

Research Paper

Long Non-Coding RNA NONHSAT076754 Promotes Invasion and Metastasis in Epithelial Ovarian Cancer

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Abstract

Although accumulating evidence suggests that long non-coding RNAs (lncRNAs) are critical determinants of ovarian cancer development and progression, reports of metastasis-associated lncRNAs are limited. Here, we focused on NONHSAT076754 and explored its expression level, clinical value, biological behavior and molecular basis in epithelial ovarian cancer (EOC) metastasis. The results showed that NONHSAT076754 expression was increased in EOC tissues and cell lines and that this expression was closely related with FIGO stage, high tumor grade and lymph node metastasis. Furthermore, NONHSAT076754 knockdown markedly inhibited EOC cell migration and invasion *in vitro*. Consistently, the *in vivo* data from both the bioluminescence imaging and tumor dissection revealed that depletion of NONHSAT076754 reduced EOC metastasis. Mechanically, the pro-metastatic activities of NONHSAT076754 were partially regulated by PTEN and HTATIP2. Further rescue assays validated that knockdown of HTATIP2 remarkably reversed NONHSAT076754 silencer-induced inhibition of EOC cell metastasis. These data indicate that NONHSAT076754 is a vital regulator of EOC metastasis, laying the foundation for lncRNA-based clinical management of EOC aggressiveness and metastasis.

Key words: long non-coding RNA, NONHSAT076754, invasion, metastasis, ovarian cancer

Introduction

Epithelial ovarian cancer (EOC) is among the most common gynecological malignancies and causes the most deaths in women with these malignancies. The high mortality is attributed to the frequent spread of metastases at time of diagnosis in patients with generally limited symptoms [1, 2]. Despite progresses in clinical management with effective surgery and chemotherapy, overall survival remains at an uncomfortable rate of 30% largely because of abdominal or other systemic metastases [3]. Hence, the identification of an available metastasis-associated biomarker and exploration of its molecular mechanism will be great beneficial for the clinical management of ovarian cancer (OC).

Recently, high-throughput sequencing efforts

have revealed that long non-coding RNAs (lncRNAs), which are longer than 200 nucleotides in length, account for the majority of the human genome rather than "transcription noise" [4, 5]. Growing evidence has showed that lncRNAs are abnormally expressed in various cancers and participate in multiple biological processes, including apoptosis, proliferation, cell migration, and invasion [6]. Furthermore, several lncRNAs, such as MALAT1 [7], H19 [8], HOTAIR [9], AFAP1-AS1 [10], CCAT2 [11], lncRNA-ATB [12] and lincRNA-RoR [13] have emerged as promising candidates for gene regulation during tumor progression. In the case of EOC, existing evidence, including our previous findings, have unveiled a handful of lncRNAs are implicated in EOC

metastasis. For example, the long non-coding RNA ANRIL exhibited great clinical significance in predicting the aggressive prognosis of OC and stimulated the invasion and metastasis of serous ovarian cancer [14]. In another study, we revealed that HOTAIR also acted as a pro-metastasis lncRNA in EOC, and its overexpression was highly connected with poor prognosis in patients with EOC [15]. However, despite these findings, the essential roles and mechanisms of metastasis-associated lncRNAs in EOC are unclear. Based on the current situation on metastasis-associated lncRNAs research, it is necessary to identify novel lncRNAs involved in metastasis process and to further explore their molecular mechanisms.

In the present study, the expression of four metastasis-associated lncRNAs (NONHSAT076754, HEIRCC, lncTCF7 and ZEB2NAT) was examined, and the novel OC-related lncRNA NONHSAT076754 captured our attention due to its remarkably consistently high expression in EOC [16-21]. NONHSAT076754, which is located on chromosome 2, is 695 bp in length and transcribed from fibronectin 1, which is closely associated with cells' adhesive and migrative capabilities [22-24]. NONHSAT076754 was originally found to promote migration and invasiveness in papillary thyroid cancer (PTC) and could be a valuable biomarker and predictor of lymph node metastasis [16]. However, other than these studies, no data regarding the role of NONHSAT076754 in the invasion and metastasis of EOC are currently available.

The present investigation aimed to illustrate the involvement of NONHSAT076754 in EOC metastasis. Thus, we first detected the expression level of NONHSAT076754 in EOC clinical samples and cell lines and analysed the association between NONHSAT076754 expression and EOC clinicopathological characteristics. Additionally, NONHSAT076754 was silenced to evaluate its effect on invasion and metastasis both *in vitro* and *vivo*. Moreover, the mechanism by which NONHSAT076754 modulated EOC metastasis was explored. The major objective of the present study is to highlight the instructive significance of NONHSAT076754 in EOC metastasis.

Materials and Methods

Patients and tissue samples

In total, 70 EOC tissue samples were gained from the Tissue Bank of the Obstetrics and Gynecology Hospital of Fudan University. Inclusion criteria were as follows: (1) Patients were diagnosed with first-time EOC and samples were confirmed by postoperative pathology; (2) patients underwent surgery between

August 2013 and September 2016. Exclusion criteria were as follows: (1) Patients had two or more different malignancies; (2) patients had received preoperative chemotherapy, radiotherapy, or hormonal therapy; (3) patients had coexisting autoimmune diseases or evidence of active infection; (4) there were metastatic or non-epithelial tumors in ovary. Ten normal ovarian epithelial tissues were collected from the uterine fibroids participants scheduled to experience hysterectomy and oophorectomy. Inclusion criteria were as follows: All samples were confirmed by postoperative pathology as uterine leiomyoma with no abnormality in adnexa. Exclusion criteria were the same as EOC group apart from those patients who had previous ovarian pathology or surgery and who had history of malignancy. This study was approved by the Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University (No. [2017]82).

Cell lines and transfection

The human EOC cell lines (SKOV3, OVCAR5, OVCAR3, OVCAR8, HO8910) and a normal human ovarian epithelial cell line (HOSEPIC) were offered by the University of Texas MD Anderson Cancer Center (Houston, TX, USA). The cells were grown in RPMI-1640 medium (Genom, Hangzhou, China) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 100 u/ml penicillin streptomycin in a humidified 5% carbon dioxide incubator at 37 °C.

The control small interfering RNA (siRNA) and siRNAs targeting NONHSAT076754 and HTATIP2 (GenePharma, Shanghai, China) were applied in combination with Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA, USA) to SKOV3, OVCAR5 cells based on the manufacturer's protocol. The target sequences of the NONHSAT076754 siRNAs were 5'-UGAUGUGGUGGUCUGGUCUC-3' (siRNA-1), 5'-GACCAGACCACCACAUCAUTT-3' (siRNA-2) and 5'-GCAUACUGUAACCUCUGCATT-3' (siRNA-3). The target sequences of the HTATIP2 siRNAs were 5'-GGAGGGAUUUGUUCGUGUUTT-3' (siRNA-1), 5'-GCAGAAUAAAUCCGUCUUUTT-3' (siRNA-2) and 5'-CCAGGUGAAUGGCUGGUUATT-3' (siRNA-3). The sequence of the negative control (NC) was 5'-UUCUCCGAACGUGUCACGUTT-3'. After 48 h, the knockdown efficiency was examined using quantitative real-time polymerase chain reaction (qRT-PCR).

Establishment of stable NONHSAT076754-knockdown tumor cells

Stable knockdown tumor cells were established by using lentiviral vectors carrying NONHSAT076754-siRNA-1 and a luciferase sequence (KD-luc-SKOV3).

Accordingly, the negative control lentivirus (NC-luc-SKOV3) (Genechem, Shanghai, China) were prepared. At 72h post-infection, luciferin (0.15mg/ml) was added, and cells were imaged by Bioluminescence Imaging Facility to confirm luciferase expression. Subsequently, puromycin (4µg/ml) was applied for approximately 12 days to isolate the puromycin-resistant cell clones.

RNA extraction and qRT-PCR

The total RNA was isolated using TRIZOL reagent (TAKARA, Code No.9109, Dalian, China) and the cDNA was synthesized using the Prime-Script™ RT Master Mix (TAKARA, RR036A, Dalian, China). qRT-PCR was performed using the SYBR Green qPCR (TAKARA, RR820A, Dalian, China). All steps were conducted according to the manufacturer's instructions. The relative quantification results were displayed as fold change using $2^{-\Delta\Delta Ct}$. The primer sequences are listed in Table 1.

Table 1. The primers used in the study.

Gene	Primer	Sequence (5'-3')
GAPDH	Forward	GTCATCAATGGAAATCCCATCA
	Reverse	CCAG TGGACTCCACGACGTAC
NONHSAT076754	Forward	AAGTTTCTCACTCACCCACCTG
	Reverse	GAAGCATGTACAGTTCAGCATGTG
HEIRCC	Forward	GCTGCTATTCTGGTGCC
	Reverse	TCAACTCCGATAAACAGGTGA
IncTCF7	Forward	AGGAGTCCTTGGACCTGAGC
	Reverse	AGTGGCTGGCATATAACCAACA
ZEB2NAT	Forward	GAG AGA CGA GAG ACC CTG AA
	Reverse	TGC ACA CAC CCT AAT ACA CAT
HPSE	Forward	TTCTCACAGCAAACCTCTCC
	Reverse	AACAACAACGAGGTTCTCTAT
HRAS	Forward	GGCTTCCTGTGTGTGTTTC
	Reverse	AGTACAGGGAGCAGATCAAA
PTEN	Forward	CTGGTTCACATCCTACCCCT
	Reverse	CGAAGGGTTTTGCTACATTC
FGFR4	Forward	TCAAAGACAACGCTCTGAC
	Reverse	GAGATGGAGGTGATGAAGCT
HTATIP2	Forward	GTTTTCCAAAGTCACGCTCA
	Reverse	GTTGGATTCTGTGCTGCTGGG
NR4A3	Forward	GTCTGAAAGGGAGGAGAGGT
	Reverse	ACCTTCTCAGCCCTCTCCAC

Scratch assay

The cells were seeded onto 6-cell plates. Once approximately 80% confluency was reached, the cells were scraped with a sterile pipette tip, washed with phosphate-buffer-solution (PBS) and maintained in serum-free media at 37°C and 5% CO₂. The scratch width was photographed at 0 h, 24h and 48 h at the same positions.

Cell migration assay

The cell migration assay was conducted by using transwell chambers with a diameter of 8 µm (Millipore, Billerica, MA). The cells were seeded onto the

upper chamber with 200 µL of serum-free RPMI-1640. Then, 600 µL of RPMI-1640 containing 10% FBS were added to the bottom transwell chamber as a chemo-inducer. After 24 h, the cells in the bottom layer were fixed and stained. Further analyses were conducted through imaging and counting the number of cells in five random regions using inverted microscopy.

Invasion assay

The invasion assay was performed similar to the migration assay; however, the transwell membranes were coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) before the cell inoculation.

In vivo intraperitoneal metastasis model

All animal protocols were approved by the Institutional Animal Care and Use Committee of the Fudan University. Female 5- to 6-week-old Balb/c nude mice were obtained from Slac Laboratory Animal Co. Ltd. (Shanghai, China), maintained in a specific-pathogen free (SPF) environment and randomly assigned to the following two groups: the control group and the experimental knockdown group (six mice per group), which were intraperitoneally (i.p.) injected with NC-luc-SKOV3 and KD-luc-SKOV3 cells ($5 \times 10^6/0.2$ ml), respectively. Then, the mice were analyzed weekly at the Small Animal Bioluminescence Imaging Facility, and luciferin(150mg/kg) i.p. injected into each mouse before imaging. After 4 weeks, all mice were sacrificed, and the tumors were dissected for analysis.

Tumour metastasis real-time PCR array

The total RNA extraction was conducted on SKOV3-NONHSAT076754-siRNA1 cells and SKOV3-NC-siRNA cells with TRIZOL reagent (TAKARA, Code No.9109, Dalian, China). Then, the RNA was reverse transcribed into cDNA using an RT2 First Strand kit (Qiagen, Mississauga, ON, Canada). Following the protocol provided by the manufacturer, a cDNA library was produced and the cDNA was applied to the Human Tumour Metastasis RT2 Profiler™ PCR array (Qiagen, Mississauga, ON, Canada), which assessed 84 genes related to tumour metastatic activity. The $2^{-\Delta\Delta Ct}$ method was used to calculate the expression fold-changes.

Western blot (WB) analysis

WB was conducted as described previously [15]. The primary antibodies used were rabbit anti-GAPDH (Cell Signaling Technology, #5174, USA), anti-PTEN (Abcam, ab32199, Cambridge, UK) and anti-HTATIP2 (Abcam, ab177961, Cambridge, UK).

Data analysis

The data analysis was done through the SPSS

version 20.0 software (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism (Prism 5.0, GraphPad Software, San Diego, CA, USA). Continuous data were processed by Student's t-test (2 groups) or one-way ANOVA (>2 groups), while categorical data were analyzed by chi-square test or Fisher's exact test. P <0.05 were defined statistically significant.

Results

NONHSAT076754 expression is elevated in ovarian cancer cells

Based on existing studies [16-21], four metastasis-associated lncRNAs (NONHSAT076754, HEIRCC, IncTCF7 and ZEB2NAT) were selected and their expression levels were measured in five EOC cell lines (SKOV3, OVCAR5, OVCAR3, OVCAR8 and HO8910) and a normal human ovarian epithelial cell line (HOSEPIC). The results showed that NONHSAT076754 expression exhibited optimal consistency and that compared to its expression in HOSEPIC, NONHSAT076754 expression in ovarian cancer cells was notably higher (Fig. 1). Thus, NONHSAT076754 was selected for further clinical, functional and mechanical investigations.

Overexpression of NONHSAT076754 is associated with advanced and metastatic ovarian cancer clinical phenotype

The NONHSAT076754 expression levels in 70 EOC and 10 normal ovarian surface epithelial tissues were determined by qRT-PCR. NONHSAT076754 expression in the EOC tissue was significantly higher than that in the normal tissues (Fig. 2A). In addition, there were significant differences in NONHSAT076754 expression levels between the lymph node-positive group (with lymph node metastasis) and the lymph node-negative group (without detectable lymph node metastasis) (Fig. 2B). Additionally, EOC samples from patients with advanced FIGO stage (III-IV) cancer showed higher NONHSAT076754 expression levels than those from early FIGO stage (I-II) patients (Fig. 2C). For the clinicopathological correlation analysis, 70 EOC patients were divided into two groups, i.e., high NONHSAT076754 group (n = 35) and low NONHSAT076754 group (n = 35), according to the median relative NONHSAT076754 expression. Consequently, high NONHSAT076754 expression levels were closely associated with FIGO stage, tumor grade and lymph node metastasis while no significant differences in age, histological subtype, residual tumor diameter, CA125, ascites and distant metastasis were found (Table 2). These results illustrated that up-regulation of NONHSAT076754 might have an essential impact on EOC progression.

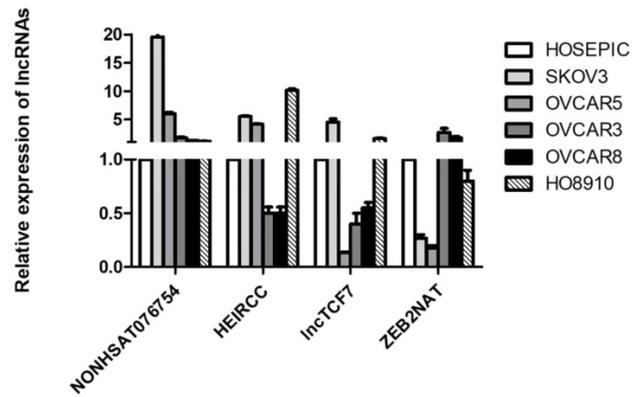


Figure 1. Expression analysis of four metastasis-associated lncRNAs in EOC cell lines (SKOV3, OVCAR5, OVCAR3, OVCAR8 and HO8910) and a normal human ovarian epithelial cell line (HOSEPIC).

Table 2. Association between NONHSAT076754 expression and clinicopathological factors in 70 EOC patients.

Variable	Low NONHSAT076754 expression (n=35) n(%)	High NONHSAT076754 expression (n=35) n(%)	P-value
Age (years)			
<50	18 (51.4)	11 (31.4)	0.090
≥50	17 (48.6)	24 (68.6)	
Histological subtype			
Serous	28 (80.0)	22 (62.9)	0.112
Others	7 (20.0)	13 (37.1)	
Tumor grade			
Low	13 (37.1)	5 (14.3)	0.029
High	22 (62.9)	30 (85.7)	
FIGO Stage			
I-II	12 (34.3)	4 (11.4)	0.023
III-IV	23 (65.7)	31 (88.6)	
Residual tumor diameter (cm)			
<1	24 (68.6)	21 (60.0)	0.454
≥1	11 (31.4)	14 (40.0)	
Lymph node metastasis			
Absent	21 (60.0)	7 (20.0)	<0.001
Present	14 (40.0)	28 (80.0)	
CA125 level (U/ml)			
<600	16 (45.7)	14 (40.0)	0.629
≥600	19 (54.3)	21 (60.0)	
Ascites			
<100	18 (51.4)	21 (60.0)	0.470
≥100	17 (48.6)	14 (40.0)	
Distant metastasis			
Absent	33 (94.3)	28 (80.0)	0.074
Present	2 (5.7)	7 (20.0)	

NONHSAT076754 silencing represses EOC cell metastasis in vitro

Because the highest NONHSAT076754 expression was observed in the SKOV3 and OVCAR5 cells (Fig. 1), the loss-of-function experiments were performed using these two EOC cell lines. Firstly, we designed three siRNAs to silence NONHSAT076754 in the SKOV3 and OVCAR5 cells. Consequently, siRNA1 and siRNA2 showed a higher knockdown efficiency, as determined by qRT-PCR (Fig. 3A).

Subsequently, to explore the role of NONHSAT076754 in cell migration and invasion, we performed wound-healing and transwell assays using NONHSA T076754-siRNA-transfected cells. The results showed that the NONHSAT076754 reduction significantly attenuated SKOV3 and OVCAR5 metastasis *in vitro* (Fig. 3B-D).

NONHSAT076754 silencing represses EOC cell metastasis *in vivo*

A model of abdominal metastasis was used to assess the metastatic role of NONHSAT076754 *in vivo*. Firstly, we established stable NONHSAT076754-knockdown 1 SKOV3 cells expressing luciferase (KD-luc-SKOV3) and the corresponding controls (NC-luc-SKOV3). Then, nude mice were i.p. injected with the above cells. The bioluminescence imaging showed that the mice injected with the KD-luc-SKOV3 cells exhibited an apparent decrease in the bioluminescence signal compared with those injected with NC-luc-SKOV3 cells (Fig. 4A and 4B). After four weeks, all mice were sacrificed, and the tumors were dissected for analysis. Consequently, the mice injected with the NC-luc-SKOV3 cells displayed multiple metastatic nodules in the abdominal cavity primarily visible on the subphrenic and mesentery spaces adjacent to the bowel. Additionally, several smaller metastatic foci were seeded throughout the abdominal cavity. In comparison, the mice injected with the KD-luc-SKOV3 cells exhibited either no or little tumour formation limited to the injection site of

abdominal wall at four weeks (Fig. 4C). Statistically, the mice injected with the NC-luc-SKOV3 cells developed more and larger metastatic nodules than those injected with the KD-luc-SKOV3 (Fig. 4D and 4E). Collectively, NONHSAT076754 silencing could repress EOC cell metastasis *in vivo*.

NONHSAT076754 affects EOC metastasis via PTEN and HTATIP2

To further investigate the potential mechanisms underlying the pro-metastatic role of NONHSAT076754 in EOC, a tumour metastasis real-time PCR array was applied to compare the metastasis-related gene alterations in SKOV3-NC with those in SKOV3-siRNA1. Consequently, six genes were tremendously dysregulated (>2-fold) after the NONHSAT076754-knockdown in the SKOV3 cells; among these genes, four genes (PTEN, FGFR4, HTATIP2, and NR4A3) were upregulated and two genes (HPSE and HRAS) were downregulated (Table 3). Subsequently, qRT-PCR and WB assays were conducted for further validation of these differentially expressed genes. Consistent with the results derived from the array, the up-regulation of PTEN and HTATIP2 was observed at both the mRNA and protein levels in the SKOV3 and OVCAR5 cells transfected with the two siRNAs (Fig. 5A and 5B). Collectively, these data indicated that the two metastasis-related genes PTEN and HTATIP2 might participate in NONHSAT076754-mediated pro-metastatic effect.

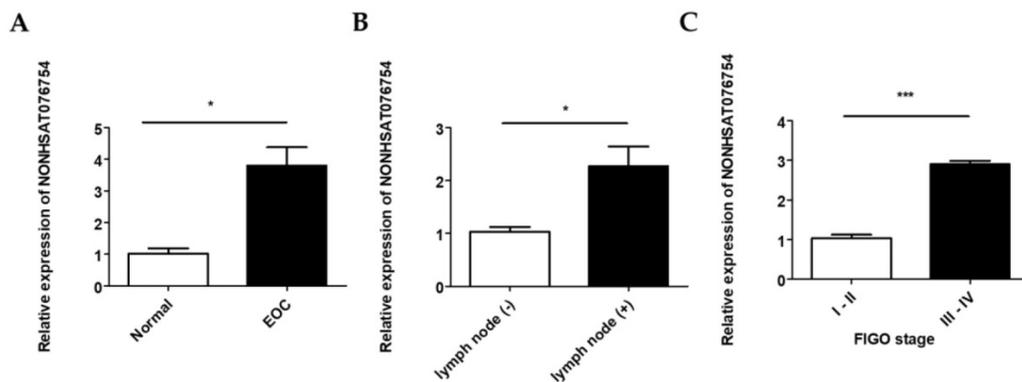


Figure 2. Relative NONHSAT076754 expression levels in EOC. **A.** Expression of NONHSAT076754 in EOC and normal ovarian tissues. **B.** Expression of NONHSAT076754 in lymph node-positive and lymph node-negative EOC samples. **C.** Expression of NONHSAT076754 in samples from EOC patients with advanced and early FIGO stage cancers. Data are shown as the mean ± SD. * $p < 0.05$, *** $p < 0.001$ vs. NC groups.

Table 3. Differential expressed genes (>2-fold) after NONHSAT076754-knockdown in SKOV3 cells as indicated by an array analysis.

Gene name	GeneBank ID	Description	Function	Fold change
HPSE	NM_006665	Heparanase	Remodeling of the extracellular matrix	0.16
HRAS	NM_005343	HRas proto-oncogene, GTPase	HRas proto-oncogene	0.17
PTEN	NM_000314	Phosphatase and tensin homolog	Tumor suppressor	5.34
FGFR4	NM_002011	Fibroblast growth factor receptor 4	Cell proliferation, cell differentiation, cell migration	4.68
HTATIP2	NM_006410	HIV-1 Tat interactive protein 2	Tumor suppressor	4.3
NR4A3	NM_006981	Nuclear receptor subfamily 4, group A, member 3	Tumor suppressor	2.26

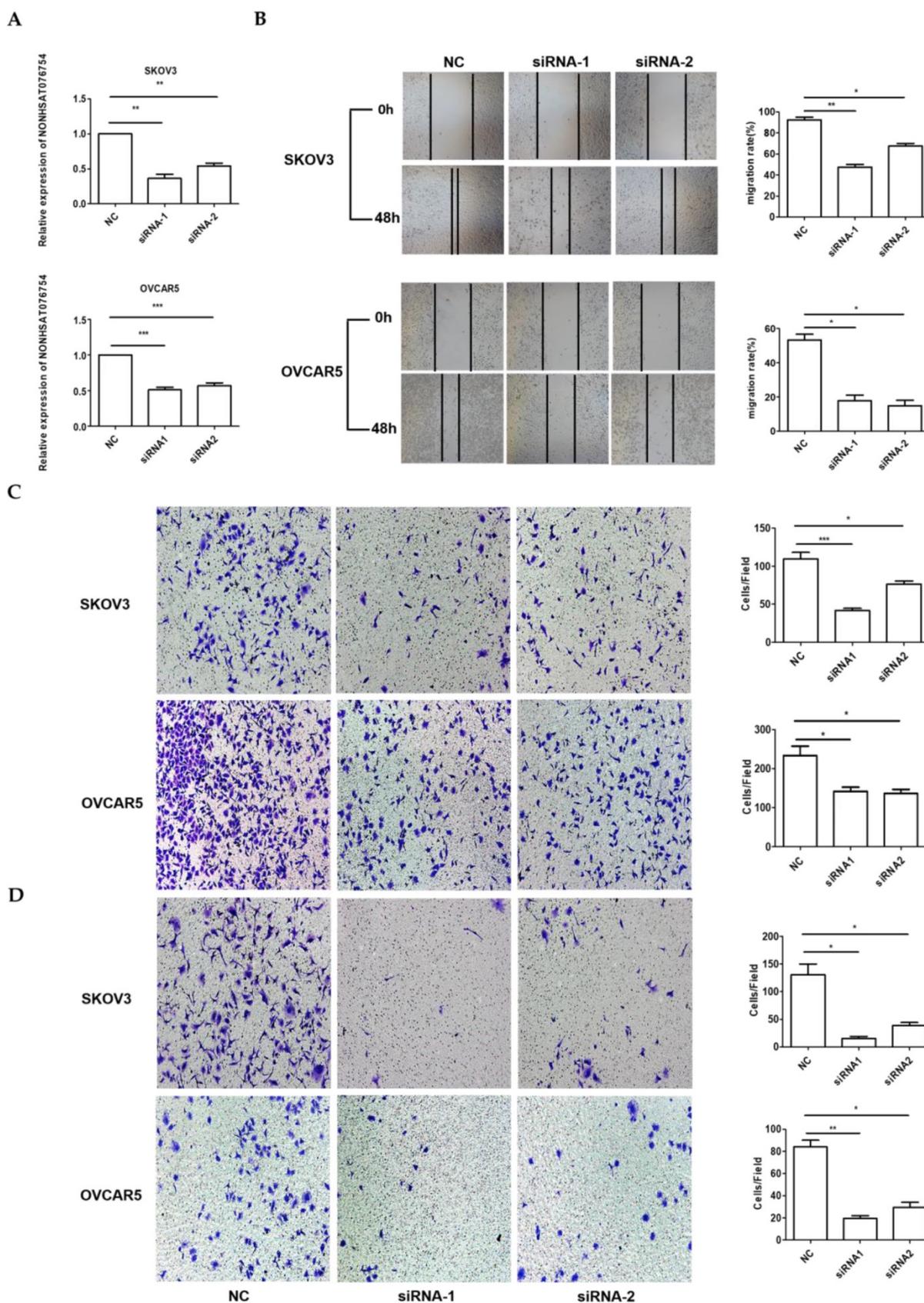


Figure 3. Knockdown of NONHSAT076754 hinders migration and invasion in SKOV3 and OVCAR5 cells. **A.** Efficiency of NONHSAT076754 silencing in SKOV3 and OVCAR5 cells transfected with siRNAs was confirmed by qRT-PCR. **B.** Migration of NONHSAT076754-knockdown cells was evaluated by wound healing assays. **C.** Transwell migration assays were conducted to confirm the cell migration activity. **D.** Invasion of NONHSAT076754-knockdown cells was detected by transwell invasion assays. Data are shown as the mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. NC groups.

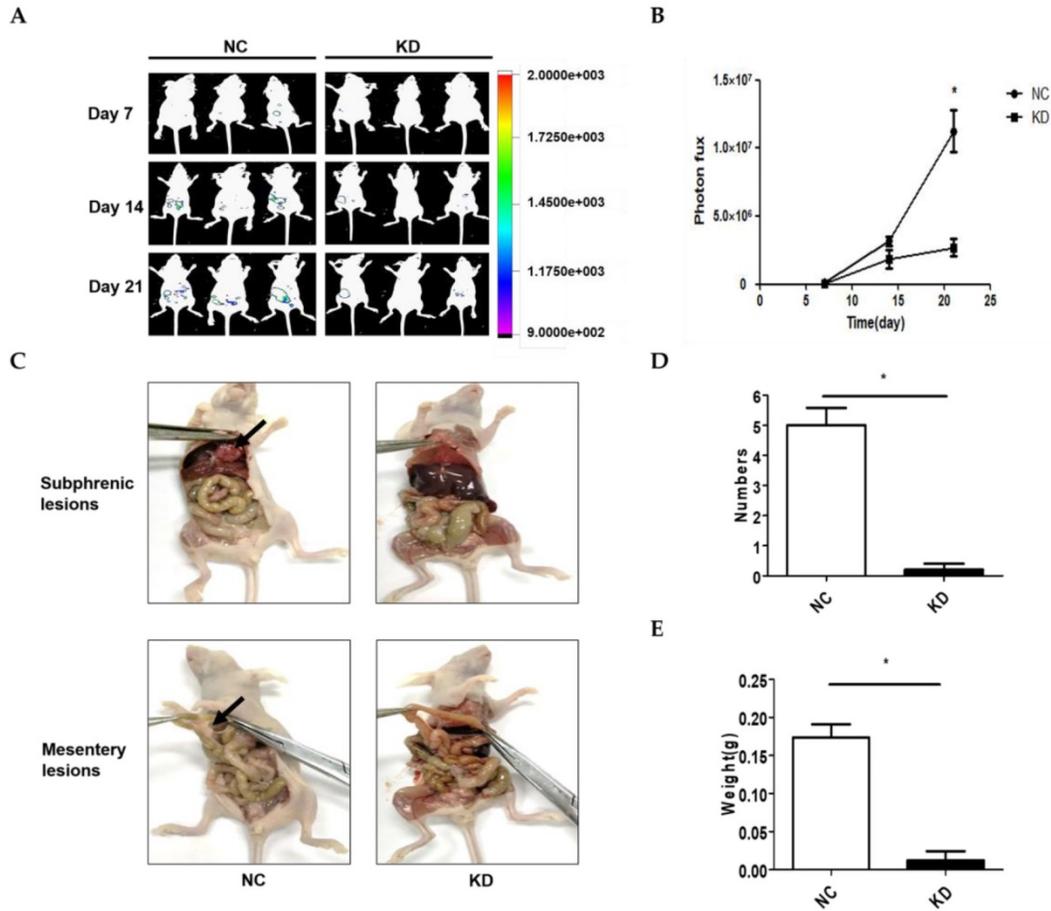


Figure 4. NONHSAT076754 silencing suppresses metastasis in an SKOV3-luc ovarian cancer intraperitoneal metastasis model. A. Representative images of luciferase signals in the KD-luc-SKOV3 groups compared with those in the NC-luc-SKOV3. **B.** Quantification of the photon flux from the metastatic lesions. **C.** Representative views of visible metastasis in the peritoneal cavity. **D.** Analysis of the numbers of dissected tumors. **E.** Analysis of the weight of dissected tumors. Data are shown as mean ± SD. * p < 0.05 vs. NC groups.

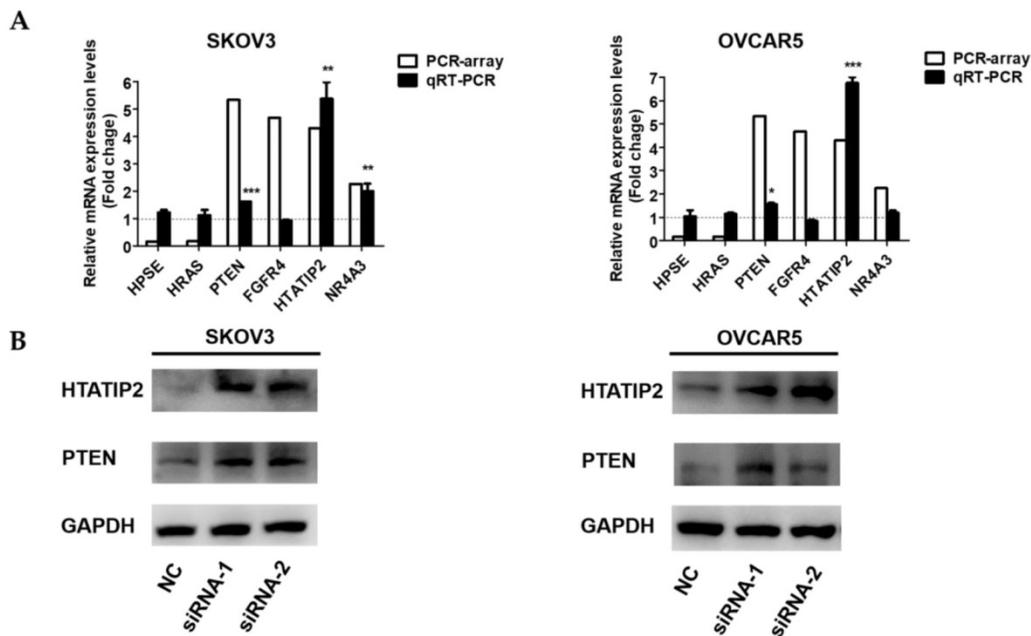


Figure 5. NONHSAT076754 regulates EOC metastasis via the downstream metastasis-related genes PTEN and HTATIP2. A. qRT-PCR examination of relative PTEN and HTATIP2 expression in SKOV3 and OVCAR5 transfected with NC or NONHSAT076754-siRNA. **B.** WB verification of PTEN and HTATIP2 expression at the protein level in SKOV3 and OVCAR5 cells transfected with NC or NONHSAT076754-siRNA. Data are shown as the mean ± SD. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. NC groups.

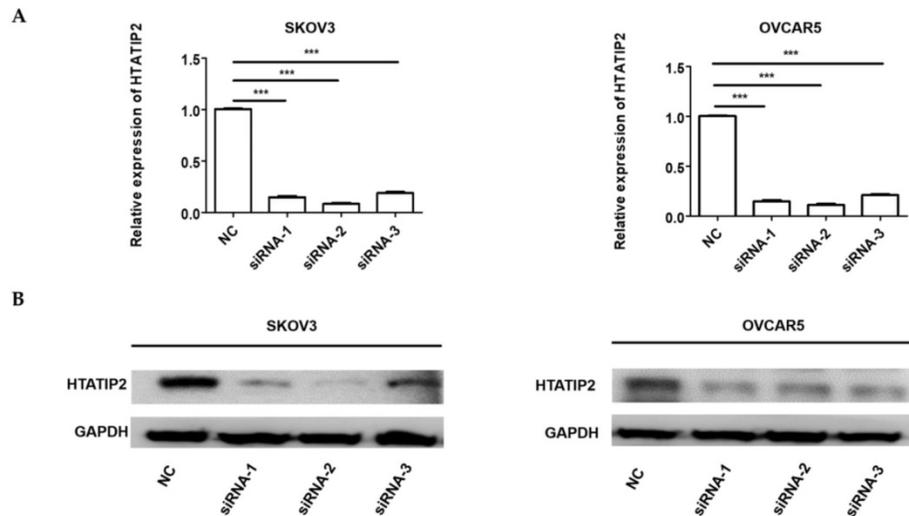


Figure 6. Expression of HTATIP2 is silenced by siRNAs in SKOV3 and OVCAR5 cells. A. The mRNA expression levels of HTATIP2 in SKOV3 and OVCAR5 cells transfected with siRNAs were examined by qRT-PCR. **B.** The protein expression levels of HTATIP2 in SKOV3 and OVCAR5 cells after transfection of siRNAs were determined by WB assays. The results are shown as the mean \pm SD. *** $p < 0.001$ vs. NC groups.

Knockdown of HTATIP2 significantly rescues the repressive effects of silencing NONHAT076754 on EOC metastasis

Since HTATIP2 was the most markedly altered genes when NONHAT076754 was silenced in both two EOC cells (SKOV3 and OVCAR5), we further attempted to ascertain whether NONHAT076754 acted its pro-metastatic effect partly through its target HTATIP2. Firstly, three siRNAs (siRNA-1, siRNA-2 and siRNA-3) targeting HTATIP2 were synthesized, among which, siRNA-2 acquired the most significant knockdown efficiency in SKOV3 and OVCAR5 cells at both mRNA and protein levels (Fig. 6A and 6B). Therefore, siRNA-2 was used for further co-transfection assays. Secondly, two EOC cells were co-transfected NONHAT076754-siRNA or NC with HTATIP2-siRNA. Consequently, the results showed that knockdown of HTATIP2 partially reversed the inhibitory effect of silencing NONHAT076754 on EOC migration (Fig. 7A-C), and invasion (Fig. 7D). These data further determined that NONHAT076754 might alternate cell migration, and invasion partly through its modulation on HTATIP2.

Discussion

Ovarian cancer ranks among the most common and lethal malignant diseases worldwide. The poor prognosis of ovarian cancer is primarily linked to the occurrence of metastasis, which is a complex process that is poorly understood [2]. Recently, accumulating evidence has indicated that lncRNAs play prominent roles in tumour metastasis [25]. Although various lncRNAs have been reported to act as crucial determinants of cancer metastasis, knowledge regarding the metastasis-associated

lncRNAs involved in EOC is still limited. Therefore, further studies investigating lncRNAs in EOC metastasis are imperative for improving the clinical diagnosis and treatment of EOC.

In this investigation, we identified four metastasis-associated lncRNAs including NONHSAT 076754, HEIRCC, lncTCF7 and ZEB2NAT, whose roles in EOC have not been explored. Among these lncRNAs, NONHSAT076754 attracted much interest as the most significantly differentially expressed lncRNA in EOC cell lines and tissues. NONHSAT076754, which is a newly functionally characterized lncRNA, was initially implicated in PTC and elevation in NONHSAT076754 led to PTC progression [16]. To the best of our knowledge, this study is the first to evaluate the contribution of NONHSAT076754 to EOC metastasis.

Here, we confirmed that high NONHSAT076754 expression was linked to aggressive clinicopathological variables including advanced tumor stage, high tumor grade and lymph node metastasis. Interestingly, there was no significant difference (P -value 0.074) between NONHSAT076754 expression and distant metastasis, which might due to the limitation of tissue bank-derived clinical samples and small sample size. Therefore, clinical observation with larger sample size was essential to ascertain the real relevance in future. Furthermore, the primary tumors with lymph node metastasis expressed higher NONHSAT076754 levels than those in the non-lymph node metastasis groups, suggesting that this lncRNA is highly related to the metastatic potential of EOC. Based on the expression and clinical features of NONHSAT076754, we speculated that this molecule might perform a critical function in the biological process of EOC metastasis.

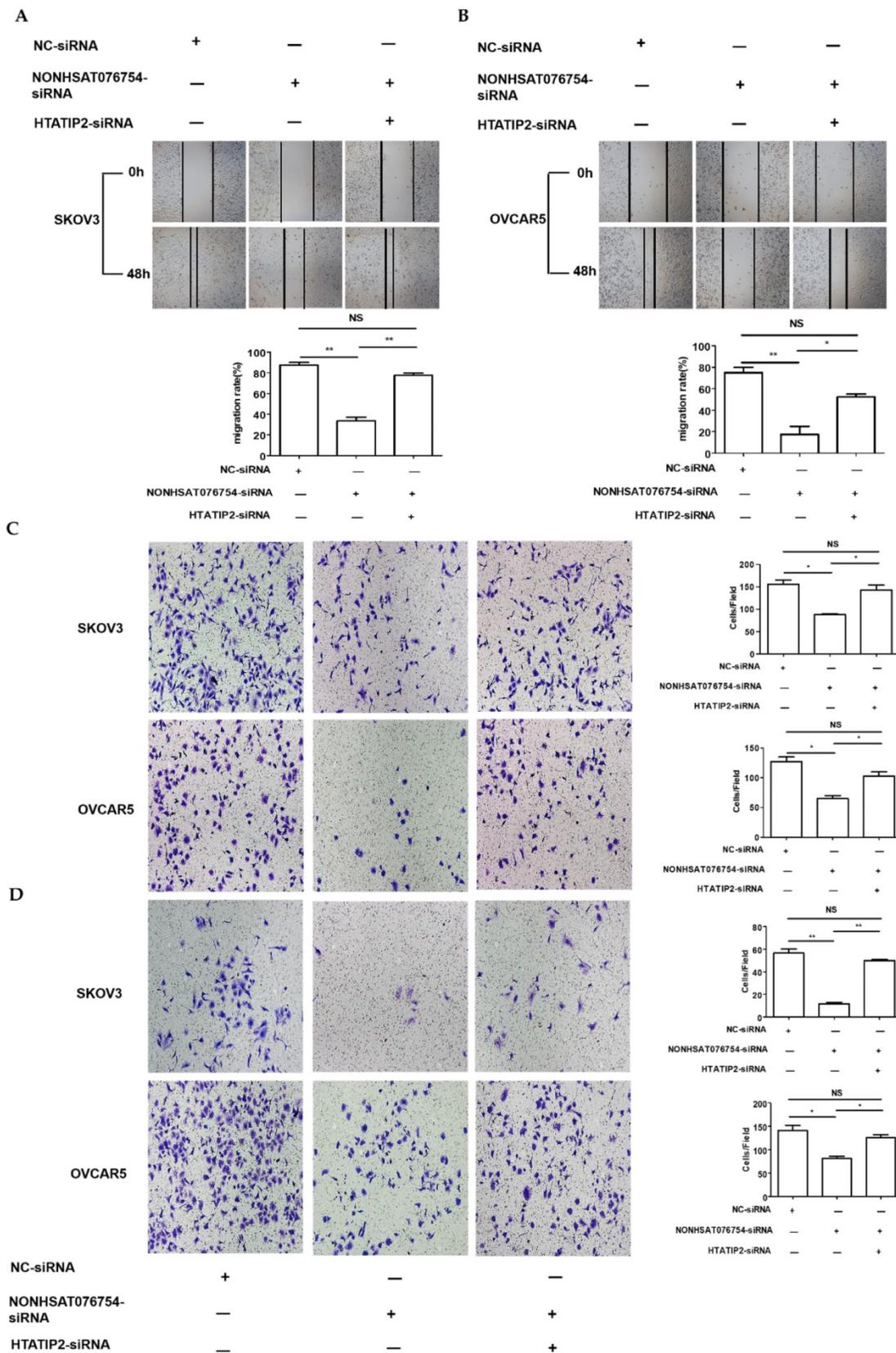


Figure 7. Knockdown of HTATIP2 partially reverses NONHAT076754 silencer-induced repressive effects on EOC metastasis. A. Knockdown of HTATIP2 partially reversed NONHAT076754 silencer-induced inhibition of migration in SKOV3 detected by wound healing assays. **B.** Knockdown of HTATIP2 partially reversed NONHAT076754 silencer-induced inhibition of migration in OVCAR5 cells detected by wound healing assays. **C.** Transwell migration assays confirmed the rescue action of HTATIP2 interference on altered migration activity caused by NONHAT076754-siRNA. **D.** Knockdown of HTATIP2 partially reversed NONHAT076754 silencer-generated anti-invasion in SKOV3 and OVCAR5 cells determined by transwell invasion assays. Data are shown as the mean \pm SD. * $p < 0.05$, ** $p < 0.01$ vs. NC groups.

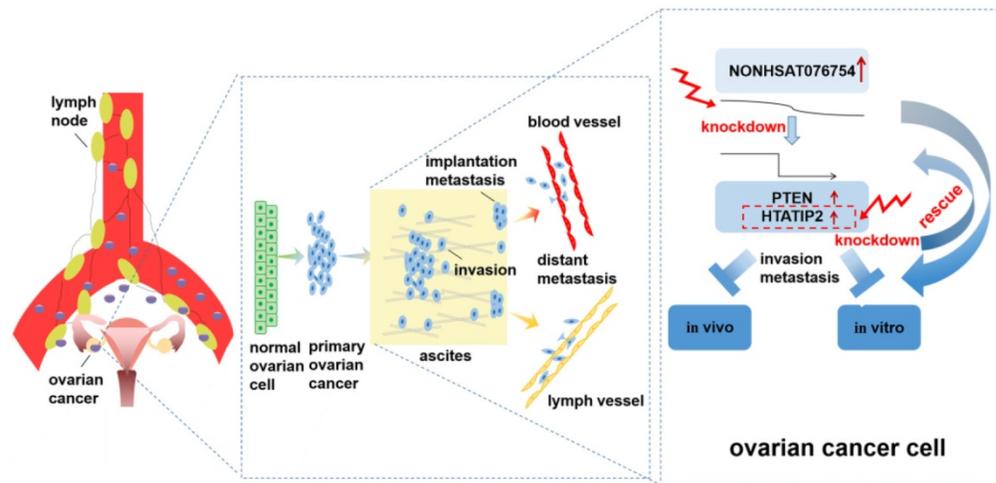


Figure 8. Schematic diagram of the mechanism proposed in this research. NONHSAT076754 was overexpressed in EOC. NONHSAT076754 knockdown markedly inhibited EOC invasion and metastasis *in vitro* and *in vivo*. Mechanically, two metastasis-related genes PTEN and HTATIP2 might participate in NONHSAT076754-mediated pro-metastatic effect. Further rescue assays validated that knockdown of HTATIP2 remarkably reversed NONHSAT076754 silencer-induced inhibition of EOC cell metastasis.

To verify our hypothesis, we subsequently performed a set of *in vitro* and *in vivo* assays. The cell function experiments investigating migration and invasion showed that the depletion of NONHSAT076754 impaired the metastatic and invasive potential *in vitro*. Consistently, by using an animal model of abdominal metastasis, the results from both the bioluminescence imaging and tumor dissection showed that NONHSAT076754 silencing in nude mice resulted in a remarkable reduction in the number, weight and range of the tumor. Taken together, these results suggest that NONHSAT076754 has a pro-metastasis effect on EOC.

Since the metastatic value of NONHSAT076754 in EOC was determined, we further supposed whether certain metastasis-related downstream genes were affected by NONHSAT076754. To date, the mechanisms underlying NONHSAT076754-mediated pro-metastasis behavior are unclear. Metastasis is a complex process when multiple events occur and plenty of genes are dysregulated [26]. To systematically explore the relevant molecular regulation of NONHSAT076754, a tumour metastasis real-time PCR array containing 84 well-known genes that are well known to be associated with tumour metastasis was applied. The subsequent validation of the array data confirmed that the expression of PTEN and HTATIP2, two well-known tumor suppressors [27-31], was altered at both mRNA and protein levels after the knockdown of NONHSAT076754. Further rescue assays determined that the repressive effects of silencing NONHSAT076754 on metastasis could be reversed by co-transfection with HTATIP2-siRNA. Collectively, this study implied that NONHSAT076754 facilitated EOC aggressiveness and metastasis

partially by modulating HTATIP2. Notably, future efforts are needed to fully understand the direct or indirect regulatory relationship between NONHSAT076754 and downstream genes. Moreover, pertaining to clinical application, more clinical data, including blood specimen and prognosis of patients inaccessible in this investigation for limitation in tissue bank-derived samples, are necessary to assess the effectiveness of NONHSAT076754 in clinical management.

Conclusion

In conclusion, the present study provides the first evidence that the overexpression of NONHSAT076754 is associated with advanced tumor stage, high tumor grade and lymph node metastasis in EOC. Furthermore, NONHSAT076754 plays a pro-metastatic role in EOC by regulating certain metastasis-associated genes. Particularly, HTATIP2 might be the target downstream gene of NONHSAT076754 due to rescue action of HTATIP2 interference on altered migration and invasion activity caused by NONHSAT076754-siRNA (Fig. 8). This data highlight the value of NONHSAT076754 in EOC progression and imply that this molecule is a potentially crucial predictor of EOC metastasis.

Abbreviations

EOC: epithelial ovarian cancer; OC: ovarian cancer; lncRNAs: long non-coding RNAs; PTC: papillary thyroid cancer; FBS: fetal bovine serum; siRNA: small interfering RNA; NC: negative control; qRT-PCR: quantitative real-time polymerase chain reaction; PBS: phosphate-buffer-solution; SPF: specific-pathogen free; WB: western blot.

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Competing Interests

The authors have declared that no competing interest exists.

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