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Research Paper

Long non-coding RNA HOXA-AS2 promotes tumorigenesis and predicts poor prognosis in ovarian cancer

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ABSTRACT

Recent reports confirms that there are some long non-coding RNAs involved in the different biological regulatories in cancer, including proliferation and apoptosis. However, the correlation between the HOXA-AS2 expression and proliferation in epithelial ovarian cancer was not completely investigated. *In vivo* expression of HOXA-AS2 in 73 epithelial ovarian cancer tissues and 57 normal tissues was determined by qRT-PCR. Results showed that not only HOXA-AS2 was overexpressed in epithelial ovarian cancer tissues, but also had a relationship with the tumor size and FIGO stage of patients. Further, the overall survival in epithelial ovarian cancer patients presenting high expression of HOXA-AS2 was lower to those presenting low expression. Next, the functional effect of HOXA-AS2 was explored via GSEA analysis in ovarian cancer datasets. The results confirmed that HOXA-AS2 was enriched in cell apoptosis and JAK-STAT pathway. Then, EOC cell lines were transfected with HOXA-AS2 siRNA. Cell proliferation was significantly inhibited after knockdown of HOXA-AS2 via promoting apoptosis both in HO-8910 and SKOV3 cells. Reversely, overexpression of HOXA-AS2 in normal ovarian cell line could promote proliferation and inhibit apoptosis. Animal study consistently indicated that lower expression of HOXA-AS2 inhibited proliferation of HO-8910 cells. Finally, Western blot results showed that expressions of procaspase-9, -8, -3, and PARP were decreased after knockdown of HOXA-AS2, while cleaved Caspase-9, -8 and -3 were increased. Meanwhile, expressions of Bcl-2 and Bid were decreased and Bax was increased. In addition, overexpression of HOXA-AS2 increased the level of Bcl-2, Bid, JAK2 and Stat3 while Bax was decreased in HO8910 and SKOV3. Besides, the IHC assay also showed the same expression changes of Bcl-2 and Bax. To sum up, this study suggested that lncRNA HOXA-AS2 acted as an oncogene in EOC that partly inhibited the intrinsic mitochondria pathway and extrinsic death receptor pathway, which

was associated with Bcl-2 protein family; HOXA-AS2 would be regarded as a new biological prognosis and potential therapeutic target of epithelial ovarian cancer.

INTRODUCTION

Epithelial ovarian cancer (EOC) is the most fatal malignancy in the female reproductive system and ranked the fifth in female cancer death [1-3]. Most patients with EOC presented advanced stages of disease when diagnosed. Although most patients with advanced disease underwent surgery followed by platinum-taxane-based chemotherapy, median survival was only about 3-4 years because of approximately 80–90% developing recurrences and metastasis [4, 5]. Therefore, it is urgent for better understanding of the molecular mechanisms of epithelial ovarian carcinogenesis, thus providing basic for the diagnosis and therapy of EOC.

LncRNA is considered to be a RNA molecule with over 200 nucleotides (nt) which dose not have the translation function into proteins [6, 7]. LncRNAs have the ability to modulate gene expressions within different biological levels included transcriptional and post-transcriptional regulation [8, 9]. Moreover, lncRNAs have been confirmed to regulate the tumorigenesis, proliferation, and metastasis via diverse mechanisms [10-12]. Recently, more and more studies have revealed that lncRNAs acted as important molecule in the development and progression of EOC. For example, the pro-metastatic effect of HOTAIR and ANRIL were regulated, at least in part, by the certain matrix metalloproteinases (MMPs) and genes related to epithelial-to-mesenchymal transition (EMT), which were associated with poor prognosis of EOC [13, 14]. LncRNA HOST2 promoted cell proliferation, migration, and invasion by sponging microRNA let-7b in EOC [15]. Although the dysregulation of lncRNAs has been shown in EOC, the overall pathophysiological effects of lncRNAs on EOC remain unknown.

In human, studies have proved that homebox (HOXA) A gene contained transcription factors which promoted embryogenesis and tumorgenesis in diverse tumors [16-20]. Besides, many studies showed that the dysregulated HOX gene played a crucial role in ovarian cancer. For example, HOXA11-AS promoted cell proliferation and invasion, it also predicted poor outcome in serous ovarian cancer [21]. HOTAIR controlled the expression of Rab22a by sponging miR-373 in ovarian cancer [22]. These findings indicated that the HOXA gene translational noncoding RNA may affect a lot in ovarian cancer.

As a long non-coding RNA as HOXA cluster antisense RNA 2 (HOXA-AS2) it is, HOXA-AS2 is positioned between the HOXA3 and HOXA4 genes in the HOXA cluster. Previous studies implied that after decreasing the HOXA-AS2 expression by shRNA transduction, the amount of viable cells were decreased but the ratio of apoptotic cells were increased determined by annexin V binding and Western blot results. HOXA-AS2 promoted proliferation of gastric cancer cells when P21/PLK3/DDIT3 expression was epigenetically silenced [11]. Unfortunately, the effects and importance of HOXA-AS2 in EOC have not been fully investigated. As this study presented, insilicoMerging and Rankprod packages were utilized to perform microarrays for gene expressions of lncRNAs profiling (GSE36668, GSE38666 and GSE52037) in one independent dataset from the Gene Expression Omnibus (GEO) by a local computer. After complete analysis on lncRNA expression profiles, HOXA-AS2 was identified as a new candidate lncRNA that was capable of promoting development of EOC. Further, HOXA-AS2 upregulation was found to be related with large tumor size, advanced clinical pathologic stage and short overall survival of EOC patients. Next, its biological effects showed that HOXA-AS2 had the ability to promote EOC cell growth via inhibiting the intrinsic mitochondria pathway and extrinsic death receptor pathway both *in vitro* and *in vivo*, which were associated with the Bcl-2 protein family. Altogether, our results indicated that HOXA-AS2 served as an oncogene in EOC development and could be a new prognosis as well as a potential therapeutic target of EOC.

RESULTS

Expression of HOXA-AS2 is upregulated in EOC tissues

First, expression of HOXA-AS2 in human EOC tissues was investigated by raw microarray data downloaded from GEO. As we pointed out, HOXA-AS2 expression was significantly increased in epithelial ovarian cancerous tissues when comparing with normal tissues via Rankprod R package ($p < 0.001$) (Figure 1A). In order to validate this finding, we detected the HOXA-AS2 expression in 73 OC tissues and 57 normal tissues by qRT-PCR, which was normalized to GAPDH. The result showed that HOXA-AS2 was up-regulated in EOC group (Figure 1B). Further, 73 OC patients were assigned into two groups based on the HOXA-AS2 expression in tumor tissues: relative low group (n = 37) and relative high group (n = 36) (Figure 1C). Relationship between the HOXA-AS2 expression and clinical pathological characteristics elucidated that overexpressed HOXA-AS2 was associated with FIGO stage ($p = 0.011$) and tumor size ($p = 0.007$), but not related to other clinical characteristics included age, histological grade, lymph node metastasis, or CA125 level (Table 1). When investigating overall survival, we found that overexpressed HOXA-AS2 was confirmed to be related with poor outcome ($p < 0.001$, log-rank test; Figure 1D). Altogether, our data suggested that the highly expressed HOXA-AS2 was an oncogene in the EOC.

HOXA-AS2 is a cell apoptosis–associated long non-coding RNA in ovarian cancer by GSEA analysis

To identify the mechanism of HOXA-AS2 involved in ovarian cancer. We divided the samples from TCGA (n=379), GSE9891 (n=285) and GSE63885 (n=101) into two groups independent by the average expression of HOXA-AS2. Then the kegg pathway analysis by GSEA was preformed, and the overlapping results in these three datasets showed that the kegg pathway was enriched in apoptosis (Figure 2A). Besides, the JAK-STAT signaling pathway was generally activated in these three datasets (Figure 2B). In addition, the kegg pathway in cancer was consistently enriched (Figure 2C). The above results underlid that HOXA-AS2 played an important role in ovarian cancer and regulated apoptosis in ovarian cancer via JAK-STAT signaling pathway.

Knockdown of HOXA-AS2 inhibits proliferation of EOC cells and results in apoptosis *in vitro*

Based on the obtained results, we next focused on the biological effect of HOXA-AS2 on EOC cells, first, the corresponding expression of HOXA-AS2 in different OC cell lines was explored by qRT-PCR. There was a more increased expression of HOXA-AS2 in three cancer cell lines (SKOV3, HO-8910, and HEY) compared with a human normal ovarian cell line (IOSE386), especially in SKOV3 and HO-8910 cell lines (Figure 3A), therefore, the two cell cells were selected for further experiments. Then HOXA-AS2 siRNA was transfected into HO-8910 and SKOV3 cell lines. QRT-PCR verified that the HOXA-AS2 expression was sharply decreased by HOXA-AS2 siRNA in contrast with si-NC, especially si-HOXA-AS2-1 in HO-8910 cell line and SKOV3 cell line (Figure 3B). Subsequently, the most effective si-HOXA-AS2-1 was selected for the further studies. CCK8 and colony-formation assay revealed that there was a significant inhibitory after knockdown of HOXA-AS2 on cell proliferation both in HO-8910 and SKOV3 cell lines than the control cells (Figure 3C, 3D). In addition, the Edu(red)/Hoechst(blue) immunostaining assay presented an inhibited cell proliferation *in vitro* in cells with knockdown of HOXA-AS2 (Figure 3F). Also, flow cytometric analysis revealed that there was a dramatic increase of apoptosis both in HO-8910 and SKOV3 cell lines after knockdown of HOXA-AS2 (Figure 3E). The above results suggested that lncRNA HOXA-AS2 may serve as an oncogene to promote proliferation and inhibit apoptosis.

Over-expression of HOXA-AS2 promotes the proliferative ability of IOSE-386 cell line

When we explored the significance of HOXA-AS2 in normal ovarian epithelial cell lines, pcDNA-NC and

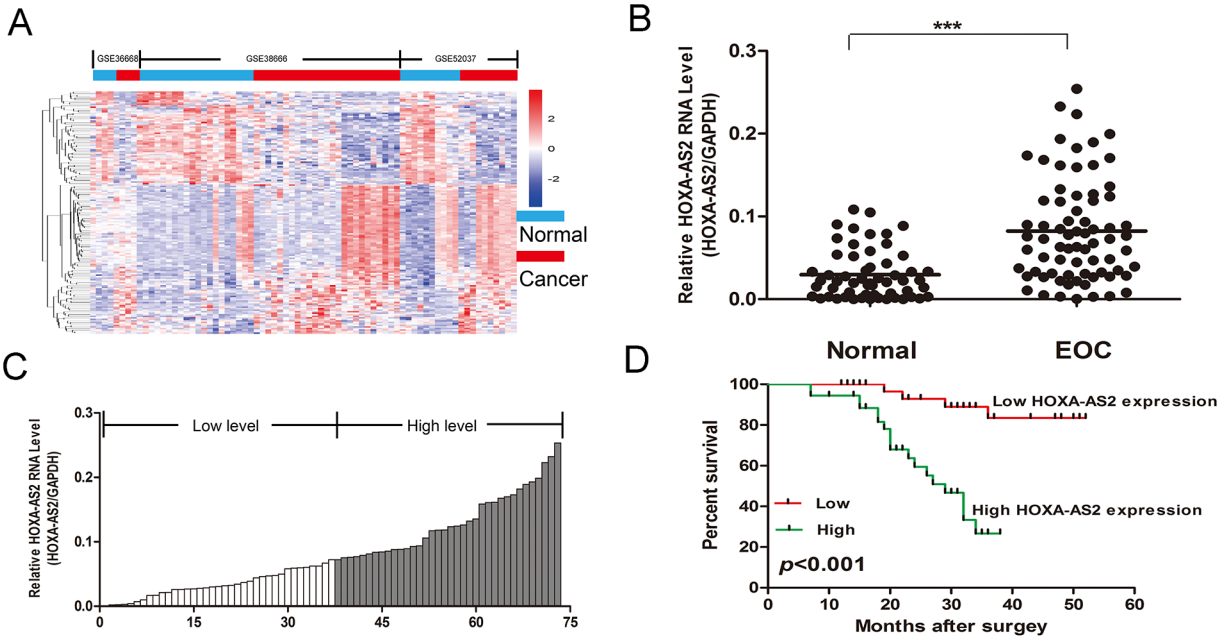

Figure 1: HOXA-AS2 expression is upregulated in epithelial ovarian cancer. (A) Screen of EOC-related lncRNAs in GEO database. LncRNA HOXA-AS2 was overexpressed in EOC tissues compared to the normal tissues in GSE36668, GSE38666 and GSE52037. **(B)** Relative expression of HOXA-AS2 in EOC tissues (n = 73) compared with normal tissues (n = 57). HOXA-AS2 expression was examined by qPCR and normalized to GAPDH expression. **(C)** Patients were divided into two groups according to the median value of relative HOXA-AS2 expression. **(D)** Postoperative Kaplan Meier analysis based on HOXA-AS2 expression in 73 EOC patients. $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001.

pcDNA-HOXA-AS2 were transfected into IOSE-386 cell line. The result showed that there was an up-regulated expression of HOXA-AS2 compared to the pcDNA-NC group (Figure 4A). Besides, the cell viability in pcDNA-HOXA-AS2 group was higher than that of pcDNA-NC group (Figure 4B). Additionally, EDU assay also indicated that HOXA-AS2 enhanced the proliferation capacity of IOSE-386 cell line (Figure 4D). At last, the flow cytometric analysis pointed out that over-expression of HOXA-AS2 could decrease the cell apoptosis in IOSE-386 cell line (Figure 4C). It was concluded that HOXA-AS2 had the ability to promote proliferation of IOSE-386 cell line.

Knockdown of HOXA-AS2 inhibits EOC cells proliferation *in vivo*

To confirm whether there was a similar effect of lncRNA HOXA-AS2 *in vivo*, HO-8910 cells transfected with sh-HOXA-AS2 or empty vector were subcutaneously injected into nude mice. Xenograft tumors were all developed at the injection site and mice were sacrificed 21 days after the transplantation. Results presented that tumors derived from cells with HOXA-AS2 knockdown developed slower than those from empty vector group (Figure 5A). The average tumor size derived from the sh-HOXA-AS2 group was significantly smaller than that from the empty vector group ($p < 0.01$, Figure 5B). Further, tumor weight in the sh-HOXA-AS2 group was significantly slower than that in the empty vector group (Figure 5C). Consistently, qRT-PCR analysis revealed a lower HOXA-AS2 expression in tumor tissues derived from sh-HOXA-AS2 cells than those derived from the empty vector group (Figure 5D). Immunostaining revealed that tumors developed from sh-HOXA-AS2 cells presented lower expression of Ki-67 and fewer PCNA staining than those formed from empty vector transfected

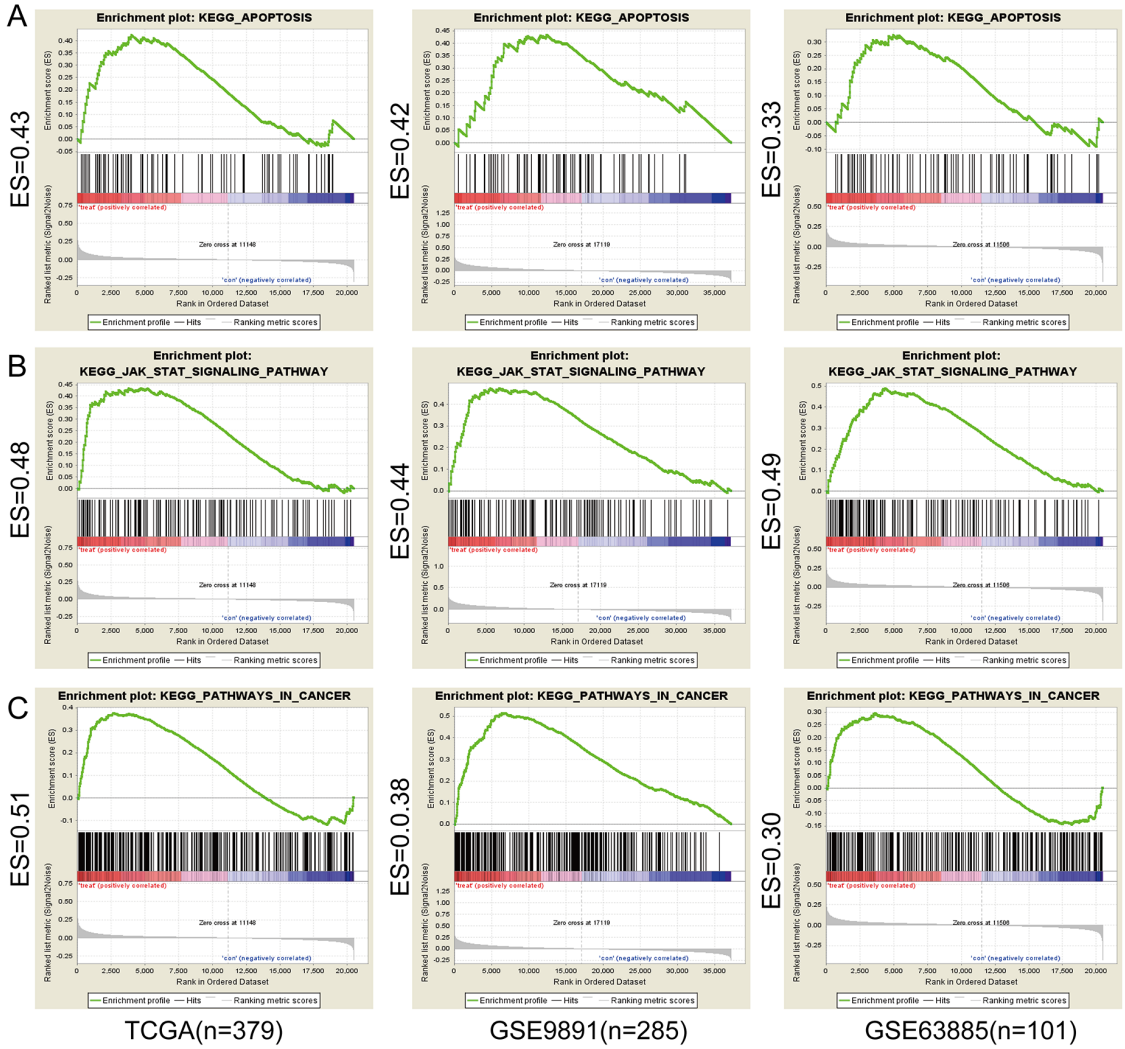

Figure 2: Gene sets enrichment analysis of HOXA-AS2 in TCGA and GEO datasets. (A) Apoptosis pathway was enriched in TCGA and GEO datasets. **(B and C)** The JAK-STAT signaling pathway and pathways in cancer were enriched in TCGA and GEO datasets.

cells (Figure 5E and 5F). These results further indicated an association between overexpressed HOXA-AS2 and the proliferative capacity of EOC cells *in vivo*.

Molecular mechanism of lncRNA HOXA-AS2

Western blot here was used to access the expressions of downstream genes of lncRNA HOXA-AS2 (Figure 6). First, we detected the corresponding protein levels of JAK-STAT signaling pathway, the results showed that this pathway was silenced in the si-HOXA-AS2 group (Figure 6E and 6F). Then, the apoptosis associated factors were detected. In accordance to the functional characterization *in vitro*, knockdown of HOXA-AS2 led to downregulation of anti-apoptosis related factors (Bcl-2, Bid, procaspase3, PARP, procaspase8, and procaspase9) in HO-8910 and SKOV3 cell lines compared with the control cells, whilst apoptosis factors (Bax, cleaved Caspase-3, cleaved

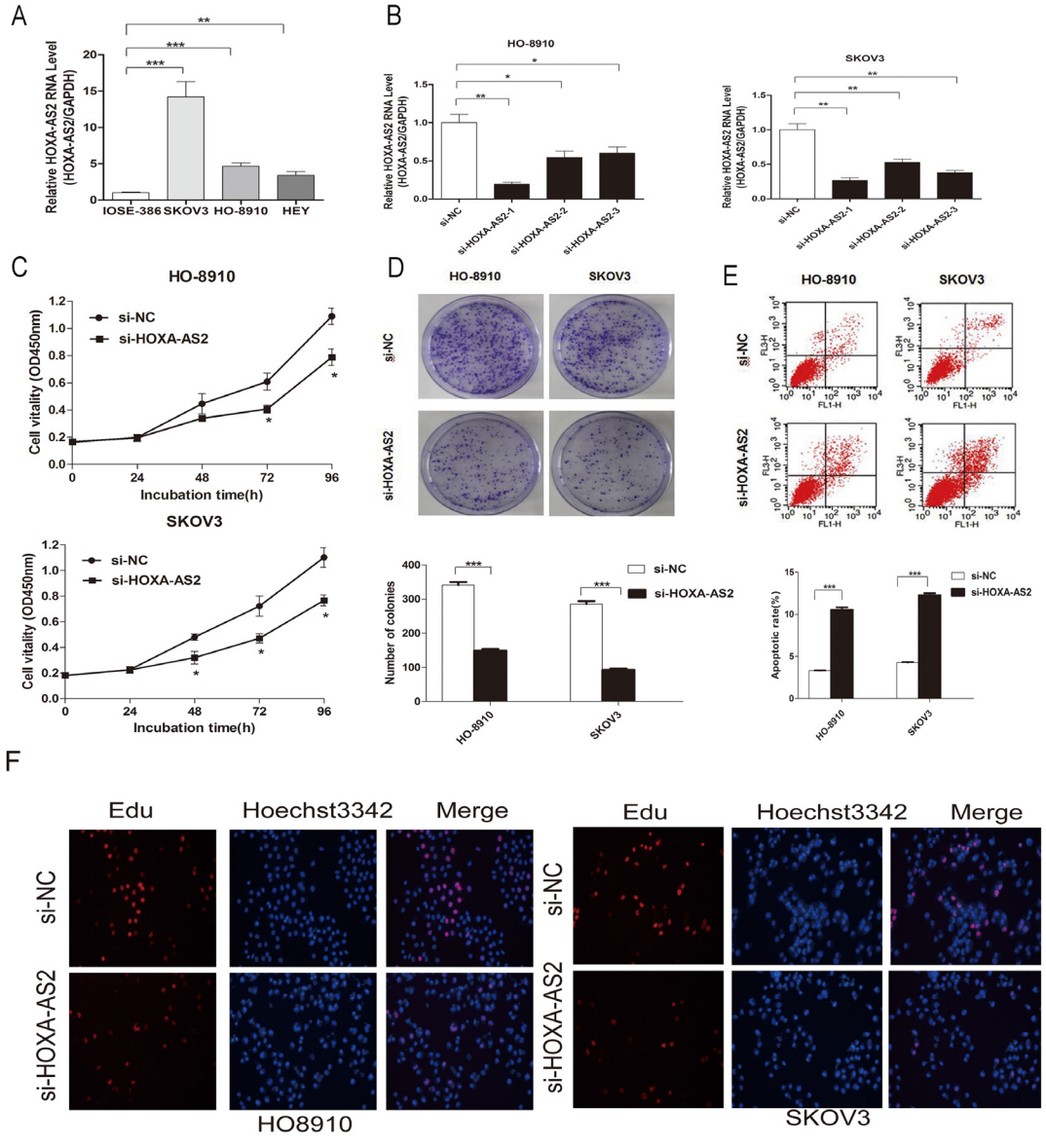

Figure 3: Knockdown of HOXA-AS2 inhibits EOC cells proliferation and promotes apoptosis *in vitro*. **(A)** qRT-PCR analysis of HOXA-AS2 expression in normal ovarian cells (IOSE-386) and EOC cell (SKOV3, HO-8910, and HEY). **(B)** Three small interfering RNAs (si-HOXA-AS2-1, si-HOXA-AS2-2, si-HOXA-AS2-3) against HOXA-AS2 were designed; knockdown efficiency was determined by qRT-PCR in HO-8910 and SKOV3 cells. **(C)** Knockdown of HOXA-AS2 in HO-8910 and SKOV3 cells significantly reduced their proliferative capacities, as determined by CCK8 assay. **(D)** Knockdown of HOXA-AS2 in HO-8910 and SKOV3 cells significantly reduced colony formation capacity. **(E)** Knockdown of HOXA-AS2 in HO-8910 and SKOV3 cells caused a dramatic increase of apoptosis. **(F)** Effect of HOXA-AS2 on proliferation of EOC cells confirmed by EdU assay. The Click-it reaction revealed Edu staining (red). Cell nuclei was stained with Hoechst 3342 (blue). Representative images and data were based on three independent experiments. $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001.

Caspase-8, and cleaved Caspase-9) were significantly upregulated (Figure 6A, 6B, 6C and 6D). It indicated that knockdown of HOXA-AS2 induced apoptosis of EOC cells through the intrinsic mitochondria pathway and extrinsic death receptor pathway, which were confirmed to be associated with Bcl-2 protein family. Finally, immunostaining of the anti-apoptosis factors (Bcl-2, Bid) in the sh-HOXA-AS2 group were fewer than those formed from cells transfected with empty vector (Figure 5E and 5F). On the contrary, the immunostaining of apoptosis factor (Bax) in the sh-HOXA-AS2 group was more than those formed from cells transfected with empty vector (Figure 5E and 5F). To clarify the specific mechanism of HOXA-AS2, we performed gain-function assay in SKOV3 and HO-8910 cell lines. Results showed that overexpression of HOXA-AS2 could decrease the expression of Bax and increase the expressions of Bcl-2 and Bid. In addition, the JAK-STAT pathway was significantly activated in the pcDNA-HOXA-AS2 group (Figure 7A and 7B).

Relationship between HOXA-AS2 and other HOX(A) gene family members

Relationship between HOXA-AS2 and other HOX(A) family genes in this study was also explored, we computed the Pearson relationship between HOXA-AS2 and all other protein coding genes in TCGA, GSE9891 and GSE63885. Then, we selected the top 5 positive related genes in these three datasets and we found that the top five positive related genes were all HOXA1, HOXA2, HOXA3, HOXA4 and HOXA5, suggesting a strong relationship between HOXA-AS2 and these five HOX(A) genes (Supplementary Table 1).

DISCUSSION

Over the past decade, abundant research have shown that lncRNA expressions were aberrant in human cancers [24]. Abnormal lncRNA expression in EOC could greatly contribute to tumorigenesis, as exemplified by HOTAIR [13], AB073614 [25], and LSINCT5 [26]. As a consequence, identification of EOC-related lncRNAs, consideration of their clinical importance and biological actions may be helpful to clarify the potential application of lncRNAs in diagnosing and treating of EOC. Previous studies showed that upregulateion of HOXA-AS2 promote proliferation and induces EMT in gallbladder cancer [27]. Besides, HOXA-AS2 also promote gastric cancer proliferation by silencing P21/PLK3/DDIT3 [28]. But the function of HOXA-AS2 remains unclear in ovarian cancer.

As we found in this study, the overexpressed HOXA-AS2 was more obvious in 73 EOC tissues and 3 cancer cell lines than normal tissues and ovarian epithelial cell lines. Additionally, higher expression of HOXA-AS2 was related to worse overall survival. Our study confirmed that lncRNA HOXA-AS2 was capable of regulating EOC progression and could serve as a new prognostic biomarker for EOC. Besides, the apoptosis pathway and JAK-STAT pathway were enriched in HOXA-AS2 higher group.

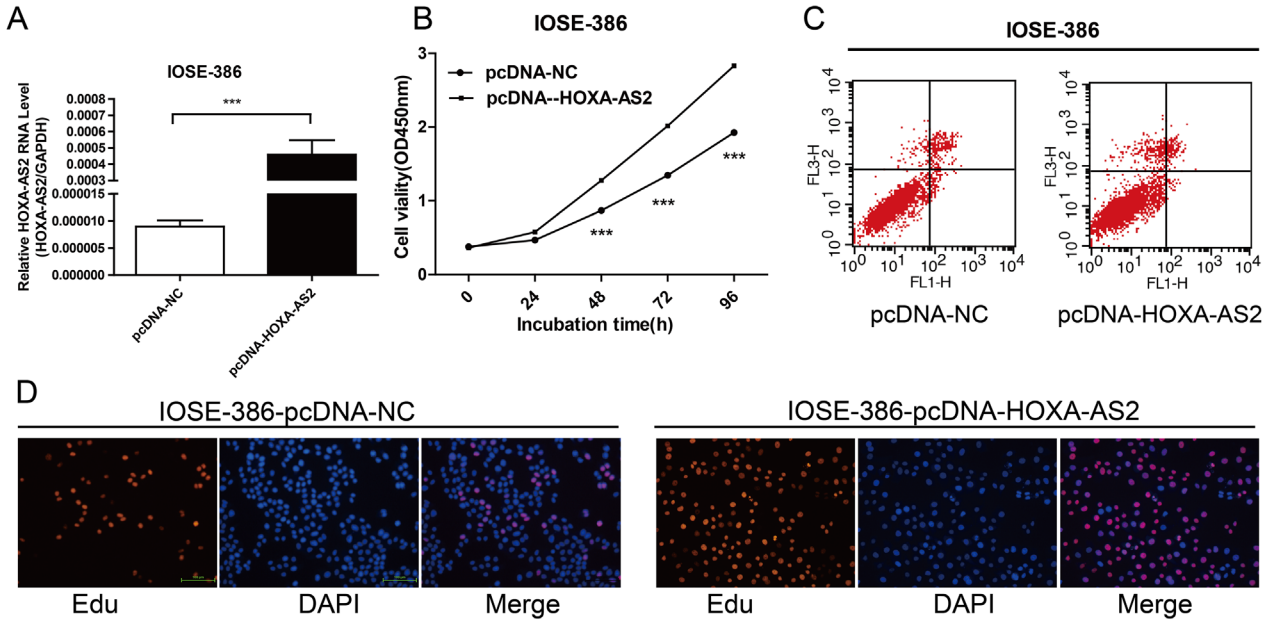

Figure 4: Overexpression of HOXA-AS2 promotes proliferation and inhibits apoptosis in IOSE-386 cell line. (A) HOXA-AS2 was up-regulated in pcDNA-HOXA-AS2 group compared to pcDNA-NC in IOSE-386. B and D HOXA-AS2 could promote proliferative capacity in IOSE-386 by CCK8 **(B)** and Edu assay **(D)**. **(C)** The cell apoptosis rate decreased in the pcDNA-HOXA-AS2 group.

To elucidate the possible function of lncRNA HOXA-AS2 in EOC, two EOC cell lines (HO-8910 and SKOV3) expressed higher HOXA-AS2 were selected. RNAi-mediated inhibitory on lncRNA HOXA-AS2 in both cell lines sharply suppressed cell proliferation but promoted apoptosis; whereas over-expression of HOXA-AS2 could promote the proliferation and inhibit apoptosis. Growth inhibition has also been observed in animal experiment as tumors derived from cells with HOXA-AS2 knockdown grew more slowly. After knockdown of HOXA-AS2, proliferation of EOC cells both *in vitro* and *in vivo* were significantly inhibited by promoting apoptosis. These data suggested that lncRNA HOXA-AS2 served as an oncogene in EOC and a potential candidate for EOC treatment.

More and more studies have revealed that lncRNAs were participated in epigenetic regulation, transcription regulation, processing of small RNAs, and other regulatory effects [29, 30]. Several studies elucidated that lncRNAs promoted cancer progression via mediating expressions of protein-coding genes [13, 25, 31]. It is now well established that differential expressed lncRNAs in specific types of tumors sensitized cancer cells to apoptotic stimuli [32]. SiRNA-mediated silencing of certain lncRNAs helped to upregulation of Caspase-3, -8, -9, and Bax but downregulation of Bcl-2 and Bcl-xl [33, 34]. Meanwhile, previous studies have found that the JAK-STAT signaling pathway functioned as apoptosis associated in diverse cancers [35-37]. Besides, researchers also found that knockdown of HOXA-AS2 induced apoptosis via Caspase-3, -8, and -9. Therefore, we speculated whether knockdown of HOXA-AS2 were mediated by the activation of intrinsic mitochondrial pathway or extrinsic death receptor pathway through regulating apoptotic factors.

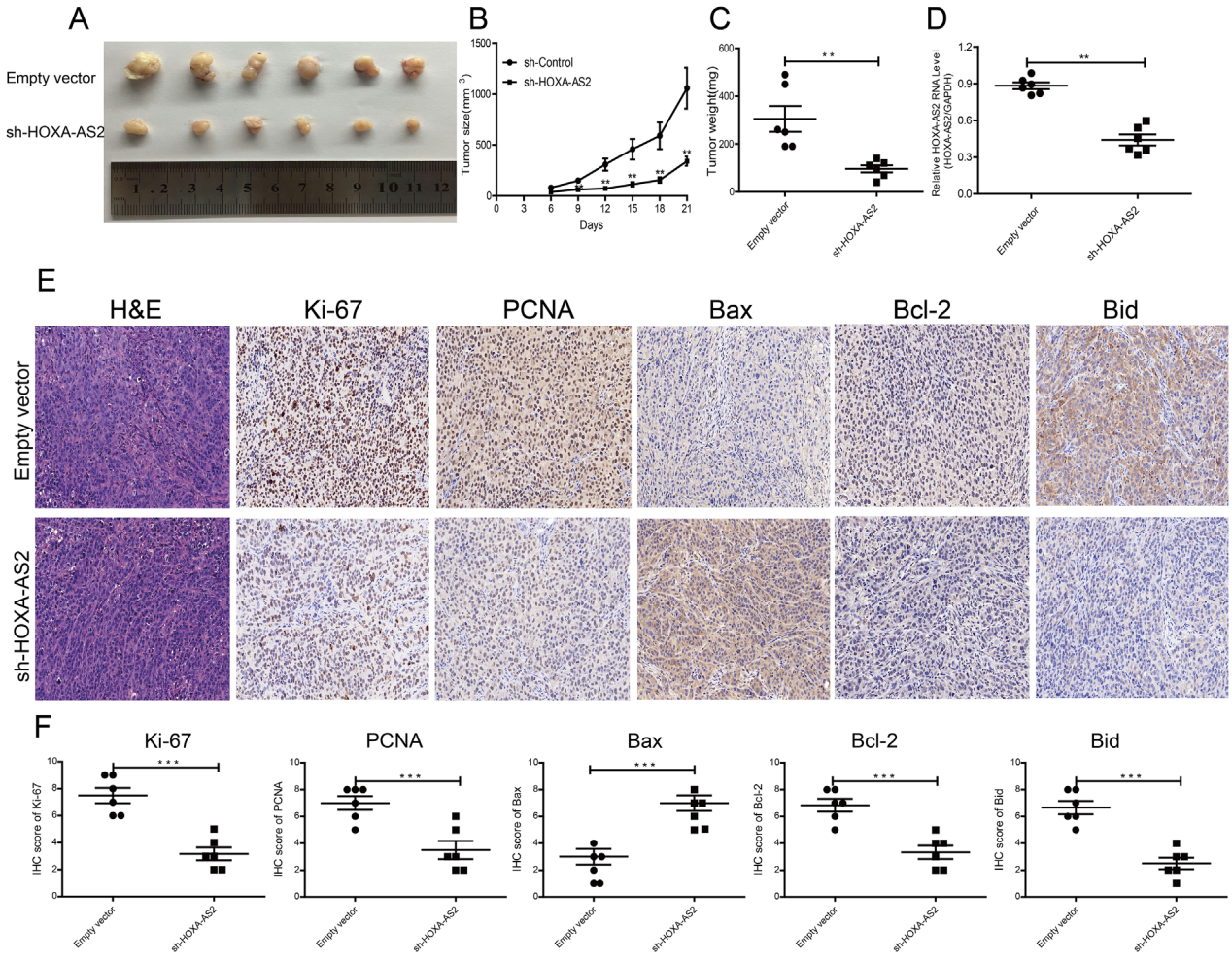

Figure 5: Knockdown of HOXA-AS2 inhibits EOC cells proliferation *in vivo*. HO-8910 cells transfected with sh-Control or sh-HOXA-AS2 were subcutaneously inoculated into nude mice (n = 6) in each groups. **(A)** Tumors formed in sh-HOXA-AS2 group were dramatically smaller relative to controls. **(B)** Tumor size was monitored every 3 days. **(C)** Tumor weight was represented as means of tumor weight ± SD. **(D)** qRT-PCR was performed to detect the average expression of HOXA-AS2 in xenograft tumors (n = 6). **(E and F)** Transplanted tumors with H&E staining. Immunostaining of Ki-67, PCNA, Bax, Bcl-2, Bid in xenograft tumors. Error bars indicated mean ± standard errors of the mean. $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001.

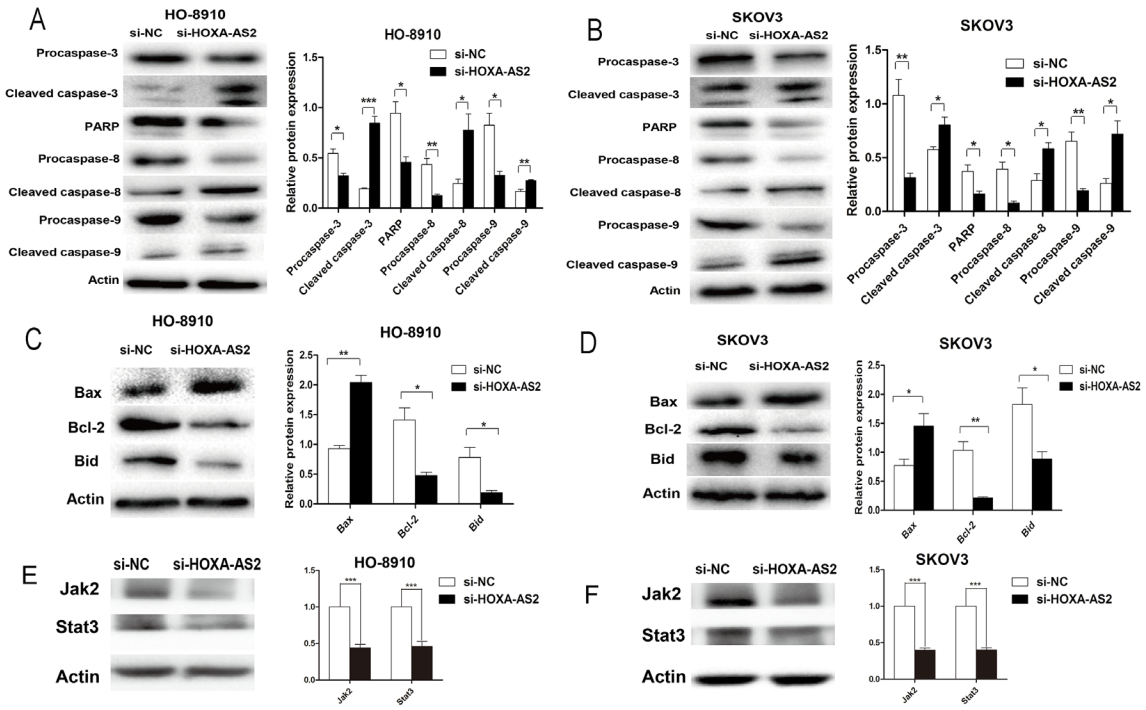

Figure 6: Mechanism of HOXA-AS2 knockdown in promoting EOC cells apoptosis. (A and B) Knockdown of HOXA-AS2 induced activation and cleavage of Caspases and PARP were determined by Western blott in HO-8910 and SKOV3 cells. **(C and D)** Knockdown of HOXA-AS2 altered the protein expressions of Bcl-2, Bax, and Bid detected by Western blot in HO-8910 and SKOV3 cells. **(E and F)** Knockdown of HOXA-AS2 decreased the expression level of Jak2 and Stat3 in both HO8910 and SKOV3 cell lines. Left panel, representative results of Western blot; right panel, protein levels relative to Actin. Data were presented as the mean value from three independent experiments ± S.D. $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001.

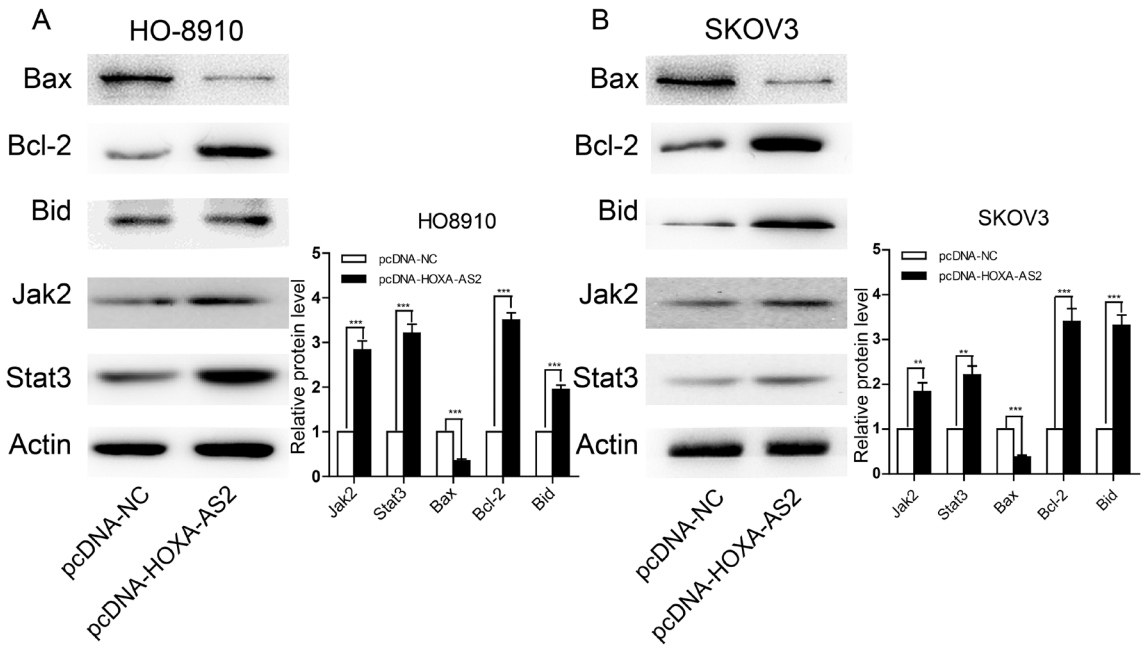

Figure 7: Mechanism of HOXA-AS2 overexpression in inhibiting EOC cells apoptosis. (A and B) Overexpression of HOXA-AS2 decreased the protein expressions of Bax, and increased the level of Bcl-2, Bid, Jak2 and Stat3 in both HO8910 and SKOV3 cell lines.

Table 1: Association between HOXA-AS2 expression and clinicopathological characteristics of patients with EOC (n = 73)

Clinicopathologic features	Number of cases	HOXA-AS2 expression		*p* value
		Low (n=37)	High (n=36)	
Age (years)				
<51	33	15	18	0.417
≥51	40	22	18	
Histological subtype				
Serous	54	29	25	0.384
Others	19	8	11	
Tumor size				
<8CM	34	23	11	0.007**
≥ 8CM	39	14	25	
FIGO stage				
I~II	15	12	3	0.011*
III~IV	58	25	33	
Histological grade				
G1~G2	36	16	20	0.293
G3	37	21	16	
Lymph node metastasis				
Absent	46	20	26	0.108
Present	27	17	10	
CA125 Level (U/ml)				
<500	36	15	21	0.128
≥500	37	22	15	
Ascites				
Absent	29	14	15	0.738
present	44	23	21	

*p <0.05.

Two important pathways were responsible for inducing apoptosis: the intrinsic mitochondria pathway and extrinsic death receptor pathway [38, 39]. Based on proapoptotic functions, the caspases were classified into two groups: initiators (e.g., Caspase-8 and -9) and effectors (e.g., Caspase-3) [40, 41]. Bcl-2 family proteins were the critical molecules responsible of the mitochondria-dependent (intrinsic) death pathway. In humans, over 20 members in Bcl-2 family have been identified to be related to apoptosis (e.g., Bcl-2 or Bax) [42, 43]. Caspase-8 was an essential initiator of apoptosis through extrinsic death receptor pathway [44, 45]. Caspase-8 could cleave cytosolic Bid protein, which in turn triggered the release of Cyt c and Caspase-9 from isolated mitochondria [46, 47]. Once activated, Caspase-9 directly processed the downstream Caspase-3 [48, 49]. Caspase-3 was accounted in the specific proteolytic breakdown of poly(ADP-ribose) polymerase(PARP) when apoptosis occurred [50, 51], then cells become unstable and thus apoptosis formed. The Caspase proteolytic signaling cascades were interconnected, thereby greatly amplifying the apoptotic signaling pathway [52].

In our study, results showed that knockdown of HOXA-AS2 led to illustrious decreased expressions of procaspase-9, -8, -3, and PARP both in HO-8910 and SKOV3 cell lines, whilist cleaved Caspase-9, -8, and -3 increased. At the same time, the expression of Bcl-

2 and Bid decreased but Bax increased, implying that these genes participated in HOXA-AS2-induced EOC progression. Besides, over-expression of HOXA-AS2 dramatically increased the protein expressions of Bcl-2, Bid, Jak2 and Stat3. Meanwhile, the expression of Bax was down-regulated. Taken together, results were summarized as the following: HOXA-AS2 was correlated with EOC proliferation through inhibiting cell apoptosis; HOXA-AS2 partially inhibited EOC apoptosis through suppressing the intrinsic mitochondria pathway and extrinsic death receptor pathway, which were associated with Bcl-2 protein family.

Apoptosis is a very complex process, further in-depth studies are needed to carry out so as to look for the specific molecular mechanism of lncRNA HOXA-AS2 in regulating the abovementioned genes and signaling pathways. To sum up, our study first pointed out that lncRNA HOXA-AS2 acted as a functional oncogene in EOC cell lines, and the upregulation of lncRNA HOXA-AS2 expression was related to EOC development. Hence, our study provided a reference that lncRNA HOXA-AS2 may be a new prognostic biomarker and potential therapy candidate of EOC.

MATERIALS AND METHODS

HOXA-AS2 expression analysis

Data of EOC gene expression were downloaded from the Gene Expression Omnibus (GEO) dataset and TCGA. Then insilicoMerging package combined several mostly used methods was used to remove unwanted batch effects in order to merge different datasets. After that, Rankprod package computed the difference of two arguments origins and classes between two groups. Differential probes were re-annoteted with blast+2.2.30 on GENCODE Release 21 sequence databases for lncRNA and mRNA. For the condition that multiple probes were corresponded to one gene, we selected the most normalized signal to detect the lncRNA and mRNA expressions.

Gene set enrichment analysis of HOXA-AS2 expression

The RNA-seq profiles of ovarian cancers samples from TCGA and GEO were analyzed by GSEA [23]. Based on the approach, HOXA-AS2 expression was evaluated as a binary variable, and samples were grouped into the low or high HOXA-AS2 expression. When analyzing the ranking genes in the GSEA, differential expressed HOXA-AS2 was compared, and other parameters were set by their default values.

Tissue samples

73 EOC tissues and 57 normal ovarian tissues were obtained from patients who underwent surgery at the Department of Gynecology and Obstetrics, the First Affiliated Hospital of Nanjing Medical University from January 2012 to May 2016. Patients enrolled in this study had no previous local or systemic treatment before the surgery. EOC patient tissues were immediately snap-frozen in liquid nitrogen as soon as possible and stored at –80°C for further study. Tumor stages and grades were in accordance to the criteria in the International Federation of Gynecologists and Obstetricians (FIGO). We recorded each patient's comprehensive follow-up information. Patients' overall survival time was recorded from the first operation day to death or the last follow-up. Our study was approved by the Research Ethics Committee of Nanjing Medical University, China. Every patient signed the informed consent for the tissue specimens used in this study. Clinical pathological characteristics of the EOC patients were presented in Table 1.

Cell lines

Three human EOC cell lines (SKOV3, HO-8910 and HEY) and a normal ovarian cell line (IOSE386) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). IOSE386 cells were cultured in DMEM medium (GIBCO) and the other ovarian cancer cell lines were cultured in RPMI 1640 medium (GIBCO) containing 10% fetal bovine serum (FBS) and were then maintained at 37°C and 5% CO_2 in a humidified atmosphere. Cells in the logarithmic growth phase were utlized for further studies.

RNA isolation and quantitative RT-PCR

Total RNA was extracted from tissues and cell lines using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The RNA level of HOXA-AS2 was accessed by quantitative RT-PCR using the SYBRGreen (TaKaRa) on ABI StepOne PCR instrument, with GAPDH as the loading control. The primers were as follows: lncRNA HOXA-AS2 5′- AACCCATCTTTGCCTTCTGC-3′ and 5′- CGGAGGAGTTTGGAGTTGG -3′; GAPDH 5′- CCCACTCCTCCACCTTTGAC -3′ and 5′- GGATCTCGCTCCTGGAAGATG -3′. Comparative Ct method was used for transcript quantification. The target genes were normalized to those of GAPDH by the formula $2^{-\Delta CT}$.

Transfection of EOC cells

SiRNA targeting human HOXA-AS2 and one negative control siRNA were designed and synthesized by Genepharma (Shanghai, China). Briefly, siRNA transfection (100 nM) was carried out by Lipofectamine2000 (Invitrogen) according to the manufacturer's protocols. The effective sequences were as follows: 5′-CUUUGCGUCUACAGACCUATT-3′ (si-

HOXA-AS2-1), 5′-CAUGCAAGGUCAAGUAUCUTT-3′ (si-HOXA-AS2-2), and 5′-GAGUUCAGCUCAAGUUG AATT-3′ (si-HOXA-AS2-3).

For stable knockdown of HOXA-AS2 in HO-8910 cells in the *in vivo* experiment, we constructed lentiviral particles that encoded for shRNA against HOXA-AS2, which was also synthesized by Genepharma (Shanghai, China). The sh-HOXA-AS2 targeting sequence was GGACACGTTTCTATGCCTTAC. A non-targeting scrambled shRNA LV3-GFP vector was conducted for negative control. Afterwards, plasmids were further co-transfected into HO-8910 cells with lentiviral packaging plasmids and a HOXA-AS2 shRNA-expressing lentivirus or a control shRNA-expressing lentivirus were conducted, respectively. For cell infection, HO-8910 cells were cultured and infected with the constructed lentiviruses expressing HO-8910 shRNA and scrambled shRNA following the manufacturer's protocol. 48 hours later, 1 mg/mL puromycin (Invitrogen) was added into the medium and puromycin-resistant clones were selected. Efficacy of HOXA-AS2 RNA knockdown was evaluated by quantitative RT-PCR.

Cell proliferation assay

For determining cell viability, cells transfected with siRNA were seeded in 96-well plates (3000 cells per well). Cell proliferation was conducting after siRNA transfection for 24, 48, 72, and 96 h. CCK8 (10 μL/well) was added to each well and incubated at 37°C for 2 h. Absorbance values at 450 nm were detected by the microplate reader (BioTek, United States). All experiments were performed in quadruplicate. For colony formation assay, 400 cells per well were seeded in 60-mm dishes for transfection with siRNA. After cell culture for two weeks, colonies were fixed with methanol and stained by 0.1% crystal violet. For those whose diameter of colonies larger than 1 mm were counted. Each treatment was separated in three wells and performed in triplicate, and each experiment was independently repeated for three times.

Flow cytometric analysis

After cells were transfected for 48 h, the transfected cells were collected and ice-cold phosphate-buffered saline (PBS) was used to wash cells. When the double staining with FITC-annexin V and propidium iodide (PI) was performed, the FITC annexin V apoptosis detection kit (BD Biosciences) was utilized according to the manufacturer's instructions, cells were then analyzed with a flow cytometry (FACScan; BD Biosciences) equipped with a CellQuest software (BD Biosciences). Next, cells were classified into viable cells, dead cells, early apoptotic cells, apoptotic cells, finally, the relative ratio of early apoptotic cells and apoptotic cells were in comparison to control transfected cells in each experiment.

Ethynyl deoxyuridine analysis

The 5-ethynyl-a-deoxyuridine labeling / detection kit (Riobio, Guangzhou, China) were used to assess the proliferating cells based on the manufacturer's instruction. In short, $5x10^3$ HO8910 and SKOV3 cells were cultured and transfected with si-NC and si-HOXA-AS2 for 48 h in 96-well plates. Then, 50 uM ethynyl deoxyuridine (Edu) labeling medium was added to each culture sample and incubated for 2 h at 37°C with 5% CO_2. Then, 4% paraformadehyde (pH 7.4) was used to fix the culture cells for 30 mins and subsequently treated with 0.5% Triton X-100 for 20 mins at room temperature. Afterwards, samples were stained with anti-Edu working solution after washing with PBS at 25°C for 30 mins. Finally, cells were incubated with 100 uL Hoechst 3342 (5ug/ml) at 25°C for 30 mins, observed under a fluorescent microscope. Edu-positive cells was counted from five random fields in three wells and the Edu-positive percentage was calculated.

In vivo experiments

In vivo experiments were approved by the Animal Experimentation Ethics Committee of the Nanjing Medical University. Female athymic BALB/c nude mice (4-week-old) were provided by Slac Laboratory Animal Co. Ltd. (Shanghai, China). Animals were raised in a pathogen-free animal laboratory and randomly divided to the control or experimental group (six mice in each group). HO-8910 cells transfected with empty vector or sh-HOXA-AS2 were collected and injected into one posterior side of every mouse (1×10^8/0.1 mL). Tumor volume was assessed with an interval of 3 days based on the formula: 0.5 × length × width. All animals were sacrificed 21 days later. Tumor tissues from mice were taken, paraffin-embedded and formalin-fixed for H&E staining and Ki-67 immunostaining.

Western blot assay

The following primary antibodies were utilized in Western blot assay: Bcl-2 (Proteintech; 1:1000), Bax (Proteintech; 1:1000), Bid (Proteintech; 1:1000), Caspase-3 (Cell signaling; 1:1000), cleaved Caspase-3 (Cell signaling; 1:1000), PARP (Cell signaling; 1:1000), Caspase-8 (Cell signaling; 1:1000), Caspase-9 (Cell signaling; 1:1000), and Actin (Millipore; 1:1000). In short, cells were lysed with RIPA buffer containing protease inhibitors; 60 μg samples of the lysates were separated on 12% SDS-PAGE gels and transferred to PVDF membranes. Next, primary antibodies were used to incubate the membranes overnight at 4°C. Anti-rabbit or anti-mouse secondary antibodies were purchased from Beyotime (Nantong, Shanghai, China). Finally, bands of the bounding antibodies were detected using an ECL substrate.

Statistical analysis

SPSS 16.0 software was used for all statistical analysis and GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA) was used to present the data results. Comparisons between groups were analyzed by Chi-squared test, student's t test, and the Kaplan-Meier method appropriately based on the specific situations. All p values <0.05 were considered statistically significant; $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts interest with the study or preparation of the manuscript.

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