LncRNA TP73-AS1 predicts poor prognosis and promotes cell proliferation in ovarian cancer via cell cycle and apoptosis regulation

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Abstract. TP73-AS1, a critical cancer-associated long noncoding RNA (lncRNA), has been identified in esophageal cancer and glioma. However, its biological role in ovarian cancer (OC) remains to be investigated. The aim of the present study was to investigate the role of TP73-AS1 in human OC cell lines and clinical tumor samples to determine the function of this molecule. Reverse transcription-quantitative polymerase chain reaction analysis was carried out to detect that TP73-AS1 was upregulated in OC tissues and cell lines. Kaplan Meier Method was applied to study the association between overall survival of patients with OC and TP73-AS1 expression. The results suggested that patients with high expression levels of TP73-AS1 had lower survival compared with patients with low expression level of TP73-AS1. MTT and colony formation assays were conducted to investigate the effects of TP73-AS1 expression on OC cell proliferation. Flow cytometry analysis was used to analyze the effects of TP73-AS1 expression on cell cycle progression and apoptosis. Loss-of-function experiments revealed that TP73-AS1 silencing was able to suppress the growth of OC cells via modulating the cell cycle and apoptosis. The results of the present study suggest that TP73-AS1 may be an oncogenic lncRNA that promotes the proliferation of OC cells and may therefore be an effective therapeutic target in patients with OC.

Introduction

Ovarian cancer (OC) is a common gynecological malignancy and the most common cause of gynecological cancer-associated death in women worldwide (1). Despite advances in surgical techniques and chemotherapeutic agents, the prognosis for OC remains poor, with a 5-year survival rate of <30% (2). The poor prognosis of OC is associated with early metastasis and recurrence (3); it is therefore important to explore the underlying molecular mechanisms associated with ovarian carcinogenesis and progression in order to develop more effective treatment strategies and personalized therapies for OC.

Long non-coding RNAs (lncRNAs), a heterogeneous group of genomic transcripts with length >200 nucleotides, have little or no protein-coding ability (4). The functional roles of lncRNAs have been underestimated, as they were initially thought to be transcriptional noise in the genome (5). However, accumulating evidence has demonstrated that they serve crucial roles in physiological and pathological processes (6), including cancer progression and metastasis (7-10). P73 antisense RNA 1T (non-protein coding), also called TP73-AS1, is a long non-coding RNA that regulates apoptosis via the modulation of p53-dependent anti-apoptotic gene and is abnormally expressed in cancer (11,12). However, the biological role of TP73-AS1 in patients with OC remains to be elucidated.

In the present study, the expression of TP73-AS1 in OC tissues and cell lines was assessed and it was investigated whether TP73-AS1 expression was associated with disease prognosis. Functional assays were also performed to determine the impact of TP73-AS1 on cell proliferation, cell cycle and apoptosis.

Materials and methods

Clinical samples. The present study was approved by the Ethics Committee of the Department of Gynecology and Obstetrics, Binzhou Central Hospital (Binzhou, China). A total of 62 pairs of OC tissues and their adjacent noncancerous tissues were collected from 62 patients (age 36-87 years; all female) who had undergone ovariectomy at the Department of Gynecology and Obstetrics, Binzhou Central Hospital, between March 2006 and April 2011. All cases were diagnosed with OC by two independent pathologists. Signed informed consent was provided by all patients enrolled in this study. Patients with other known tumors and those receiving medical

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treatment and/or previous cancer treatment were excluded from the present study. Patients' clinicopathological features are summarized in Table I.

Cell culture and cell transfection. The human OC cell lines SKOV3, A2780, HO8910 and CAOV3, as well as one normal human ovarian surface epithelial cell line, HOSEPiCs, were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured for 3 days in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc. Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml streptomycin/penicillin in a humidified atmosphere containing 5% of CO_2 at 37˚C. Full-length human TP73-AS1 transcript cDNA (NCBI Reference Sequence: NM_001204189.1) was amplified by polymerase chain reaction (PCR) and subcloned into a pcDNA3.1 vector, according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.). The pcDNA3.1 empty vector was used as the control. phi29 DNA polymerase (Pure-one Bio Technology, Co., Ltd., Shanghai, China; 10 U/μl) was used for PCR reaction. The forward and reverse primer sequences for LnRNATP73-AS1 were 5'-CCGGTTTTCCAG TTCTTGCAC-3' and 5'-GCCTCACAGGGAAACTTCATG C3-3', respectively. The following primers were used for amplification of BDH2: Forward, 5'-TTCCAGCGTCAAAGGAGT TGT-3' and reverse, 5'-TTCCTGGGCACACACAGTTG-3'. The following thermocycling conditions were used for the PCR: 94˚C for 60 sec, 37˚C for 60 sec, 72˚C for 120 sec; 25 cycles. Anza restriction enzyme cloning system (Thermo Fisher Scientific, Inc.) was used to cut DNA. Small interfering (si) RNA for TP73-AS1 (si-TP73-AS1) and negative control siRNA (neg siRNA) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). Cells ($5x10^4$ cells/well) were seeded into 6-well plates and transfected with 20 nM siRNA using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. At 48 h following transfection, the transfected cells were used in *in vitro* assays. The target siRNA sequence for TP73-AS1 was as follows: 5'-TAAGGTTATCCGAATAACGGTATCGTT-3'. The sequence of neg siRNA was as follows: 5'-TTCTCCGAACGT GTCACGT-3'. All experiments were performed three times.

Western blotting. Total protein lysates were obtained using RIPA lysis buffer (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Bicinchoninic acid method was used for protein determination. Proteins (10-15 μg/lane) were separated by 10% SDS-PAGE, and electrophoretically transferred to polyvinylidene difluoride membranes (Roche Applied Science, Penzberg, Germany). Membranes were probed with primary antibodies against anti-caspase-8 (1:2,000; cat. no. ab25901), anti-caspase-9 (1:2,000; cat. no. ab32539), anti-B-cell lymphoma 2 (Bcl-2; 1:2,000; cat. no. ab32124), anti-Bcl-2-associated X protein (Bax; 1:2,000; cat. no. ab32503) and GAPDH (1:3,000; cat. no. ab8245; all Abcam, Cambridge, UK) at 4˚C overnight. Membranes were blocked with 5% of non-fat (v/v) milk for 1 h at room temperature. Followed primary antibody incubation, membranes were probed with the corresponding goat anti rabbit immunoglobulin G Alexa Fluor® 488-conjugated secondary antibody (1:3,000; cat. no. ab150077; Abcam) at 4˚C for 1 h. Blots were washed with PBS three times for 15 min and visualized using an enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Inc.), followed by the exposure with a Tanon 5200 instrument (Tanon Science and Technology Co., Ltd., Shanghai, China). Image Lab (version 4.0; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for quantitative analysis. All experiments were repeated at least three times.

Table I. Association between lncRNA-TP73-AS1 expression and clinical features (n=62).

	LncRNA-TP73-AS1 expression		
Variable	Low	High	P-value
Age (years)			
<50	19	21	0.288
≥50	7	15	
Residual tumor diameter (cm)			
<1	17	19	0.435
≥1	9	17	
Histological grade			
G1-G2	21	13	0.001
G3	5	23	
Lymph node metastasis			
Negative	21	7	<0.001
Positive	5	29	
CA125 level (U/ml)			
<600	17	23	1.000
≥600	9	13	
International Federation of Gynecology and Obstetrics stage			
I-II	22	8	<0.001
III-IV	4	28	

RNA extraction and RT-qPCR. Total RNA was extracted from tissues using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA synthesis was carried out using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA). Reverse transcription was conducted at 70˚C for 10 min and at 4˚C for 5 min. TP73-AS1 expression was measured using SYBR-Green fluorescent dye and STEP ONE RT-PCR apparatus (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 20-30 cycles of initial denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec and final extension at 70-72˚C for 30-60 sec. The relative expression levels of genes were quantified using $2^{-\Delta\Delta Cq}$ method (13). GAPDH was used as an internal reference. The primers used were as follows: TP73-AS1 forward, 5'-CCGGTTTTCCAGTTCTTG CAC-3' and reverse, 5'-GCCTCACAGGGAAACTTCATG C-3'; GAPDH forward, 5'-GTCAACGGATTTGGTCTGTAT T-3' and reverse, 5'-AGTCTTCTGGGTGGCAGTGAT-3'. All assays were repeated at least three times.

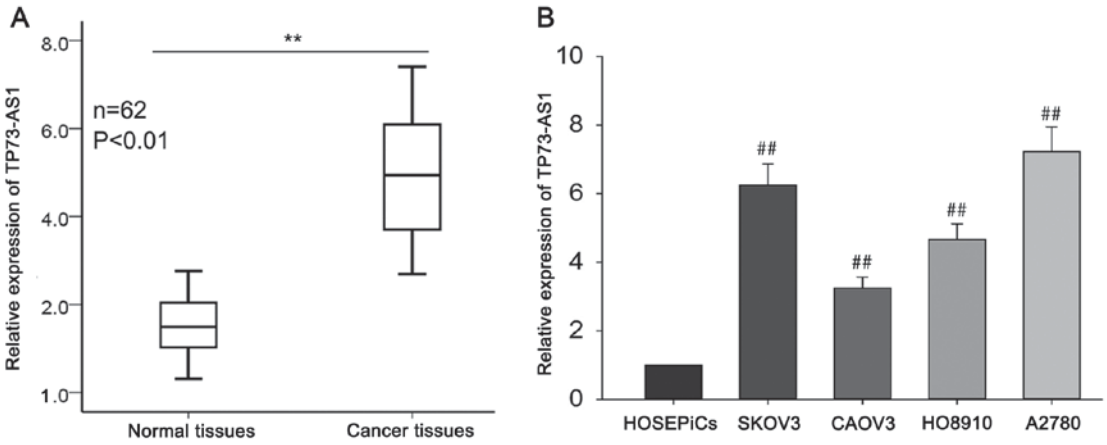

Figure 1. TP73-AS1 is upregulated in OC tissues and cell lines. TP73-AS1 expression in (A) ovarian cancer and corresponding normal tissues and (B) four ovarian cancer cell lines (SKOV3, CAOV3, HO8910 and A2780) and one normal human ovarian surface epithelial cell line (HOSEPiCs) was assessed using reverse transcription-quantitative polymerase chain reaction. **P<0.01 vs. normal tissues and $^{\#\#}$P<0.01 vs. HOSEPiCs.

Relative cell viability. Negative siRNA and si-TP73-A1 were transfected into SKOV3 and A2780 cells, while pcDNA-TP73-AS1 and control pcDNA vector were transfected into HOSEPiC cell by Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Ovarian cancer cells ($3x10^3$ to $6x10^3$ cells/μl) were inoculated in a 96-well plate (200 μl per well, 6 repeated wells) at 37˚C in an atmosphere containing 5% CO_2 for 24-72 h and 20 μl MTT solution (5 mg/ml; Sigma-Aldrich; Merck KGaA) was added into each well. Following 4 h of incubation at 37˚C, the incubation was terminated and the culture medium was discarded. 10% dimethyl sulfoxide (150 μl; Sigma-Aldrich; Merck KGaA) was added to each well and plates were gently shaken for 10 min dissolve formazan crystals. Absorbance values were determined with a plate detector at 0, 24, 48, 72 and 96 h at a wavelength of 450 nm. The DMSO was used as the control. The experiment was performed in triplicate.

Colony formation assay. Negative siRNA and si-TP73-A1 were transfected into SKOV3 and A2780 cells, while pcDNA-TP73-AS1 and control pcDNA vector were transfected into HOSEPiCs using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Transfections were terminated at a final concentration of 50 nM, as described above. Cells (500 cells/well) were plated in 6-well plates and incubated in RPMI-1640 with 10% FBS at 37˚C for two weeks and the medium was changed every 3 days. Then, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature and stained with 0.1% crystal violet for 30 min at a room temperature. The number of visible colonies was counted manually through observing the violet dots. Cell clone formation was observed under a light microscope, the cell number was observed at a magnification of x10 in one field of view. All independent assays were repeated at least three times.

Flow cytometric analysis of apoptosis. Apoptosis was determined in transfected cells using flow cytometric analysis with an Annexin V: Fluorescein isothiocyanate Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. The apoptotic cells were analyzed by using FlowJo 10 software and Attune® NxT cytometer (both Thermo Fisher Scientific, Inc.). All samples were assayed in triplicate.

Flow cytometric analysis of cell cycle distribution. Cells were collected directly or 48 h following transfection, washed with ice-cold PBS and fixed with 70% ethanol overnight at -20˚C. Fixed cells were rehydrated in PBS for 10 min and incubated in 1 mg/ml of RNase A (Thermo Fisher Scientific, Inc.) for 30 min at 37˚C. Subsequently, the cells were subjected to propidium iodide/RNase staining at 4˚C for 30 min followed by flow cytometric analysis with a FACScan instrument (BD Biosciences) and Cell Quest software version 3.0 (BD Biosciences) as previously described (14). All samples were assayed in triplicate.

Statistical analysis. SPSS version 19.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Data were presented as the mean ± standard deviation. The Pearson χ^2 test was used to assess the association between TP73-AS1 expression and clinicopathological factors. Data between two groups were analyzed using the Student's t-test. Multiple comparisons were made by one-way analysis of variance with the LSD post hoc test. Survival analysis was performed using the Kaplan-Meier method and the log-rank test was used to compare differences among patient groups. A cox proportional hazards regression model was generated to identify factors associated with overall survival through a multivariate survival analysis of OC. P<0.05 was considered to indicate a statistically significant difference.

Results

TP73-AS1 is upregulated in OC tissues and cell lines. To determine the biological function of TP73-AS1, RT-qPCR was performed to measure the relative expression of TP73-AS1 in OC tissues (n=62) compared with corresponding normal tissues (n=62). TP73-AS1 was significantly overexpressed in OC tissues compared with the corresponding adjacent non-tumorous tissues (Fig. 1A). TP73-AS1 expression in four OC cell lines (SKOV3, CAOV3, HO8910 and A2780) and one normal human ovarian surface epithelial cell line (HOSEPiCs)

was assessed using RT-qPCR. The results revealed a significant increase in TP73-AS1 expression in OC cell lines compared with HOSEPiCs (Fig. 1B). Of the four OC cell lines, TP73-AS1 expression was highest in the SKOV3 and A2780 cells, and so SKOV3 and A2780 cells were chosen for the following experiments. These findings suggest that TP73-AS1 may act as an oncogene and contribute to the progression of OC.

High expression of TP73-AS1 predicts poor prognosis in OC patients. To determine the clinical relevance of TP73-AS1 expression in OC, the association between the levels of TP73-AS1 and clinicopathological parameters was analyzed. According to the mean value of TP73-AS1 expression (4.99) in OC tissues, TP73-AS1 expression was divided into two groups (high group, n=36; and low group, n=26). TP73-AS1 expression was significantly associated with histological grade, lymph node metastasis and International Federation of Gynecology and Obstetrics (FIGO) stage (Table I); however, no significant associations with age, residual tumor diameter or CA125 levels were observed (Table I). A Kaplan-Meier method analysis (log-rank test) was performed and revealed that higher expression of TP73-AS1 was associated with a lower overall survival in patients with OC (Fig. 2). Proportional hazards method analysis revealed that high levels of TP73-AS1 may be a prognostic factor in addition to the independent prognostic impact of histological grade and FIGO stage (Table II). These results suggest that TP73-AS1 serves a role in OC and may be considered as a specific biomarker of poor prognosis.

Dysregulation of TP73-AS1 affects OC cell proliferation. To explore the biological role of TP73-AS1 in OC cell proliferation, SKOV3 and A2780 cells were transfected with TP73-AS1-specific siRNA and HOSEPiCs cells were transfected with a TP73-AS1 expression vector (pcDNA/TP73-AS1). Transfection efficiency was determined after 48 h (Fig. 3A). MTT assays were performed to determine the relative viability of SKOV3 and A2780 cells transfected with si-TP73-AS1, and HOSEPiCs cells transfected with pcDNA/TP73-AS1. TP73-AS1 silencing reduced the viability of OC cells compared with cells transfected with negative siRNA, whereas TP73-AS1 overexpression enhanced the viability of HOSEPiCs cells (Fig. 3B). Consistent with the MTT assays, the results of colony formation assays also demonstrated a growth-inhibition effect of TP73-AS1 knockdown and a pro-proliferation effect of TP73-AS1 overexpression (Fig. 3C). Collectively, these findings suggest that TP73-AS1 may function as an oncogene via regulating cell proliferation.

Growth-modulation mediated by dysregulation of TP73-AS1 is attributed to its effect on cell cycle and apoptosis. As cell cycle dysregulation serves a role in tumor proliferation (15), it was hypothesized that growth modulation mediated by TP73-AS1 dysregulation may occur by affecting the cell cycle and apoptosis. To verify this hypothesis, flow cytometric analysis was performed. SKOV3 and A2780 cells transfected with si-TP73-AS1 and exhibited a high percentage of G1 cells compared with the negative siRNA group, whereas HOSEPiCs cell transfected with pcDNA/TP72-AS1 had a higher percentage of S phase cells compared with the pcDNA group (Fig. 4A). In addition, flow cytometric analysis of apoptosis revealed that TP73-AS1 knockdown increased the percentage of apoptotic cells in SKOV3 and A2780 assays. By comparison, forced overexpression of TP73-AS1 significantly reduced the number of apoptotic cells in the HOSEPiCs experiment (Fig. 4B). Results of western blotting revealed that the expression of apoptosis-associated proteins (caspase-8, caspase-9, Bcl-2 and Bax) was affected by TP73-AS1 (Fig. 4C). The protein levels of caspase-8, caspase-9 and Bax were upregulated by knockdown of TP3-AS1 in SKOV3 and A2780 cells, and they were also upregulated by silencing of TP3-AS1 in HOSEPiCs cell. By contrast, the protein level of Bcl-2 was downregulated by TP73-AS1 knockdown in SKOV3 and A2780 cells, and downregulated by TP73-AS1 silencing in HOSEPiCs cells. These data suggest that the pro-growth effect of TP73-AS1 in OC cells may be attributed to its influence on the cell cycle and apoptosis.

Table II. Multivariate analysis of prognostic parameters in patients with ovarian cancer by Cox regression analysis.

Variable	Category	P-value
Age (years)	<50	0.264
	≥50	
Residual tumor diameter (cm)	<1	0.211
	≥1	
Histological grade	G1-G2	0.026
	G3	
Lymph node metastasis	Negative	0.852
	Positive	
CA125 level (U/ml)	<600	0.430
	≥600	
International Federation of Gynecology and Obstetrics stage	I-II	<0.001
	III-IV	
TP73-AS1 expression	Low	0.003
	High	

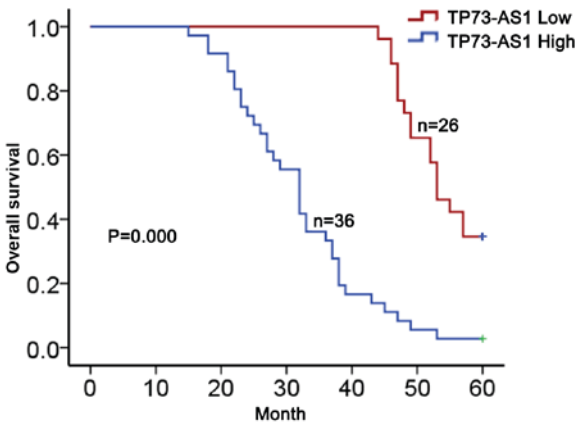

Figure 2. Association between TP73-AS1 expression and prognosis in patients with ovarian cancer, as analyzed using Kaplan-Meier method analysis (log-rank test).

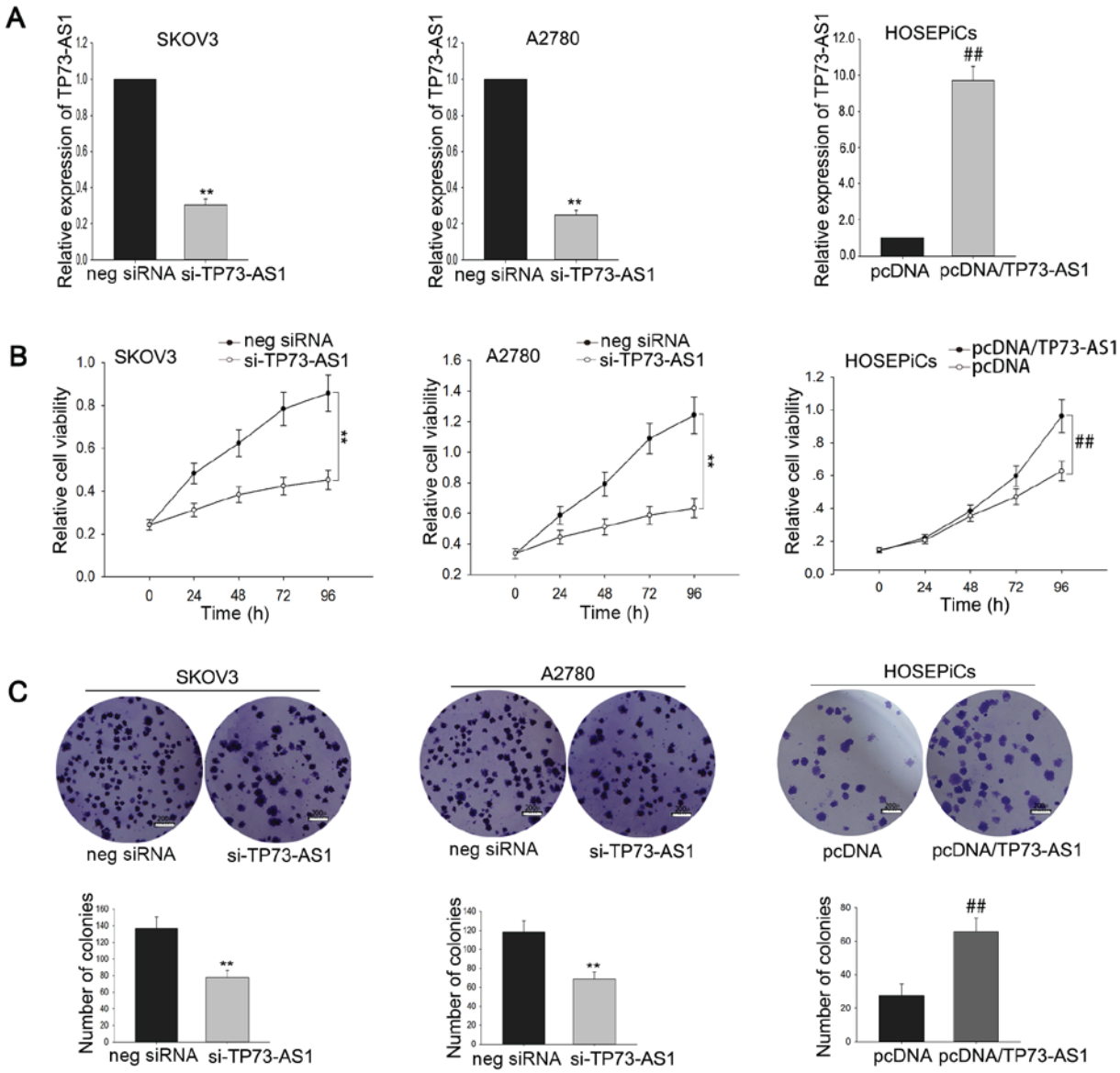

Figure 3. Dysregulation of TP73-AS1 affects ovarian cancer cell growth. (A) SKOV3 and A2780 cells were transfected with TP73-AS1-specific siRNA and HOSEPiCs cells were transfected with pcDNA/TP73-AS1. Transfection efficiency was determined using reverse transcription-quantitative polymerase chain reaction. The viability of SKOV3 and A2780 cells transfected with si-TP73-AS1 and HOSEPiCs cells transfected with pcDNA/TP73-AS1 was assessed using (B) MTT and (C) colony formation assays (Scale bar, 200 μm). **P<0.01 vs. neg siRNA and $^{\#\#}$P<0.01 vs. pcDNA.

Discussion

OC is the most common cause of cancer-associated death in women worldwide (16). As there is no effective method for early diagnosis, the majority of patients with OC are diagnosed at an advanced stage, and so the prognosis of OC is poor (17,18). The molecular mechanism underlying the initiation and progression of OC is generally complex, involving dysregulated oncogenes and tumor suppressor genes (19-21).

Previous reports have identified that the dysregulation of lncRNAs contributes to a range of biological processes and is associated with a variety of cancers (22-30), including OC. For example, in 2016, Kuang *et al* (31) reported that lncRNA TUG1 regulated OC proliferation and metastasis via affecting epithelial-mesenchymal transition. Richards *et al* (32) demonstrated that a functional variant of HOXA11-antisense, a novel long non-coding RNA, inhibited the oncogenic phenotype of epithelial OC. Furthermore, Liu *et al* (33) reported that overexpression of long non-coding RNA PVT1 in OC cells promoted cisplatin resistance by regulating apoptotic pathways. However, the exact molecular mechanisms underlying the tumorigenesis of OC remain unclear. TP73-AS1, transcribed from chromosome 1p36, has been reported to be associated with cell proliferation and tumor progression in glioma and esophageal squamous cell carcinoma (11,12). However, its biological roles in OC remain unknown.

The results of the present study demonstrate that TP73-AS1 was overexpressed in OC tissues and cell lines compared with controls and increased TP73-AS1 expression was associated with histological grade, lymph node metastasis, FIGO stage and poor prognosis in patients with OC. Furthermore, proportional hazards method analysis revealed that high

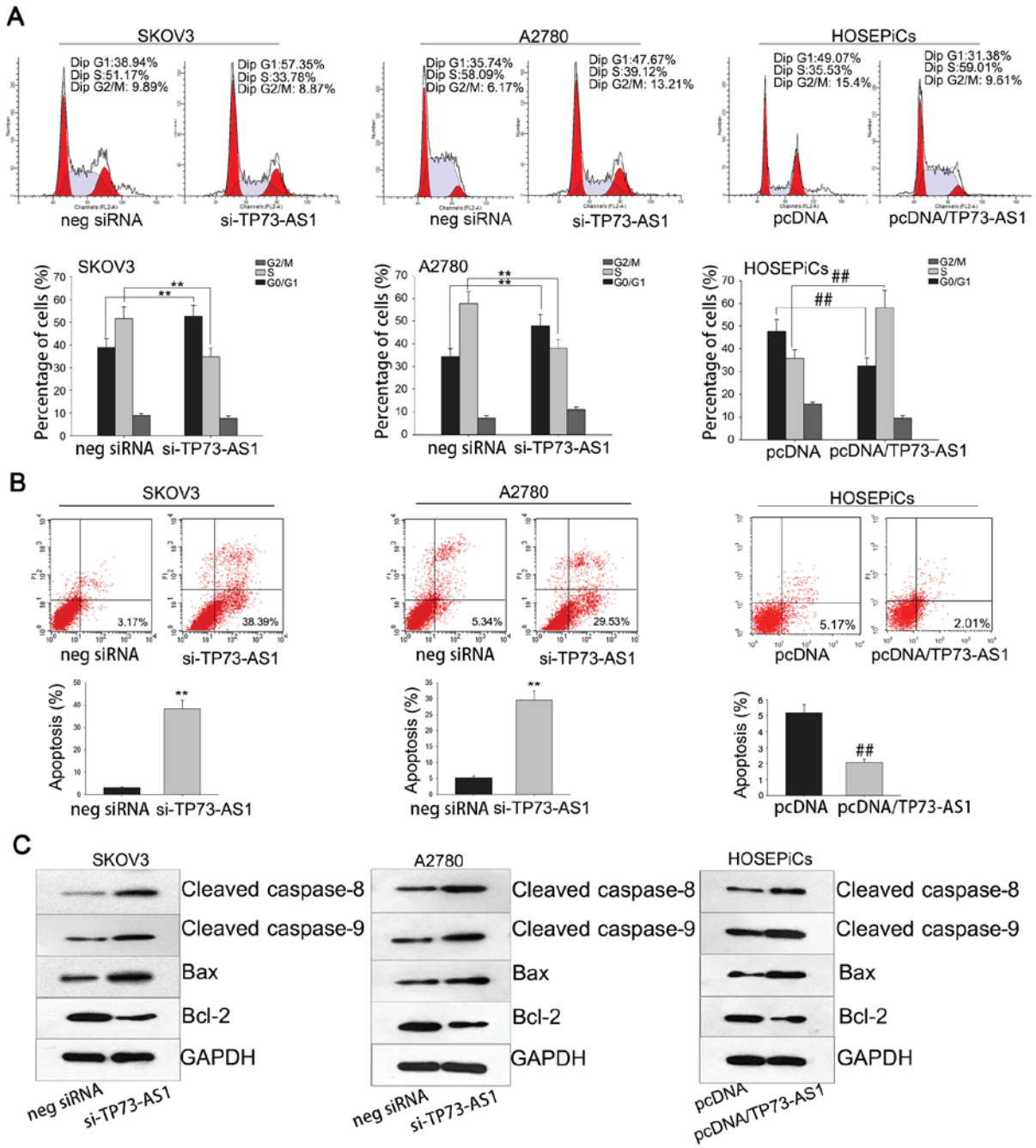

Figure 4. Cell cycle and apoptosis analysis. Flow cytometric analysis was performed to evaluate (A) cell cycle distribution and (B) the percentage of apoptotic cells in SKOV3 and A2780 cells transfected with si-TP73-AS1 and HOSEPiCs cells transfected with pcDNA/TP73-AS1. (C) Western blotting were performed to measure the expression of apoptosis-associated proteins. **P<0.01 vs. neg siRNA and $^{\#\#}$P<0.01 vs. pcDNA.

TP73-AS1 expression may be a specific biomarker of poor prognosis for OC. Loss-of-function assays revealed that TP73-AS1 knockdown inhibited the proliferation ability of OC cells. Additionally, flow cytometric analysis revealed that the proliferation-inhibition mediated by TP73-AS1 knockdown may be due to changes in the cell cycle and apoptosis. These results suggest that TP73-AS1 expression may be used as a biomarker for predicting OC prognosis. TP73-AS1 may therefore be used as a potential prognostic and therapeutic target of OC. Future research should focus on analyzing other pathways and signaling molecules associated with TP73-AS1.

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Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XL and XW analyzed and interpreted the patient data. LM, SZ and HW analyzed and interpreted the *in vitro* data obtained using cell lines. All authors contributed to experiments. XL was the major contributor in writing the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Signed informed consent to participate in the study was obtained from all participants. This study has approved by the ethics committee of Binzhou Central Hospital (Binzhou, China).

Consent for publication

All patients have provided written informed consent for publication.

Competing interests

The authors declare that they have no competing interests.

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