct target of miR-532-5p that regulates its expression in OC cells.

Overexpressing CXCL2 reverses the influence of miR-532-5p enrichment on invasion, proliferation, cloning, and apoptosis of OC cells

To study whether miR-532-5p interacts with CXCL2, miR-532-5p, vector, miR532-5p + CXCL2 or miR-532-5p + vectors was introduced into SKOV3 cells. As a result, CXCL2 was inhibi

Figure 3. Effect of ACTA2-AS1 knockout on the proliferation, apoptosis, and invasion of ovarian cancer cells. A: Knockout results of ACTA2-AS1; B: Proliferation of OC cells after knockout of ACTA2-AS1 according to the MTT assay; C: Apoptosis of SKOV3 cells after down-regulation of ACTA2-AS1 according to the flow cytometry assay; D: Invasion of cells according to the Transwell assay (200×). Compared with si-NC group, *P<0.05.
Figure 4. The effect of si-ACTA2-AS1 on ovarian cancer cells can be partially offset by anti-miR-532-5p. A: Expression of miR-532-5p in cells determined by the qRT-PCR assay; B: Proliferation of cells according to the MTT assay; C: Apoptosis of cells according to the flow cytometry; D: Invasion of cells according to the Transwell assay (200×). Compared with the anti-NC group, *P<0.05; compared with the si-ACTA2-AS1 + anti-NC group, #P<0.05.
Mechanism of long non-coding RNA (lncRNA) ACTA2-AS1


...transfection with miR-532-5p, but up-regulated by transfection with miR-532-5p + CXCL2 (both P<0.05) (Figure 6A). The MTT assay revealed that in SKOV3 cells, the cell proliferation suppressed by miR532-5p overexpression was recovered by synchronous upregulation of CXCL2 (P<0.05) (Figure 6B). According to the flow cytometry assay, the apoptosis rate induced by miR-532-5p overexpression was inhibited by synchronous upregulation of CXCL2 (P<0.05; Figure 6C). In addition, the Transwell assay revealed that invasive cells in cells transfected with miR-532-5p decreased, while invasive cells in those transfected with miR-532-5p + CXCL2 increased (Figure 6D). In a word, overexpressing miR-532-5p can prevent the progression of OC cells through inhibiting the expression of CXCL2.

Discussion

In this study, we disclosed that ACTA2-AS1 in cases with OC was up-regulated. Functionally, knockout of ACTA2-AS1 gene inhibited the
Figure 6. The effect of miR-532-5p on ovarian cancer cells can be partially reversed by CXCL2. A: Expression of CXCL2 in transfected cells determined using the qRT-PCR assay; B: Proliferation of cells determined by the MTT assay; C: Apoptosis of cells according to the flow cytometry assay; D: Invasion of cells according to the Transwell assay (200×). Compared with the vector group, *P<0.05; compared with the miR-532-5p + vector group, †P<0.05.
malignant behavior of OC cells and tumor growth. Mechanically, it was verified that as a target of ACTA2-AS1, miR-532-5p directly interacted with CXCL2. In addition, we verified through analysis that ACTA2-AS1 regulated CXCL2 in OC by competitively binding miR-532-5p.

One study has verified that ACTA2-AS1 promotes the development of cervical cancer [9]. Understanding the influence of ACTA2-AS1 on the progression of OC may be helpful to discover new occurrence and metastasis mechanisms of OC. We confirmed that ACTA2-AS1 in OC tissues and cells was up-regulated, and the interference of ACTA2-AS1 suppressed the proliferation, invasion as well as cloning of OC cells, but promoted their apoptosis to a certain extent. All the data indicate the oncogenic role of ACTA2-AS1 in OC.

MiR-532-5p was verified as the target of ACTA2-AS1. One earlier study has pointed out that miR-532-5p expression in epithelial OC tissues decreased [21]. The same potential function of miR-532-5p lies in other cancers including breast cancer, colorectal cancer, as well as bladder cancer [22-24]. Overall, miR-532-5p in these cancer cases is down-regulated, and its enrichment effectively inhibits the cell malignant behavior. Similar to previous studies, our study showed that the expression of miR-532-5p in OC tissues and cells also decreased and inhibiting miR-532-5p reversed the inhibitory effect of ACTA2-AS1 gene knockout on the development of OC. The above findings suggest that miR-532-5p is a tumor suppressor in nearly all cancers, such as OC.

As a member of LIM domain protein, CXCL2 plays a crucial role in cell growth, differentiation, cytoskeleton construction, as well as cell fate [25]. Lately, the role of CXCL2 in cancers including OC is increasingly clear. For example, the high abundance of CXCL2 is related to the unfavorable prognosis of patients with OC, silencing of CXCL2 suppresses the growth and metastasis of OC cells, and CXCL2 overexpresses in OC cells [26-30]. Our research revealed that with increased expression in OC tissues and cells, CXCL2 was the target of miR-532-5p, and it was regulated by ACTA2-AS1 through miR-532-5p. Additionally, overexpression of CXCL2 destroyed the inhibition of miR-532-5p enrichment on OC cell growth. The results suggest that CXCL2 acts as a tumor promoter in OC.

In conclusion, ACTA2-AS1 is highly expressed in OC tissues and cells. The functional analysis shows ACTA2-AS1 may up-regulate CXCL2 through competitive combination with miR-532-5p, thus playing a part in promoting the progression of OC. However, this study has conducted only in vitro experiments, but has not conducted in vivo experiments, which will be a focus of our future research.

Disclosure of conflict of interest
None.

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Mechanism of long non-coding RNA (IncRNA) ACTA2-AS1


