Long non-coding RNA THOR promotes Ovarian Cancer cells progression via STAT3 pathway

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Abstract

Background Ovarian cancer (OC) is one of the most common malignant tumors in the world. The prognosis of OC remains poor due to the advanced stage and distant metastasis at the time of diagnosis. Recently, a novel IncRNA, THOR (testis-associated highly conserved oncogenic long non-coding RNA), was characterized in human cancers and shown to exhibit an oncogenic role. However, the role of THOR in OC was still unknown.

Methods RT-PCR and western blot analysis were used to detect the expression of THOR and p-STAT3. The impact of THOR on OC proliferation, metastasis and self-renew was investigated in vitro and in vivo. The prognostic value of THOR was determined in OC patient cohorts.

Results In this study, our results found that THOR was markedly upregulated in human OC tissues and predict the poor prognosis of OC patients. THOR knockdown resulted in significant inhibition of the growth, metastasis and self-renewal of OC cells. Mechanistically, THOR drives OC cell progression via the STAT3 signaling. Moreover, the specific STAT3 inhibitor S3I-201 diminished the discrepancy in the growth, metastatic and self-renewal capacity between THOR-silenced OC cells and control cells, which further confirmed that STAT3 was required in THOR-driven OC cells progression.

Conclusion Our findings revealed that THOR could promote OC cells growth, metastasis and self-renew by activating STAT3 signaling and may be a good predictive factor and therapeutic target.

Background

Ovarian cancer (OC) was one of the most common deadly tumors in the world (1, 2). More than 70% OC patients were diagnosed at advanced stage and lost their best operative chance (3). Numerous researches reported that tumor suppressor genes or proto-oncogenes, chromosomal and microsatellite instability, and epigenetic modifications have important roles in OC initiation and progression (4). While the molecular and cellular mechanisms underlying the development of OC remains unclear. The lack of complete understanding of the molecular pathogenesis of OC has prevented the design of mechanism-based therapeutic recipes. Therefore, elucidation of the underlying mechanisms of initiation and progression of OC and identification of new therapeutic targets for OC are urgently needed.
Long non-coding RNAs (IncRNAs) are a heterogeneous class of transcripts with a length of more than 200 bases without protein-coding potential (5, 6). Accumulating evidence has indicated that IncRNAs can affect disparate cellular functions and participate in diverse physiological and pathological processes (7). The aberrant expression of IncRNAs has been demonstrated in multiple malignancies, including liver cancer, breast cancer, lung cancer and colorectal cancer (8), providing new insights into the pathogenesis of cancer (9). The IncRNA THOR was reported as an oncogene, and transgenic THOR knockout produced fertilization defects in zebrafish and conferred resistance to melanoma initiation. The researchers also discovered a conserved interaction of THOR with IGF2BP1 and showed that THOR contributes to the mRNA stabilization activities of IGF2BP1 (10). Previous studies also reported that THOR was upregulated in liver cancer and promoted HCC cells progression (11).

However, the role of THOR in OC initiation and progression remains unknown.

In the present study, we for first found that THOR was highly expressed in OC patients and associated with poor prognosis of OC patients. Next, by using loss-of-function analysis in OC cells, we demonstrated that THOR promotes the proliferation, metastasis and self-renew of OC cells. Further mechanistic analysis revealed that the STAT3 pathway was activating by THOR in OC cells. Moreover, the special STAT3 inhibitor S3I-201 diminished the discrepancy in growth, metastatic and self-renewal capacity between THOR-silenced OC cells and control cells. Collectively, the data showed that THOR promotes the progression of OC cells via the STAT3 cascades and could be a good predictive factor and a potential therapeutic target of OC patients.

Methods

Patients and tissue samples

The OC patients’ tumor tissues and paired non-tumor tissues were obtained from patients who underwent curative surgery at General Hospital of Northern Theater Command. The specimens were frozen in liquid nitrogen immediately and then stored at -80°C. Overall survival (OS) was defined as the time interval between the date of surgery to the date of death or the last follow-up. Cumulative recurrence was defined as the time interval between the dates of surgery to the date of diagnosis of recurrence. A total of 90 tissue samples were also used for clinical prognosis analysis. Patient
informed consent was also obtained and the procedure of human sample collection was approved by the Ethic Committee of General Hospital of Northern Theater Command.

**Cell lines and culture**

HO8910 and HGSOC cells were purchased from Chinese Academy of Sciences, Shanghai, China. The ovarian cancer cells were cultured in RPMI-1640 medium supplemented with 10 % fetal calf serum (FCS; Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO₂ incubator. The cultured cells were trypsinized with 0.5% trypsin and moved to a new six-well plate three times a week.

HO8910 or HGSOC cells were seeded into a six-well plate until they reached 60-70% confluence. The cells were infected with shTHOR lenti-virus and control virus as described previously (12). The sequence of shTHOR is as follows: 5’- GGUGAACACAAUACUGAGCAATT-3’. The shRNA lenti-virus was purchased from Shanghai GenePharma (Shanghai, China).

HO8910 shTHOR or HGSOC shTHOR cells and their control cells were treated with S3I-201 (100 nM) or not and then subjected to CCK8, Transwell (24 h) and invasion assays (48 h).

**Cell proliferation assays**

HO8910 shTHOR or HGSOC shTHOR cells and their control cells were seeded in 96-well plates (3x10³ cells/well). ATP activity was measured using CCK8 assays at the indicated time points. The procedure was as follows: The cell suspension (100 μl/well) was inoculated in a 96-well plate, and the plate was pre-incubated in a humidified incubator at 37°C for 1 hour. This was followed by the addition of 10 μl of the CCK-8 solution to each well of the plate, and incubation of the plate for 1 h in the incubator. Finally, the absorbance was measured at 450 nm using a microplate reader (Synergy H1; BioTek Instruments, Inc., Winooski, VT, USA).

**Colony formation assay**

For colony formation assays, the HO8910 shTHOR or HGSOC shTHOR cells and their control cells were
seeded in 12-well plates (3000 cells/well). The cells were incubated for 7 days and then fixed with 10% neutral formalin for more than 4 hours. The cells were dyed with crystal violet (Beyotime, Haimen, China). The cells were then photographed under a microscope (Olympus, Tokyo, Japan).

**EdU immunofluorescence staining**

For cell EdU immunofluorescence staining, HO8910 shTHOR or HGSOC shTHOR cells and their control cells were seeded into 96-well plates and examined using the EdU kit (RiboBio, Guangzhou, China). The results were quantified with a Zeiss Axiophot photomicroscope (Carl Zeiss, Jena, Germany) and Image-Pro Plus 6.0 software.

**Cell migration assays**

For cell migration experiments, $2 \times 10^5$ OC cells were seeded into the upper chamber of a 24-well polycarbonate transwell in serum-free DMEM. The lower chamber was supplemented with DMEM containing 20% FBS as chemoattractant. The cells were incubated for 24 hours, and the chamber was fixed with 10% neutral formalin for more than 4 hours. The cells were dyed with crystal violet (Beyotime). Then the cells were counted under a microscope (Olympus), and the cell number is expressed as the average number of the cells in 5 fields.

**Cell invasion assays**

For cell invasion experiments, $2 \times 10^5$ OC cells were seeded into the upper chamber of a Matrigel-coated Boyden chamber in serum-free DMEM. The lower chamber was supplemented with DMEM containing 20% FBS as a chemoattractant. The cells were incubated for 48 hours, and the chamber was fixed with 10% neutral formalin for more than 4 hours. The cells were dyed with crystal violet (Beyotime). Then the cells were counted under a microscope (Olympus), and the cell number is expressed as the average number of cells in each field.
**Spheroid formation assay**

HO8910 shTHOR or HGSOC shTHOR cells and their control cells were cultured in a 6-well or 96-well ultra-low attachment culture plate for one week, and the total number of spheres was counted under the microscope.

**Real-time PCR**

The total RNA from OC cells or the OC patient tissues was extracted by using TRizol reagent (Invitrogen, 15596-018). Total cDNAs were synthesized by a ThermoScript™ RT-PCR system (Invitrogen, 11146-057). The total mRNA amount present in the cells was measured by RT-PCR using the ABI PRISM 7300 sequence detector (Applied Biosystems). The THOR primer sequences were as follows: forward: 5'-'ACAATCGAGCAAGGCAGTGA-3', reverse: 5'-'TGGCCAAGACCTGCTGTTAG-3'. β-actin was used as reference for relative expression calculation and its primer sequences were as follows: forward: 5'-'GGCCCAGAATGCAGTTCGCCTT-3', reverse: 5'-'AATGGCACCTGCTCAGCA-3'. The PCR cycling conditions were as follows: 94°C degeneration for 10 min; 94°C modification for 30 sec, 60°C annealing for 30 sec, 72°C extension for 40 sec, total of 40 cycles, 72°C terminal extension for 10 min.

**Western blotting assays**

The OC cells or OC patients' tissues were lysed with cell lysis buffer (Beyotime) followed by supersonic splitting as described previous (13). The total protein was quantified using a BCA Protein Quantification kit. A total of 25 μg of protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes. The membranes were blocked with 10% non-fat milk and incubated with primary antibodies overnight. The protein band, specifically bound to the primary antibody, was detected using an IRDye 800CW-conjugated secondary antibody and the LI-COR imaging system (LI-COR Biosciences, Lincoln, NE, USA). The primary antibodies were p-AKT (1:1000; #4060, Cell Signaling Technology), p-STAT3 (1:1000; #9145, Cell Signaling Technology), p-MEK (1:1000; #9127, Cell Signaling Technology) p-SMAD3 (1:1000; #9520, Cell Signaling Technology), and GAPDH (1:5000; #5174, Cell Signaling Technology).
**In vivo animal models**

The NOD-SCID mice were purchased from Slake Company, Shanghai academy of sciences. All mouse experiments were performed according to the guidelines of the animal care and use committees at Shanghai Baoshan District Hospital of Integrated Chinese and Western medicine.

For xenograft formation assay, HO8910 shTHOR or its control cells (2 X 10^6) were injected subcutaneously into nude mice. Mice were sacrificed six weeks post inoculation and tumors were collected and examined.

For pulmonary metastasis assay, HO8910 shTHOR or its control cells (2 X 10^6) were injected into the tail vein of nude mice. The mice were sacrificed 12 weeks post inoculation and consecutive sections of the whole lung were subjected to hematoxylin and eosin (H&E) staining. All metastatic foci in the lung were calculated microscopically to evaluate the development of pulmonary metastasis.

For *in vivo* limiting dilution assay, HO8910 shTHOR cells and its control cells were mixed with Matrigel (BD) at a ratio of 1:1 and injected subcutaneously at various cell doses per mouse. The tumor formation was evaluated at 8 weeks.

**Statistical analysis**

GraphPad Prism (GraphPad Software, Inc., La Jolla, USA) was used for all statistical analyses.

Statistical analysis was carried out using t tests or Bonferroni multiple comparison tests: *p*<0.05. A *p* value of less than 0.05 was considered statistically significant.

**Results**

THOR expression was elevated in human OC tissues and predicted the poor prognosis of OC patients. To explore the function of THOR in OC progression, the expression of THOR was checked in a large number of human OC tissues. As shown in Fig. 1A, THOR expression was dramatically upregulated in OC tumor tissues compared with the paired non-tumorous tissues. OC patients have high rates of metastasis, and the metastatic focus is a prognostic factor of poor prognosis in the patients (14). As expected, THOR expression was increased in metastatic foci compared with the primary OC tissues,
indicating THOR has important role in OC metastasis (Fig. 1B).

The cohort of 90 OC patients were equally divided into a “THOR high” group and a “THOR low” group based on the THOR expression. Univariate analysis demonstrated that the patients with low THOR levels possessed a lower risk of OC recurrence and a longer survival time after surgical resection than those with high levels (Fig. 1C&D).

THOR promotes OC cells proliferation.

To elucidate the role of THOR on OC cell behavior, HO8910 and HGSOC cells were infected with shTHOR or control lenti-virus and the stable transfectants were obtained (Fig. 2A). As shown in Fig. 2B, THOR knockdown suppressed the proliferation of OC cells. In addition, HO8910 shTHOR and HGSOC cells shTHOR cells formed fewer and smaller colonies compared with their control cells (Fig. 2C). Furthermore, 5-ethynyl-2’-deoxyuridine (EdU) staining confirmed that THOR depletion inhibited OC cells growth (Fig. 2D). More importantly, THOR knockdown also inhibited the xenograft growth of OC cells in vivo (Fig. 2E–F). Taken together, the above results indicated that THOR promoted OC cell growth in vitro and in vivo.

THOR drives OC cells metastasis.

Next, we explored the role of THOR in OC cells metastasis; transwell assays and showed that migration ability was impaired in THOR-silenced OC cells (Fig. 3A&B). In addition, Matrigel invasion assays also revealed that THOR interference weakened the invasiveness of OC cells (Fig. 3C&D). Moreover, THOR also inhibited the lung metastasis foci of OC cells in vivo (Fig. 3E–F). Collectively, our results showed that THOR promoted OC cell metastasis.

THOR promotes ovarian CSCs expansion.

It was reported that CD133 was well-accepted ovarian cancer stem cells (CSCs) marker (15). As shown in Fig. 4A, THOR expression was dramatically increased in CD133-positive ovarian CSCs. Consistently, THOR expression was also upregulated in the self-renewing spheroids compared with the attached cells (Fig. 4B). Intriguingly, THOR level could be partially recovered when the spheroids cells reseeded in attached plates (Fig. 4C). Then we used THOR stably interference OC cells to explore the potential role in ovarian CSCs. As expected, THOR knockdown OC cells formed fewer spheroids.
compared with its control cells (Fig. 4D). Moreover, the expression of ovarian CSC markers was also suppressed in THOR interference spheroids (Fig. 4E). To further determine the effect of THOR on the tumorigenicity of ovarian CSCs, sphere-derived shTHOR or its control cells were inoculated into NOD-SCID mice. In vivo limiting dilution assay revealed that suppression of THOR significantly reduced tumor incidence (Fig. 4F).

THOR promotes OC cell progression through the STAT3 signaling.

Numerous studies shown that TGF-β/SMAD, PI3-K/Akt, MAPKs and JAK/STAT3 signaling pathways have pivotal roles in various cellular processes, including cell growth, metastasis and apoptosis (16-19). Herein our data showed that TGF-β/SMAD, PI3-K/Akt or MAPKs pathway was not influenced by THOR interference. However, STAT3 phosphorylation was inactivated by THOR knockdown in HO8910 and HGSOC cells (Fig. 5A). Moreover, the STAT3 downstream molecular factor was impaired in THOR knockdown OC cells (Fig. 5B). Then the special STAT3 inhibitor S3I-201 was used to confirm the role of STAT3 in THOR promoting OC cells growth, metastasis and self-renew. As expected, the inhibitor S3I-201 dramatically abolished the growth difference between THOR knockdown OC cells and control cells (Fig. 5C). Consistently, the inhibitor S3I-201 also eliminated the discrepancy in metastasis between THOR-silenced OC cells and their control cells (Fig. 5D&E). In addition, the inhibitor S3I-201 also abrogated the self-renewal ability between THOR-silenced OC cells and their control cells (Fig. 5F), suggesting that THOR promoted OC cell progression by activating STAT3 signaling.

Discussion

Ovarian cancer is one of in the female reproductive system and has a poor prognosis, which is related to its complex pathogenesis. The early symptoms of OC are not obvious and most patients are diagnosed at advanced stage (20). The current treatments used for OC include some combination of surgery, radiation therapy, chemotherapy and targeted therapy (21). While OC patients diagnosed at a late stage are usually incurable, and in that case the main goal of treatment is to improve the quality of life and prognosis (22). In the present study, our results showed that THOR expression was upregulated in OC patients and might be a potential therapeutic target.

Emerging evidence showed that IncRNAs were involved in embryonic development, stem cell self-
renewal and differentiation, adipocyte differentiation and vascularization (23). It was also accepted that IncRNAs participating in the initiation and progression of numerous human cancers and may be novel diagnosis markers and therapeutic targets (24). For instance, Inc-DILC was reduced in liver cell stem cells and suppressed their expansion through inhibiting autocrine IL6/STAT3 pathway (25). It has been reported that THOR, a IncRNA with a cancer/testis expression pattern that exhibits a conserved interaction with IGF2BP1, potentially promoted oncogenesis. Moreover, ectopic expression of human THOR in zebrafish accelerated the initiation of melanoma. Latest studies also showed that THOR promoted hepatoma cells growth and metastasis via PTEN/AKT cascades. However, the potential role of THOR in OC was unknown. In our above study, we for first demonstrated that THOR was upregulated in OC tissues and predicted the poor prognosis of OC patients. Knockdown THOR inhibited OC cells proliferation and metastasis both in vivo and in vitro.

The existence of CSCs has been confirmed by numerous studies, and these cells have the ability to self-renew and the potential for generating heterogeneous malignant progenies (26, 27). It was well-accepted that CD133 as the ovarian CSCs marker. In this study, our data showed that HTOR levels increased in CD133⁺ ovarian CSCs. Spheroid culture of cancer cells is a routine approach to enrich CSCs. We also found that THOR expression was upregulated in OC spheroids. Moreover, knockdown THOR inhibited the self-renewal ability and tumorigenesis capacity of ovarian CSCs.

STAT3 acts as a mediator of the signals initiated by the inflammation factor IL-6 cytokines, which regulate cell proliferation, differentiation and death (28, 29). STAT3 expression has been reported to correlate with poor patient outcome in several types of cancer (30, 31). In mice, STAT3 mutation has been linked to colorectal adenocarcinoma, increased systemic inflammation, and accelerated wound healing (32). The altered activity of STAT3 was linked to chronic inflammation and somatic mutations that contribute to chronic colitis and the development of colorectal cancer (33). In this study, we found that THOR played a positive role in OC cells and facilitated OC cell growth, metastasis and self-renew by activating STAT3 signaling. The STAT3 downstream molecular factor was also downregulated by THOR interference. In addition, these growth, metastasis and self-renewal effects could be diminished by the STAT3 inhibitor S3I-201.
we demonstrated for the first time that THOR expression is upregulated in OC tissues, and THOR shRNA silencing suppresses the growth, metastasis and self-renewal of OC cells. Moreover, THOR promoted OC cell progression by activating STAT3 signaling. The findings of the present study not only shed new light on the mechanisms responsible for OC progression but also suggest that THOR may be a novel prognostic marker and a potential therapeutic target for OC.

**Declarations**

**Ethics approval and consent to participate**

Informed consent was obtained from all individual participants included in the study. Patient informed consent was also obtained and the procedure of human sample collection was approved by the Ethic Committee of General Hospital of Northern Theater Command.

**Consent for publication**

Agreed.

**Availability of data and materials**

Data generated from the study are available from the corresponding author on reasonable request.

**Competing interests**

All authors declare no competing interests.

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**Authors’ contributions**

Jing Ge, Tao Han and Lili Shan designed the experimental plan and wrote the manuscript with input from all other authors. Jing Na and Ya Li provided clinical samples and analyzed clinical data. Jing Ge, Tao Han and Lili Shan performed the experiments. Jun Wang conceived the project and supervised all
experiments. All authors read and approved the final manuscript.

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Figures

Figure 1
Figure 1

Upregulation of THOR in OC patient tissues. A. The expression of THOR in 90 pairs of OC tumor (T) and normal tissues (N) was assessed by real-time PCR analysis, p<0.05. B. Comparison of THOR transcripts in paired peri-tumor normal tissues, OC tissues, and metastatic lesions using real-time PCR (n=15), p<0.05. C&D. The total of 90 OC patients were divided into low THOR group (n = 45) and high THOR group (n = 45), and the recurrence and overall survival of the patients in the two groups were compared, p<0.05.
Figure 2
Knockdown THOR suppressed OC cells proliferation. A. HO8910 and HGSOC cells were infected with shTHOR or its control lenti-virus, and then the cells were subjected to RT-PCR assays. B. Cell proliferation of HO8910 shTHOR or HGSOC shTHOR cells and their control cells was measured by using CCK8 assays. C. Colony formation assays of HO8910 shTHOR or HGSOC shTHOR cells and their control cells. D. Cell proliferation was assessed using Edu immunofluorescence staining of HO8910 shTHOR or HGSOC shTHOR cells and their control cells. E. HO8910 shTHOR and its control cells were subcutaneously injected into nude mice (n = 6) for xenograft assay. F. The above tumor average weight in each group was shown, p < 0.05.
THOR depletion inhibited OC cells metastasis. A. The migration of HO8910 shTHOR and its control cells was analyzed using polycarbonate membrane inserts in a 24-well plate, p<0.05. B. The migration of HGSOC shTHOR and its control cells was analyzed using polycarbonate membrane inserts in a 24-well plate, p<0.05. C. The invasive capacity of HO8910 shTHOR and its control cells was analyzed using Matrigel-coated Boyden chambers, p<0.05. D. The invasive ability of HGSOC shTHOR and its control cells was analyzed using Matrigel-coated Boyden chambers, p<0.05. E. Lung morphology and H&E staining of nude mice inoculated HO8910 shTHOR and its control cells via tail vein for 12 weeks. F. The number of lung metastatic foci in each group (n = 7) were also calculated, p <0.05.
Figure 4

A

HO8910  HGSOC
Relative THOR Expression
CD153  CD155

C

HO8910  HGSOC
Relative THOR Expression
Attached cells  Spheroids  Re-attached cells

B

HO8910  HGSOC
Relative THOR Expression
Attached cells  Spheroids

D

HO8910
GFP  shTHOR

E

HO8910 GFP  HO8910 shTHOR
Relative mRNA Expression
ALDH  CD153  CD44  CD84

F

| Cells Injected | HO8910 GFP | HO8910 shTHOR |
|----------------|------------|---------------|
| $1 \times 10^4$ | 4/10       | 1/10          |
| $5 \times 10^4$ | 5/10       | 2/10          |
| $1 \times 10^5$ | 6/10       | 3/10          |
| $5 \times 10^5$ | 8/10       | 4/10          |
| Total          | 23/40 (57.5%) | 10/40 (25%)   |
THOR promotes ovarian CSCs expansion. A. RT-PCR analysis of THOR expression in flow cytometry sorted CD133+ OC cells relative to CD133- OC cells. B. The level of THOR in OC spheroids and attached cells was examined by real-time PCR assay. C. OC cell-derived spheroids were trypsinized and cultured in attachment conditions. THOR level in spheroids versus reattached cells was compared by RT-PCR assay. D. Representative image of spheres formed from shTHOR and control OC cells. The number of the formed spheres was counted and compared. E. The ovarian CSCs markers in shTHOR and control OC cells were determined by real-time PCR. F. In vivo limiting dilution assay of shTHOR and control sphere-derived OC cells. (n=10)
THOR promotes OC cells progression via STAT3 pathway. A. The phosphorylation status of STAT3, SMAD3, AKT, and MEK in HO8910 shTHOR or HGSOC shTHOR cells and their control cells was determined by Western blot assays. B. The mRNA expression of IL-6, C-myc, Survive and VEGF in HO8910 shTHOR or HGSOC shTHOR cells and their control cells was checked by real-time PCR. C. The proliferation of HO8910 shTHOR or HGSOC shTHOR cells and their control cells in the presence of S3I-201 (100 nM) or not was measured using CCK8 assays. D. Migration assays were performed using OC shTHOR cells and its control cells in the presence of S3I-201 (100 nM) or not. E. Invasion assays were performed using OC shTHOR cells and its control cells in the presence of S3I-201 (100 nM) or not. F. Spheroid formation assays were performed using OC shTHOR cells and its control cells in the presence of S3I-201 (100 nM) or not.