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Original Article

High expression of ZNF93 promotes proliferation and migration of ovarian cancer cells and relates to poor prognosis

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Abstract: Ovarian cancer (OC) is most common type of gynecologic cancer and is frequently lethal. It is important to determine the pathologic mechanisms underlying OC. ZNF93 is a member of the zinc finger protein family. Abnormal expression of ZNF93 has been observed in various tumor cells. However, its clinical significance and biologic function in ovarian cancer remain unclear. In the present study, we established that ZNF93 expression was highly up-regulated in OC samples and was closely correlated with clinical stage, indicating poor prognosis. We then established that ZNF93 promoted OC cell proliferation and migration. The results of our study may provide insight into the use of ZNF93 as a marker of clinical outcome and as a potential therapeutic target in OC.

Keywords: Ovarian cancer, transcription factor ZNF93, small interfering siRNA, cell proliferation, cell migration

Introduction

Ovarian cancer is one of the leading causes of death among afflictions of the female reproductive system. Every year, 22,530 patients are diagnosed with ovarian cancer, and 13,980 patients die from ovarian cancer, making it fifth in mortality in the world [1]. In China, ovarian cancer is seventh out of the 10 most common malignant tumors in women, by incidence, but the mortality of ovarian cancer has not decreased from 2003 to 2011. Rather, it has increased by 1.7% [2]. The overall prognosis of patients with ovarian cancer is poor because the onset often goes unnoticed and the disease progresses rapidly. The five-year survival rate of early stage patients can reach 92%, but for advanced ovarian cancer it is about 29%; and among all patients it is under 45% [3]. Traditionally, epithelial ovarian cancer has been mainly classified into four types by histology: serous tumors, clear cell tumors, mucinous tumors, and endometrioid tumors. Serous tumors are the most common epithelial ovarian cancer [4]. The main risk factors for ovarian cancer include the number of ovulations, which is related to early menarche, lifelong lack of pregnancy, late menopause, and also to smoking, family history of ovarian cancer, and benign gynecologic disease (including polycystic ovary syndrome and endometriosis) [3]. Surgery and chemotherapy are currently the main methods of treating ovarian cancer. The main reason for the high five-year mortality rate is that the pathogenesis of ovarian cancer is unclear and early diagnosis is difficult. It is very important to explore the pathogenesis of ovarian cancer and find biomarkers to evaluate the prognosis.

Transcription factors are important in maintaining biological balance and in regulating biological processes. Transcription factors activate stem cells, leading to the formation of terminally differentiated cells; and they alter the dedifferentiation of terminally differentiated cells into stem cells to form new differentiation characteristics. For example, octamers binding transcription factor 4 (Oct4) is expressed in stem cells, which is necessary for

controlling stem cell pluripotency and self-renewal. It also plays an important role in tumor-initiating cells and embryonic stem cancer cells [5]. In recent years, it has also been reported that transcription factors can be used as tumor detection markers. For example, the transcription factors A2P-2alpha and c-Ets-1 are closely related to the poor prognosis of ovarian cancer [6].

Zinc finger protein is the most common classical zinc finger and protein-forming sequence-specific binding protein among transcription factors [7]. Zinc finger proteins play an important role in gene regulation, and they are mainly involved in cell differentiation and embryo development [8]. Recent studies have shown that abnormal expression of zinc finger protein is helpful for the development of ovarian cancer. For example, the expansion and overexpression of ZKSCAN3 (also known as ZNF306 or ZNF309) can promote aggressive colorectal cancer. ZNF322A promotes the proliferation, migration, and invasion of lung cancer through transcription-activated cyclin D1, and it is an independent risk factor for poor prognosis in patients with lung cancer [9]. Zinc finger protein 93 (ZNF93) is a member of the zinc finger protein family. The molecular weight of ZNF93 is about 71 KDa. It is 620 amino acids in length and its gene is located on human chromosome 19q12 [10]. It is mainly found in the nucleus and cytoplasm. ZNF93 mainly recognizes and binds to the long-dispersed nuclear element-1 (L1) sequence and suppresses the expression of L1 sequence by recruiting an inhibitor complex containing TRIM28/KAP1 [11]. Recent studies have shown that ZNF93 can enhance the resistance of human cancer cell lines to ET-743 or PM00104 drugs, so it may play an important role in chemical resistance [12]. However, there are few reports on ZNF93 in ovarian cancer.

In this paper, we found that not only is ZNF93 more highly expressed in ovarian cancer than in normal tissues, but it is also associated with poor prognosis, as indicated by analysis conducted across several databases. Our studies have shown ZNF93 expression levels to be higher in ovarian cancer tissue than normal tissue by immunohistochemistry and it affects the proliferation and migration of ovarian cancer cells. We analyzed the possible mechanism and role of ZNF93 in ovarian cancer and assessed the value of its clinical use using GESA.

Materials and methods

Biological information database

Data were obtained from the American Cancer Gene Profile (http://tcga-data.nci.nih.gov/tcga/, TCGA) and High-throughput gene expression data GEO (https://www.ncbi.nlm.nih.gov/geo/). Kaplan-Meier Plotter database (https://kmplot.com/analysis/) was used to predict the relationship between ZNF93 and the prognosis of ovarian cancer. GSEA (Gene Set Enrichment Analysis) (http://software.broadinstitute.org/gsea/login.jsp) was used to detect the signaling pathways that ZNF93 may be involved with in ovarian cancer. The TCGA database mainly stores basic information about various types of tumors, including RNAseq, miRNAseq, DNA methylation, CNV, SNP, and other information. The GEO database is a high-throughput chip expression profile database used for high-throughput gene expression, genomic hybridization, and antibody array experiments. The Kaplan-Meier Plotter database is a public database containing mRNA expression profiling chips; it covers 5 types of cancer, breast, ovarian, lung, stomach, and liver, from which gene expression and disease prognosis can be obtained. GSEA is a whole-genome expression profiling chip data analysis. It first builds a molecular tag database based on existing knowledge of gene properties, localization, biologic significance, function, and other information. The database contains multiple functional genes.

Cell culture reagents

Human ovarian cancer cell lines SKOV3, OVCAR8, OVCAR3, and A2780 were purchased from the Cell Bank of the Chinese Academy of Sciences. Fetal bovine serum and crystal violet were sourced from Gibco (United States). RPMI 1640 medium and trypsin were from Solibao Biotechnology, and Lipofectamine™ 3000 Transfection Reagent was purchased from Thermo Fisher Scientific (China). Scramble control siRNA duplex and siRNA-ZNF93-1 and siRNA-ZNF93-2 were purchased from Shanghai Tuoran Biotechnology. ZNF93 polyclonal antibody was purchased from Beijing Boaosen Biotechnology. GAPDH and tubulin monoclonal

antibodies were purchased from Abcam. ECL luminescent substrates were purchased from Shenger Biological. The CCK-8 kit was purchased from Shanghai Dongren Chemical Technology, and the Transwell cell (nitrocellulose filter membrane) was purchased from Millipore, US.

Clinical sample materials

We recruited consecutive patients with ovarian cancer into a discovery cohort from 2012 to May 2017. There were 172 human ovarian tissue samples that were obtained from Fengxian Hospital, which is affiliated with Anhui University of Science and Technology, and Changzhou Women's Insurance Hospital. These recruits included 138 patients with serous ovarian cancer, ranging in age from 18-82 years, and 34 patients with benign serous cystadenoma, ranging in age from 21-71 years. Sample clinical information was complete. The study passed ethical review from the ethics committee of Fengxian Hospital, Anhui University of Science and Technology.

Immunohistochemical staining

All of the ovarian tissues were dewaxed using routine methods, followed by sodium citrate antigen retrieval. They were then treated with anti-human polyclonal antibody for ZNF93 overnight at 4°C in a moist chamber. After washing with PBST they were incubated with the secondary antibody (Thermo Scientific, US) labeled with HRP (rabbit) for 1 h at room temperature. The sections were treated with diaminobenzidine and counterstained with hematoxylin. Scoring was conducted according to the ratio and intensity of positive-staining cells: 0-25% scored 0, 25-50% scored 1, 50-75% scored 2, 75-100% scored 3. Then score 0-1 was designated as low expression and score 2-3 as high expression. All the ZNF93 expression levels were quantified double-blindly by two independent pathologists.

Quantitative real-time PCR

Total RNA was extracted from cells using Trizol reagent and reverse transcribed using a PrimeScript RT Reagent Kit according to the manufacturer's instructions. We used ZNF93 forward: 5'-ACCTGACTGCCAAAACATGC-3'; reverse: 5'-AGGTTGCTGGGTCTGCACTA-3'; 18S forward: 5'-ATCACCATTATGCAGAATCCACG-3'; reverse: 5'-GACCTGGCTGTATTTTCCATCC-3'. qRT-PCR reaction conditions were 40 cycles of 95°C for 5 min in the first stage, 95°C for 10 s in the second stage, 60°C for 60 s in the second stage; calculations were performed using the $2^{-\Delta\Delta Ct}$ method.

siRNA transfection

Small interfering RNAs duplexes for ZNF93 were as follows: siRNA1 sense, 5'-GGAAUGUGAUGUUAGAGAAdTdT-3', anti-sense, 5'-UUCUCUAACAUCACAUUCCdTdT-3'; siRNA2 sense, 5'-GGUGUGAAAGUGUAGAUGAdTdT-3', anti-sense, 5'-UCAUCUACACUUUCACACCdTdT-3'. The scramble control siRNA duplex were sense, 5'-UUCUCCGAACGUGUCACGUTT-3', antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. Transfection was performed using Lipofectamine™ 3000 Transfection Reagent according to the manufacturer's instructions.

Western blotting

Whole cell lysates were prepared by lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton-X 100, 1 mM each of $MgCl_2$, $MnCl_2$, and $CaCl_2$, 1 mM PMSF, and 10 mM sodium fluoride). Proteins were separated using SDS-PAGE and were transferred to nitrocellulose membranes. Then the electroblotted membranes were blocked in phosphate-buffered saline/Tween-20 containing 1% BSA. The primary antibodies for ZNF93, and GAPDH/Tubulin were used (1:1000). The samples were incubated with anti-mouse or anti-rabbit secondary antibodies (1:2000) for 1 h at room temperature, and the bands were assessed using an Odyssey infrared imaging system.

Cell viability assay (CCK8 assay)

Cells were seeded into a 96-well plate at 3×10^3 cells per well with 100 µl culture medium supplemented with 10% FBS and cultured at 37°C. 10 µl Cell Counting Kit-8 was added to each well after 24 h, 48 h, and 72 h, respectively. In viable cells, CCK8 was metabolized to produce a colorimetric dye detectable at 450 nm using a microplate reader (SpectraMax M5, Molecular device). The experiment was performed in triplicate and then repeated for a total of 6 rounds.

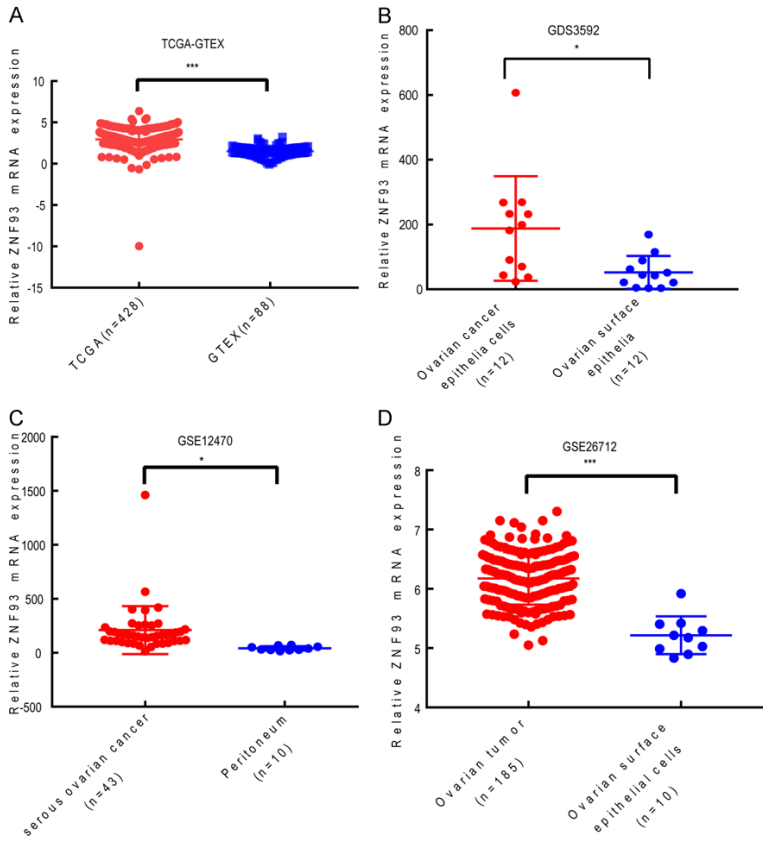

Figure 1. Analyzing the expression of ZNF93 in ovarian cancer samples in four databases. A. Expression of ZNF93 in ovarian cancer and normal ovaries in TCGA-GTEx (***$P < 0.001$). B. The expression of ZNF93 in ovarian cancer and normal ovary in GDS3592 (*$P < 0.05$). C. Expression of ZNF93 in ovarian cancer and normal peritoneum in GSE12470 (*$P < 0.05$). D. The expression of ZNF93 in ovarian cancer and normal tissue in GSE26712 (***$P < 0.001$) (Student's t-test, *$P < 0.05$).

Colony formation assay

Interfering with ZNF93 in a 6-well plate with Small interfering RNAs duplexes, then digesting the cells with trypsin, taking 3,000 cells into a 6-well plate, and 2 mL of complete culture solution was added. The old medium was replaced at 5-day intervals. After 10-15 days, fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and washed with PBS. Cell colonies were counted and photographed.

In vitro migration

Cells were suspended in medium without FBS on the upper chamber and medium with 10% FBS in the lower chamber for the in vitro transwell migration assay. After incubation for 24 h, the migrating cells were counted. All of the assays were performed independently three times.

Statistical analysis

Data are presented as the means ± standard error of the mean (SEM). Statistical analyses were performed using SPSS 19.0 for Windows (IBM). The chi-square test and Student's t-test were used for comparison between groups. Values of $P < 0.05$ were considesignificant.

Results

ZNF93 expression is increased in ovarian cancer tissues

We analyzed the expression of ZNF93 in ovarian cancer samples in two databases: TCGA (428 cases) and GTEx normal ovarian database (88 cases). We found that levels of ZNF93 were significantly higher in ovarian cancer tissues than in normal samples ($P < 0.0001$) (**Figure 1A**). We also found that the level of expression of ZNF93 was significantly higher compared to normal samples as indicated by analysis of GDS3592, GSE12470, and GSE26712 in the GEO database ($P < 0.05$, $P < 0.05$, $P < 0.0001$, respectively) (**Figure 1B-D**). Then we analyzed the ZNF93 expression in clinical samples, including 138 serous ovarian cancer tissues and 34 benign serous cystadenoma tissues, using immunohistochemistry. We found that 62 cases expressed ZNF93 at high levels and 76 expressed it at low levels in ovarian cancer tissue. However, among the benign serous cystadenoma tissues, 5 cases showed high expression and 29 cases showed low expression. Overall, ZNF93 was more highly expressed in serous ovarian cancer tissue than in benign serous cystadenoma tissue ($P = 0.001$) (**Figure 2**). Then, according to the level of pathology, we divided the samples into high-grade serous ovarian cancer and low-grade serous ovarian cancer. Among them, 97 cases were high-grade serous with a positive rate of 58.8%; 41 cases were low-grade serous with a positive rate of 46.3%. The expression level

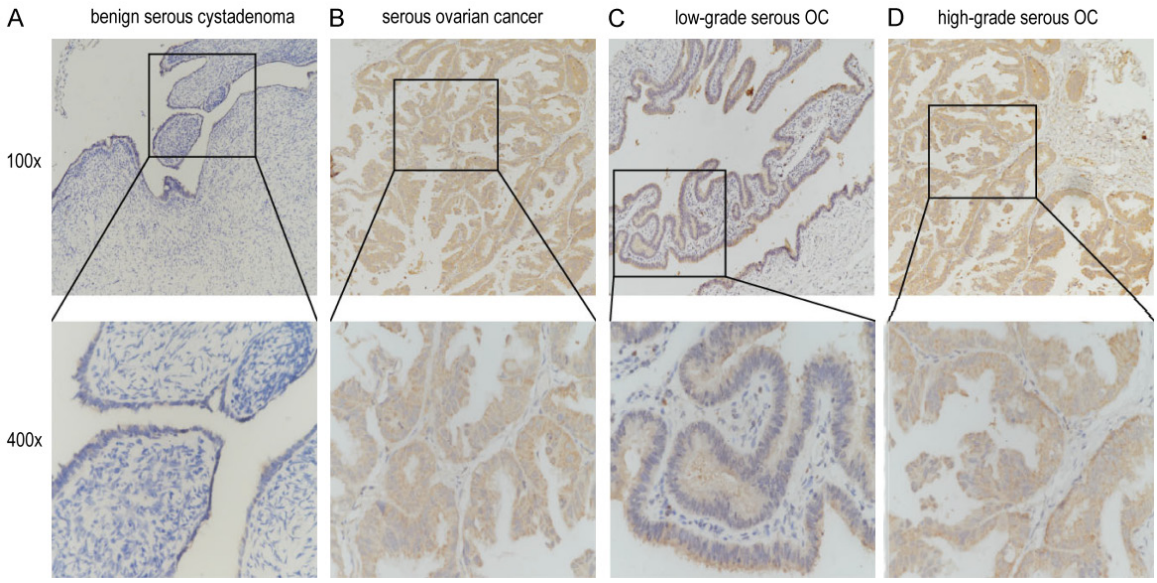

Figure 2. Immunohistochemical expression of ZNF93 in (A) benign serous cystadenoma, (B) serous ovarian cancer, (C) low-grade serous ovarian cancer, and (D) high-grade serous ovarian cancer. The top images represent 100×, the bottom images represent 400×.

Table 1. Association of ZNF93 expression level with clinical findings

Variable	High	Low	Total	X^2	*P*
Tissue Group					
Serous carcinoma	62 (44.9%)	76 (55.1%)	138		
Serous benign cystadenoma cystadenoma	29 (85.3%)	5 (14.7%)	34	10.478	0.001
Age					
≤ 50	20 (43.5%)	26 (56.5%)	46		
> 50	42 (45.7%)	50 (54.3%)	92	0.059	0.477
Grade of Serous Cancer					
High	40 (41.2%)	57 (58.8%)	97		
Low	22 (53.7%)	19 (46.3%)	41	1.797	0.124
Stage					
I	34 (68%)	16 (32%)	50		
II	5 (35.7%)	9 (64.3%)	14		
III	18 (28.1%)	46 (71.9%)	64		
IV	5 (50%)	5 (50%)	10	18.644	0.000
Stage					
I+II	39 (60.9%)	25 (39.1%)	64		
III+IV	23 (31.1%)	51 (68.9%)	74	12.364	0.000

χ^2 tests were applied for all other analysis.

of ZNF93 was not related to grade ($P = 0.124$). According to FIGO staging (2014), the samples were divided into 50 cases in stage 1 (positive rate 32%); 14 cases in stage 2 (positive rate 64.3%); 64 cases in stage 3 (positive rate 71.9%), and 10 cases in stage 4 (positive rate 50%). Expression was significantly different by stage ($P < 0.0001$). The samples were divided into 64 cases of early (stage 1+2) serous ovarian cancer (positive rate of 39.1%) and 74 cases of late stage (stage 3+4) serous ovarian cancer (positive rate of 68.9%). We observed a significant distinction between the two groups ($P < 0.0001$). These results show that the expression of ZNF93 is closely related to clinical stage (**Table 1**).

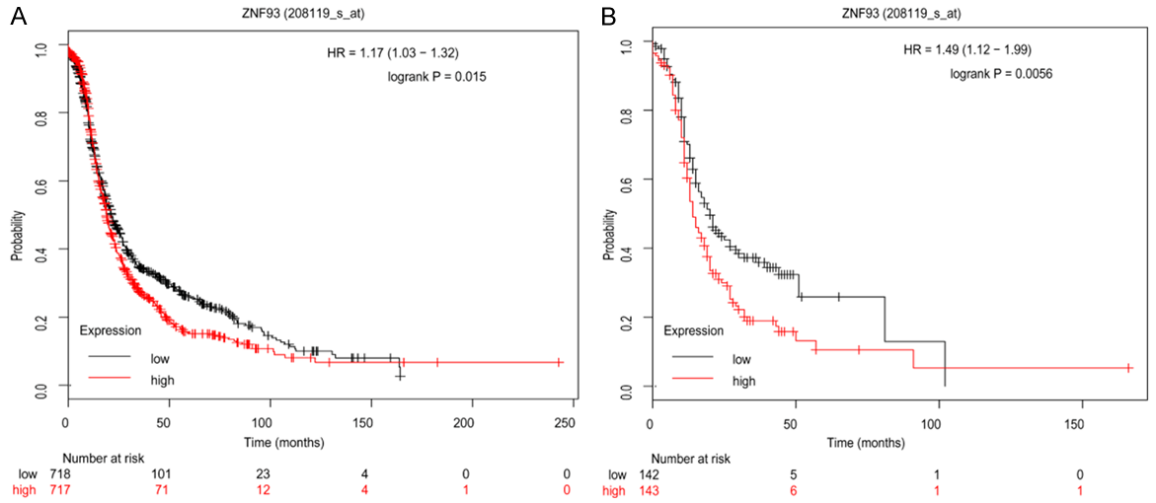

Figure 3. Kaplan-Meier analysis of the correlations between ZNF93 expression and survival rate of PFS patients based on an online database. A. Kaplan-Meier analysis of the correlations between ZNF93 expression and metastasis free survival rate of 1436 PFS patients (Gene symbol: 208119_s_at) (TCGA and GEO), B. Kaplan-Meier analysis of the correlations between ZNF93 expression and metastasis free survival rate of 285 PFS patients (Gene symbol: 208119_s_at) (GSE9891) (Logrank P, $^{*}P < 0.05$).

We observed high expression of ZNF93 (717 cases) to be associated with a worse FPS than low expression (718 cases) using Kaplan-Meier Plotter ($P = 0.015$) (**Figure 3A**). High expression (150 cases) of ZNF93 was found to be associated with a worse progression-free survival than low expression (148 cases) ($P = 0.0056$) (**Figure 3B**). These results indicate that ZNF93 may play an important role in ovarian cancer.

Knockdown of ZNF93 inhibited OC cell proliferation and migration

ZNF93 expression in OC cells was detected in four cell lines, namely, OVCAR3, OVCAR8, A2780, and SKOV3, by qRT-PCR and western blot. We found that ZNF93 expression was highest in OVCAR8 of the four cell lines (**Figure 4A**). We then knocked down the ZNF93 expression using small interfering RNA (siRNA). Results showed that the ZNF93 expression was much lower in RNA and protein levels, especially si-1 ($P = 0.001$) and si-2 ($P = 0.001$) (**Figure 4B**). We selected si-1 and si-2 as our experimental group.

In order to assess the effect of ZNF93 on the proliferation in OVCAR8 cells, we used the CCK8 assay and colony formation assay. We found that, after knockdown of ZNF93 by siRNA, cell proliferation ability was detectably more inhibited in siZNF93 groups than in the NC group at 24 h, 48 h, and 72 h, respectively ($P < 0.05$) (**Figure 4C**). We also found that the numbers of siZNF93 groups (si-1: n = 23 ± 5, si-2: n = 17 ± 3) were significantly lower than in the NC group (n = 71 ± 3) ($P = 0.0034$, $P = 0.0019$). This indicated that knockdown of ZNF expression inhibited the proliferation of OC cells (**Figure 4D**).

We used a transwell assay to determine whether ZNF93 is correlated with motility in OVCAR8 cells. We found that, after knockdown of ZNF93 by siRNA, the cell number on the upper membrane in the siZNF93 groups (n = 24 ± 2, n = 17 ± 3) was markedly lower than in the NC group (n = 98 ± 5) ($P < 0.001$, $P < 0.001$). This indicated that knockdown of ZNF93 inhibited the migration of OC cells (**Figure 4E**).

ZNF93 may be related to the MYC signaling pathway in ovarian cancer as indicated by gene set enrichment analysis

A total of 428 cases of ovarian cancer samples related to ZNF93 were retrieved from the TCGA database and divided into high expression groups (214 cases) and low expression groups (214 cases). The analysis results are based on $P < 0.05$ and FDR < 0.25. We found 6 signal pathways have a high degree of enrichment, in which the MYC and G2/M pathways took the first and second positions (NES: 1.72; 1.69,

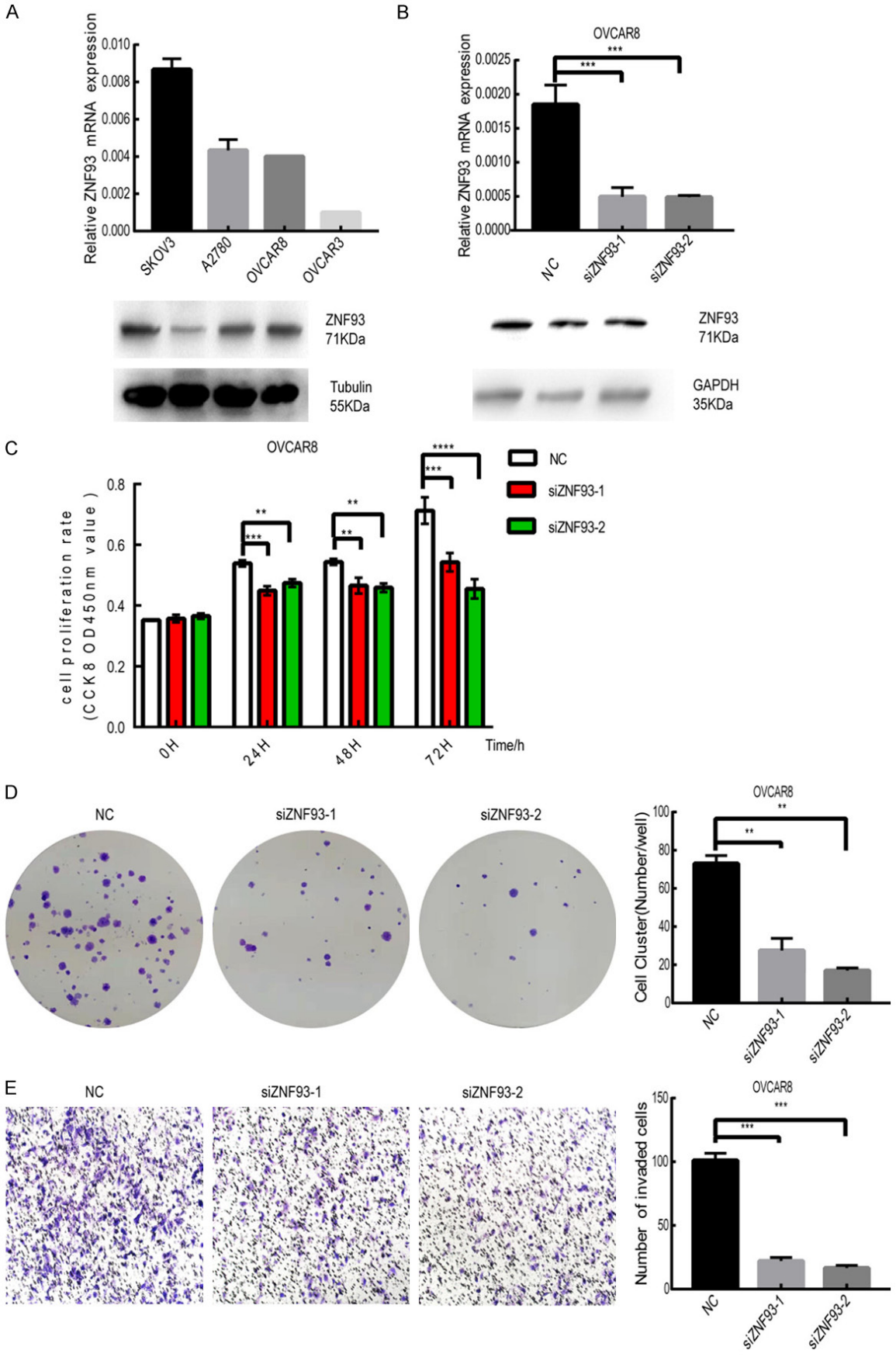
A
Relative ZNF93 mRNA expression
0.010
0.008
0.006
0.004
0.002
0.000
SKOV3
A2780
OVCAR8
OVCAR3
ZNF93
71KDa
Tubulin
55KDa
B
OVCAR8
***
***
Relative ZNF93 mRNA expression
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
NC
siZNF93-1
siZNF93-2
ZNF93
71KDa
GAPDH
35KDa
C
OVCAR8
NC
siZNF93-1
siZNF93-2
cell proliferation rate (CCK8 OD450nm value)
0.8
0.6
0.4
0.2
0.0
0H
24H
48H
72H
Time/h
D
NC
siZNF93-1
siZNF93-2
OVCAR8
Cell Cluster(Number/well)
100
80
60
40
20
0
E
NC
siZNF93-1
siZNF93-2
OVCAR8
Number of invaded cells
150
100
50
0

Figure 4. Effects of ZNF93 on cell proliferation, colony formation, and migration in ovarian cancer cells. A. The expression level of ZNF93 was detected by real-time PCR and western blot in different OC cell lines. B. The expression level of ZNF93 was detected by real-time PCR and western blot in ZNF93 knockdown OVCAR8 cells. C. The cell proliferation of control and siZNF93-1, siZNF93-2 groups in OVCAR8 cells was determined by CCK8 assay at 0, 24, 48, and 72 h, respectively. Values are mean ± SD, n = 5 ($^*P < 0.05$). D. Knockdown of ZNF93 suppressed proliferation of OS cells using colony formation assay. Representative photographs of the colony formation assay were shown in the left panel. Scale bars = 5 mm ($^*P < 0.05$). E. Representative migration images of ZNF93 silenced and control cells ($^*P < 0.05$) (Student's t-test, $^*P < 0.05$).

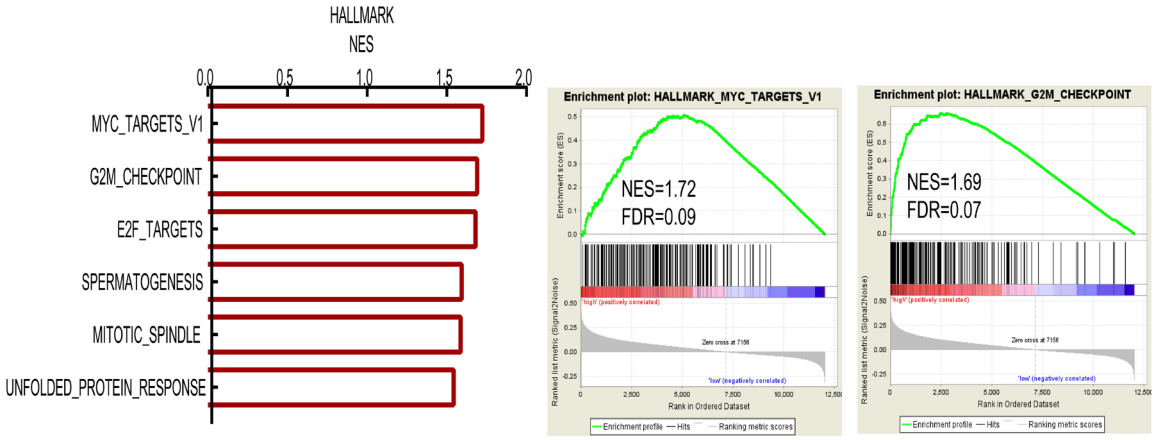

Figure 5. Downstream pathway of ZNF93 in ovarian cancer. Gene set enrichment analysis showing that ZNF93 expression positively correlated with MYC and G2/M regulated gene signatures in The Cancer Genome Atlas (TCGA) dataset.

FDR: 0.09, 0.07). ZNF93 may promote the occurrence and development of ovarian cancer through MYC and G2/M signaling pathways (**Figure 5**).

Discussion

Cancer is a disorder of cell proliferation. Over a long lifespan, a vertebrate needs a large number of cells to develop and continue to proliferate. The proliferation of normal cells can also inhibit the generation of mutant cells. If the two are not in balance, cancer develops [13]. The major reason for the high death rate among cancer patients is widespread metastasis. The process of tumor metastasis requires the ability of individual cancer cells to migrate and invade and detach from the primary tumor. Many studies have shown that the main cause of a low five-year survival rate among ovarian cancer patients is its biologic potential, with migration and proliferation together facilitating tumor development [14]. Our research indicated that ZNF93 can significantly promote the proliferation and migration of ovarian cancer cells.

Ovarian cancer is not only insidious but also highly lethal. Although a large number of studies have shown that CA125 is a sensitive biomarker for the detection of ovarian cancer, it lacks specificity [15]. This necessitates the identification of molecular biomarkers with good specificity and sensitivity. Studies have shown that zinc finger protein plays an important role in human cancer. For example, inhibition of ZNF677 in thyroid cancer can promote the proliferation and colony formation of thyroid cancer cells, and the low expression of ZNF677 is associated with poor patient prognosis [16]. A search of several databases and immunohistochemical analysis showed that ZNF93 is highly expressed in serous ovarian cancer. The prognosis of patients with high levels of ZNF93 expression is significantly worse than that of patients with low expression. This indicates that ZNF93 may be used as a molecular biomarker of poor epithelial prognosis.

It has been reported that ZNF143 may cause colon cancer [17]. Zinc finger protein 32 can protect cancer cells from oxidative-stress-

induced apoptosis by regulating C1QBP transcription [18]. It can also increase breast cancer stem cell resistance by increasing GPER expression [19]. ZFPL1 induces RL95-2 cell proliferation in endometrial cancer, and it participates in the promotion of cancer development though the PI3K/Akt pathway [20]. Zinc finger protein 521, through the negative regulation of microRNA-204-5p, promotes the proliferation, migration, and invasion of gastric cancer cells [21]. However, the specific pathogenesis of ZNF93 in tumors is still unclear.

Our GSEA enrichment analysis showed that the transcription factor ZNF93 is highly enriched in six signaling pathways in ovarian cancer, in which MYC and G2/M pathways rank first and second, respectively. This indicates that ZNF93 may be linked to MYC and G2/M relating to signaling pathways. The MYC transcription factor family includes c-MYC, L-MYC, and N-MYC. The MYC family proteins regulate a variety of cellular processes, including proliferation and differentiation [22]. Dysregulation of MYC activity occurs in a variety of cancers and greatly promotes cancer progression and metastatic capacity [23]. MYC works in both normal cells and cancer cells. It promotes glycolysis by inducing activation of genes encoding glycolytic enzymes and GLUT, which causes cell growth and proliferation and so promotes cancer progression [24]. At the same time, MYC up-regulates the expression of glutamine transporter and promotes the metabolism of glutamine in carcinogenesis [25]. The interaction of miR-145 and c-myc/GLS1 inhibits the development of ovarian cancer cells through glutamine metabolism [26]. We therefore speculate that the transcription factor ZNF93 may cause the occurrence and development of ovarian cancer through MYC and promote the proliferative ability of ovarian cancer cells through G2/M.

In summary, transcription factor ZNF93 promotes cancer proliferation and migration in serous ovarian cancer, and it may have potential as a prognostic marker of ovarian cancer. ZNF93 may promote the occurrence and development of ovarian cancer through the MYC and G2/M signaling pathways.

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Disclosure of conflict of interest

None.

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