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Sam68 is Overexpressed in Epithelial Ovarian Cancer and Promotes Tumor Cell Proliferation

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Statistical Analysis C
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Funds Collection G

ABCDF 1 Lijuan Dong*
BCDF 1 Hailuo Che*
BCD 2 Mingmei Li
ACDEG 1 Xuepeng Li

1 Department of Obstetrics and Gynecology, Zaozhuang Municipal Hospital, Zaozhuang, Shandong, P.R. China
2 Health and Family Planning Bureau in Shanting District, Zaozhuang, Shandong, P.R. China

* Lijuan Dong and Hailuo Che contributed equally to this study

Corresponding Author: Xuepeng Li, e-mail: xuepengli_zzmh@163.com
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Background: Epithelial ovarian cancer (EOC) is the deadliest gynecological malignancy, and evidence is accumulating on how molecular markers may be associated with the origin and process of EOC. Sam68 (Src-associated in mitosis, of 68 kD), is a K homology domain RNA-binding protein that has been investigated as a risk factor in multiple types of tumors. The aim of the present study was to investigate the contribution of the Sam68 gene in the pathogenesis of EOC.

Material/Methods: Western blot assay and real-time quantitative PCR methods were performed to examine Sam68 expression in EOC tissue specimens. The association of Sam68 expression with clinic-pathologic variables of EOC was evaluated. Then gain-of-function and loss-of-function strategies were adopted to examine the regulation of Sam68 on the proliferation of EOC OVCAR-3 cells using CCK-8 and colony forming assays.

Results: Sam68 was overexpressed in both mRNA and protein levels in EOC tumor tissue (n=152) in an association with malignant factors of EOC such as International Federation of Gynecology and Obstetrics (FIGO) stage, residual tumor size (cm), histological grade, and lymph node metastasis. *In vitro* results demonstrated that Sam68 overexpression was upregulated while Sam68 knockdown downregulated the proliferation of EOC OVCAR-3 cells via regulation of cell growth and colony formation.

Conclusions: Sam68 was overexpressed in EOC tissue in association with such cancer malignant factors of FIGO stage, histological grade, and lymph node metastasis, and also positively regulated the proliferation of EOC cells. Our research suggests that Sam68 might accelerate cell cycle progression, and present as a prognostic marker for EOC.

MeSH Keywords: Biological Markers • Cell Proliferation • Ovarian Neoplasms

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Background

Epithelial ovarian cancer (EOC) is the deadliest gynecological malignancy [1], with a median overall survival of only 2–4 years [2]. Multiple genetic variants in a number of genes may be involved in the origin and process of EOC [3], such as RAD51C [4], RAD51D [5], CASP8 [6], LIN28B [7], or BRCA [8]. However, only a few genetic variants exhibited strong evidence of an association with ovarian cancer, and the identification of genes associated with a predisposition to ovarian cancer requires further investigation [9]. Recently, molecular markers have been recognized to be oncogenic in EOC: lysophosphatidic acid (LPA)/Yes-associated protein (YAP) oncogenic signaling [10], Sirtuin 7 (SIRT7) [11], Interleukin-33 [12], Fli-1, the tyrosine kinase adaptor protein FRS2 [13] and other markers, high lightening the complexity of the oncology of EOC.

Sam68 (Src-associated in mitosis 68 kD) is a member of the signal transduction and activation of RNA metabolism (STAR) family of proteins, with a K homology RNA-binding domain [14]. Sam68 is involved in a wide range of cellular processes including signal transduction, transcription, RNA metabolism, cell cycle progression, and apoptosis [14–16]. Moreover, Sam68 has been recently recognized to be oncogenic in glioblastoma [15], lung cancer [17], breast cancer [18,19], prostate cancer [20], and cervical cancer [21]. Sam68 deregulates the cell cycle in tumorigenesis, by promoting the conversion of G1/S or G2/M phase [16,18,22] by modulating cancer-relevant splicing events, such as cyclin D1 and Bcl-xl, both of which are involve in cell cycle progression and apoptosis [23,24]. More recently, it was reported that the expression of Sam68 correlated with cell proliferation and survival in epithelial ovarian cancers [25].

The aim of this present study was to investigate the expression of Sam68 in EOC tissues, and to analyze the correlation of it with clinic-pathological characteristics of EOC patients. Then we examined the prognostic role of Sam68 for EOC patients, and determined the regulation of it on the proliferation of EOC OVCAR-3 cells. Our study confirmed the oncogenic role of Sam68 in EOC, and confirmed the positive regulation of it on the proliferation of EOC cells.

Material and Methods

Patients and samples

This study included 152 patients diagnosed with ovarian cancer (mean age of 48±9.82 years, from 31–62 years old, with a median age of 48 years) at Zaozhuang Municipal Hospital (Zaozhuang City, Shandong, China) between March 2011 and April 2014. Clinical characteristics, including age at diagnosis, International Federation of Gynecology and Obstetrics (FIGO) stage, residual tumor size, histological grade, lymph node metastasis, and histological type, were obtained from the patients' medical records (Table 1). Most of the patients were of Han Chinese background and resided in Shandong Province, China. All of the participants provided written informed consent to participate in this study. The Ethical Committee of Zaozhuang Municipal Hospital approved this research (zzh2013-2-11). All 152 human EOC tissue specimens and the peritumor specimens were obtained by surgical resection, and were stored at –80°C before use.

Cell lines and cell culture

The human EOC cell line OVCAR-3 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). OVCAR-3 cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA), which was supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% streptomycin/penicillin (Sigma-Aldrich, St. Louis, MO, USA) in an incubator with 5% CO_2 at 37°C. The medium was changed on alternate days and cells were split before they reached more than 90% confluence. Expression vectors pRC/CMV-Sam68-FLAG (Sam68 Up) and pcRC/CMV (Con Up, as control) were purchased from Promega (Promega, Madison, WI, USA). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was utilized to transfect plasmids into OVCAR-3 cells. Sam68 knockdown was conducted by using siRNAi-Sam68 (5'-tga gtt aaa ata gat tta gga a-3') (as control siRNA, 5'-uuc uca gaa cgu gug acg u-3'), which were transfected into OVCAR-3 cells with Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA).

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cancer tissues or OVCAR-3 cells using TRIzol reagent (Life Technologies, Grand Island, NY, USA) based on the product's suggested protocol. Reverse transcription PCR was performed using a PrimeScript RT-PCR kit (TaKaRa, Dalian, China), and qRT-PCR was performed using the Applied Biosystems 7900HT machine (Applied Biosystems, Carlsbad, CA, USA). The following primers were used to amplify the Sam68 or β-actin. Forward primer for Sam68: 5'- tgc ccg aac tca tgg ccg ag-3', reverse primer for Sam68: 5'- caa gta att ctc ctc atc at -3'. Forward primer for β-actin: 5'-tgc gtc tgg acc tgg ctg gc-3', reverse primer for β-actin: 5'-acg tag cac agc ttc tcc tt -3'. The following primers were used for the qRT-PCR analysis of Sam68 and β-actin. Forward primer for Sam68: 5'-cct gcc cga act cat ggc cg-3', and reverse primer for Sam68: 5'-tgc atg gcg tga gtg aag gac-3'. Forward primer for β-actin: 5'-cgg gac ctg act gac tac ct-3', and reverse primer for β-actin: 5'-gct cgg ccg tgg tgg tga ag-3'. β-actin was used as the internal control. The one-step qRT-PCR was performed as following: 48°C for 15 minutes followed by 94°C for 5 minutes; and then 94°C for 20 seconds, 60°C for 30 seconds, with 40 cycles.

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Table 1. Correlation of Sam68 expression with clinicopathological characteristics of EOC patients.

Characteristics	No. (n=152)	High Sam68		Low Sam68		*P* value
		N	%	N	%	
Age at diagnosis (years)						0.834629
<50	80	48	53.33	32	51.61	
≥50	72	42	46.67	30	48.39	
FIGO stage						0.009707
I	25	11	12.22	14	22.58	
II	49	23	25.56	26	41.94	
III	56	42	46.67	14	22.58	
IV	22	14	15.56	8	12.90	
Residual tumor size (cm)*						0.035284
<2	80	41	45.56	39	62.90	
≥2	72	49	54.44	23	37.10	
Histological grade						0.012075
1	40	19	21.11	21	33.87	
2	54	28	31.11	26	41.94	
3	58	43	47.78	15	24.19	
LN metastasis		90				0.020853
Positive	76	52	57.78	24	38.71	
Negative	76	38	42.22	38	61.29	
Histological type						0.676589
Serous	93	57	63.33	36	58.06	
Endometrioid	18	10	11.11	8	12.90	
Mucinous	26	13	14.44	13	20.97	
Clear cell	15	10	11.11	5	8.06	

Western blot assay

Cellular protein samples were extracted from cancer tissues or OVCAR-3 cells with Nuclear/Cytosol Fractionation Kit (BioVision, San Diego, CA, USA), separated via 10% SDS-PAGE electrophoresis, and then transferred onto polyvinylidene fluoridehydrophobic membrane (Millipore, Bedford, MA, USA). Western blotting was performed using rabbit polyclone antibody against human Sam68 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or rabbit polyclone antibody against β-actin antibody (Sigma-Aldrich, St. Louis, MO, USA). Horseradish peroxidase-linked goat anti-rabbit IgG (Pierce, Rockford, IL, USA) and the enhanced chemiluminescence (Thermo Scientific, Rockford, IL, USA) were utilized to analyze Sam68 level in cancer tissues or OVCAR-3 cells.

Cell proliferation and migration assay

Cell Counting Kit-8 (CCK-8) was utilized to examine the growth curve of the OVCAR-3 cells, in which Sam68 was overexpressed or knocked down. In brief, OVCAR-3 cells with Sam68 overexpressed or knocked down were incubated at 37 °C for 24, 48, or 72 hours, and then were incubated with CCK-8 solution (DOJINDO, Kumamoto, Japan). The 450 nm absorbance was detected after visual color occurrence. For cell colony formation assay, 4×10^2 OVCAR-3 cells, post Sam68 overexpressed or knocked down, were incubated in 6-well plates at 37°C containing 5% CO_2. After an incubation of 48 hours (for Sam68 overexpression) or 96 hours (for Sam68 knockdown), cells were stained with crystal violet (0.005%) for 25

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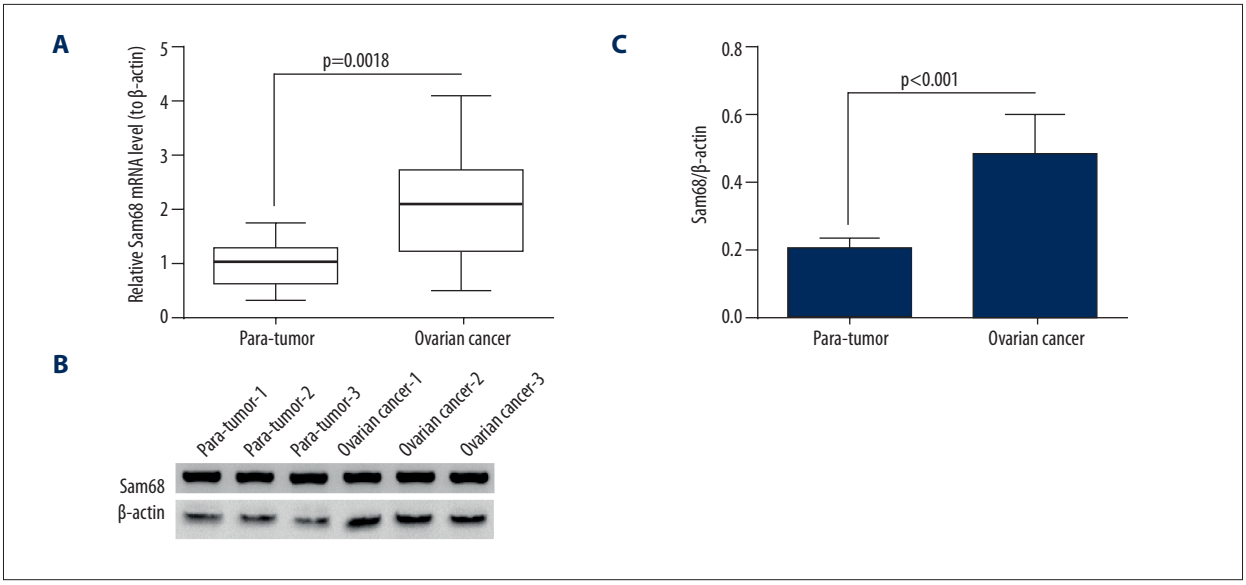

Figure 1. Overexpression of Sam68 in ovarian cancers. (**A**) Relative mRNA level of Sam68 in the ovarian cancer specimens (n=152) with para-tumor tissues as control. (**B**) Representative Western blot analysis of Sam68 protein level in ovarian cancer and para-tumor specimens. (**C**) Relative protein level of Sam68 to β-actin in the ovarian cancer and para-tumor tissues (n=30). Paired *t*-test was performed to examine the difference in either mRNA or protein level of Sam68, and statistical significance was considered when $p<0.05$.

minutes, and the colony in each group was counted by imaging J software.

Statistical analysis

Quantitative data was presented as mean ± standard deviation. Student's *t*-test was performed to compare differences between two groups. Comparisons among multiple samples were made by ANOVA analysis. Statistical analyses were conducted by SPSS 17.0 software (IBM SPSS, Armonk, NY, USA). Statistically significant difference was considered when $p<0.05$ or less.

Results

Sam68 was overexpressed in epithelial ovarian cancer (EOC) in association with cancer malignancy factors

The Sam68 expression in mRNA levels in cancer tissue and in para-tumor tissue (more than 1 cm from tumor tissue) of each patient was examined via RT-qPCR method. As shown in Figure 1, the relative Sam68 mRNA level (compared to para-tumor sample) was significantly higher in the tumor specimens using the two-tailed paired *t*-test ($p=0.0018$). We then re-grouped those samples according to their Sam68 levels as high Sam68 group (n=90) and low Sam68 group (n=62). The patients with relative Sam68 mRNA levels higher than "1" in the tumor sample than in the para-tumor sample ("1" was the average Sam68 mRNA level in the para-tumor tissues) were classified into the high Sam68 group. The unpaired *t*-test indicated a significant difference between the two group ($p<0.0001$, data not shown). We examined the Sam68 expression in protein levels in the EOC tissues with Western blot assay. As shown in Figure 1B and 1C, the protein level of Sam68 was markedly higher in the EOC specimens than in the para-tumor specimens ($p<0.001$). Therefore, we found the overexpression of Sam68 in EOC tissues.

To recognize the association between the Sam68 high expression and each clinic-pathological characteristic, we analyzed the statistical difference in age, FIGO stage, residual tumor size, histological grade, lymph node metastasis, and histological type between the high Sam68 and low Sam68 groups. Univariate analysis (Table 2) found no significant difference in the age at diagnosis (<50 *vs.* ≥50) and histological type (serous *vs.* non serous); however, there was significant difference found in the FIGO stage (I–II *vs.* III–IV, $p=0.0145$), residual tumor size (cm) (<2 *vs.* ≥2, $p=0.0431$), histological grade (1+2 *vs.* 3, $p=0.02874$) and lymph node metastasis (positive *vs.* negative, $p=0.0342$). The hazard ratio (HR) and 95% confidence interval (CI) for each variable are shown in Table 2. Moreover, the Sam68 expression was also a significant prognostic factor for EOC patients (high *vs.* low, $p=0.0013$, HR 2.347, 95% CI 1.635–3.254). In addition, the multivariate analysis for each significant variable in the univariate analysis demonstrated that FIGO stage ($p=0.0210$, 95% CI 1.26–2.27), histological grade ($p=0.0352$, 95% CI 1.24–2.33) and Sam68 expression

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Table 2. Univariate analysis of prognostic factors in EOC patients.

Variables	Univariable analysis		
	Hazard ratio	95% CI	P value
Age at diagnosis (years)			
<50 *vs.* ≥50	0.641	(0.326–1.264)	0.9362
FIGO stage			
I–II *vs.* III–IV	1.965	(1.306–2.542)	0.0145
Residual tumor size (cm)			
<2 *vs.* ≥2	1.730	(1.135–2.438)	0.0431
Histological grade			
1+2 *vs.* 3	1.792	(1.251–2.542)	0.02874
LN metastasis			
Positive *vs.* Negative	1.642	(1.214–2.265)	0.0342
Histological type			
Serous *vs.* others	0.695	(0.380–1.464)	0.7522
Sam68 expression			
High *vs.* Low	2.347	(1.635–3.254)	0.0013

Table 3. Multivariate analysis of prognostic factors in OC patients.

Variables	Multivariable analysis		
	Hazard ratio	95% CI	*P* value
FIGO stage			
I–II *vs.* III–IV	1.74	(1.26–2.27)	0.0210
Residual tumor size (cm)			
Positive *vs.* Negative	1.56	(1.13–1.88)	0.0514
Histological grade			
1+2 *vs.* 3	1.63	(1.24–2.33)	0.0352
Sam68 expression			
High *vs.* Low	1.97	(1.52–3.06)	0.0073

(p=0.0073, 95% CI 1.52–3.06) were independently associated with poor prognostic variables for EOC patients with EOC (Table 3). However, the residual tumor size (cm) was not an independent prognostic variable for EOC patients (p=0.0514).

Manipulation of Sam68 in ovarian cancer OVCAR-3 cells

To investigate the regulatory role of Sam68 in ovarian cancer OVCAR-3 cells, we first manipulated the Sam68 expression with gain-of-function and loss-of-function strategies. We found that both mRNA (p<0.001, Figure 2A) and protein (p<0.01, Figure 2B) levels of Sam68 were significantly upregulated in the OVCAR-3 cells post transfection with pRC/CMV-Sam68-FLAG (Sam68 Up). However, the Sam68-specific siRNA was used to knock down the Sam68 expression in both mRNA and protein levels. Figure 2C and 2D demonstrate that the siRNA transfection (20 nM and 40 nM) reduced both mRNA (p<0.01) and protein (p<0.01) levels of Sam68 in the OVCAR-3 cells.

Sam68 promotes the proliferation of OVCAR-3 cells.

To investigate the regulation by Sam68 on tumor growth of EOC, we determined the proliferation of OVCAR-3 cells, in which Sam68 was upregulated or was knocked down. As shown in Figure 3A, there was significant difference in the growth curve between OVCAR-3 cells (Sam68 Up) and OVCAR-3 cells (Con Up), OVCAR-3 cells (Sam68 Up) grew more efficiently (p<0.05 or p<0.01). We also performed the colony formation of OVCAR-3

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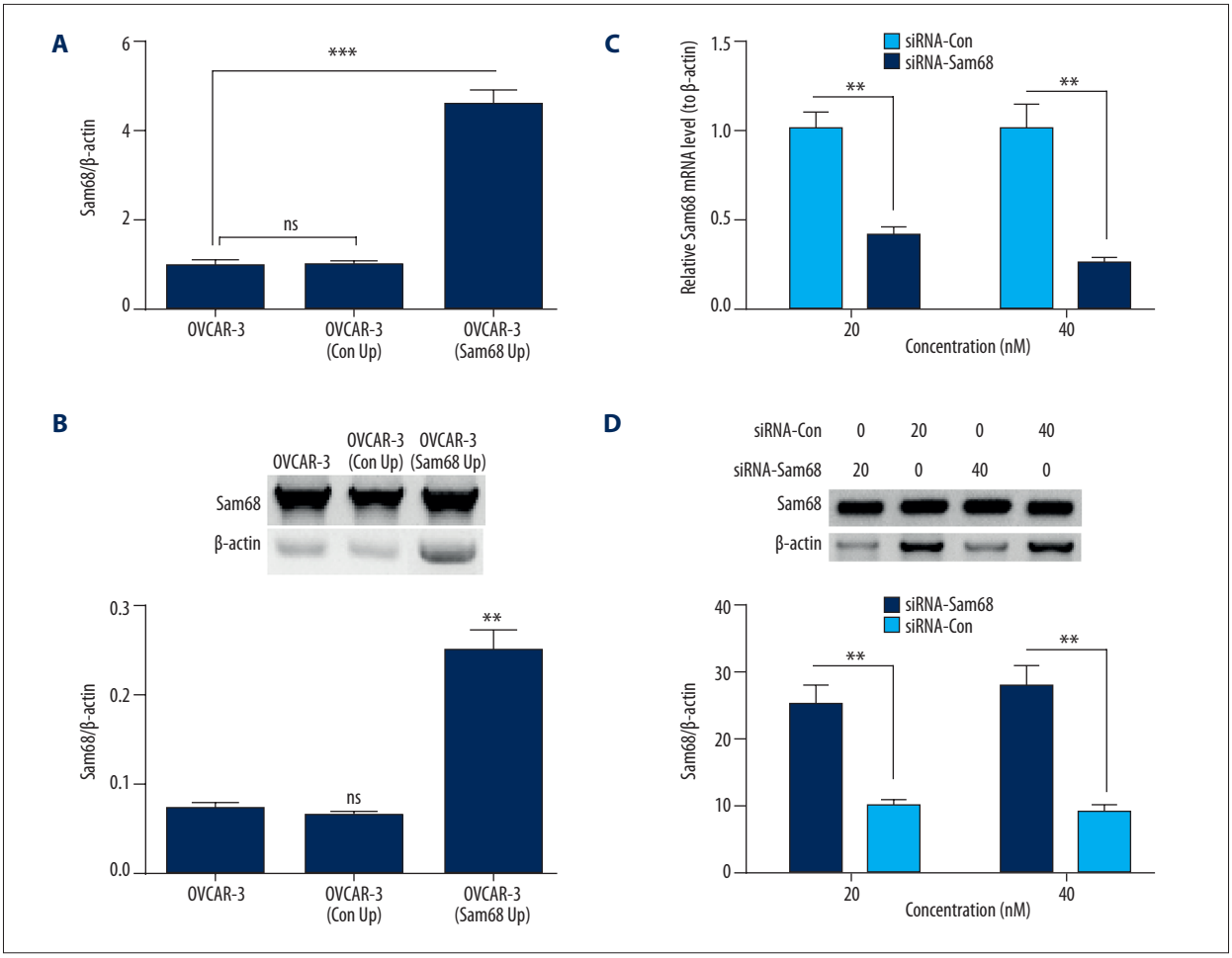

Figure 2. pRC/CMV-mediated upregulation and siRNA-mediated knockdown of Sam68 in ovarian cancer OVCAR-3 cells (**A**) mRNA level of Sam68 in OVCAR-3 cells, which were transfected with pRC/CMV-Sam68-FLAG (Sam68 +) or pcRC/CMV (Con +, as control); (**B**) Western blot analysis of Sam68 in the blank, Sam68 +, or Con + OVCAR-3 cells; (**C**) mRNA level of Sam68 in the OVCAR-3 cells, which were transfected with siRNA-Con or siRNA-Sam68 (20 or 40 nM); (**D**) Western blot analysis of Sam68 in the siRNA-Con- or siRNA-Sam68-transfected (20 or 40 nM) OVCAR-3 cells. Data was averaged for triple independent results. ns represented no significance, ** represented $p<0.01$, and *** represented $p<0.001$.

cells (Sam68 Up) and OVCAR-3 cells (Con Up) and found more colony numbers in the Sam68 Up group than in the Control group ($p<0.05$, Figure 3B, 3C). We also found that the OVCAR-3 cells (Sam68 Up) formed colonies with larger size ($p<0.01$, Figure 3B, 3C). In addition, we knocked down the Sam68 expression and re-examined the proliferation of OVCAR-3 cells. As shown in Figure 4A, there was a significant reduction in the growth efficiency of OVCAR-3 cells, post Sam68 knockdown ($p<0.05$). Colony forming results also indicated that the Sam68 (KD) OVCAR-3 cells formed less and smaller colonies than the Con (KD) OVCAR-3 cells ($p<0.05$ or $p<0.001$, Figure 4B, 4C). These results, taken together, confirmed that Sam68 promotes the proliferation of OVCAR-3 cells.

Discussion

The poor prognostic role of Sam68 has recently been identified in non-small cell lung cancer [17], bladder cancer [26], esophageal squamous cell carcinoma [27], colorectal cancer [28], and aggressive neuroblastoma [29], and is associated with the tumor progression. Sam68-deficient mice have delayed development of sexual organs [30], and display a reduction in the number of developing ovarian follicles, alteration of estrous cycles, and impaired fertility [31]. More recently, the role of Sam68 in the tumorigenesis of ovarian cancers has been reported [25].

In the present study, we found overexpression of Sam68 in EOC tissues in association with the stage of malignancy, suggesting it is a significant prognostic factor for EOC patients, along with the FIGO stage, residual tumor size, histological grade, and

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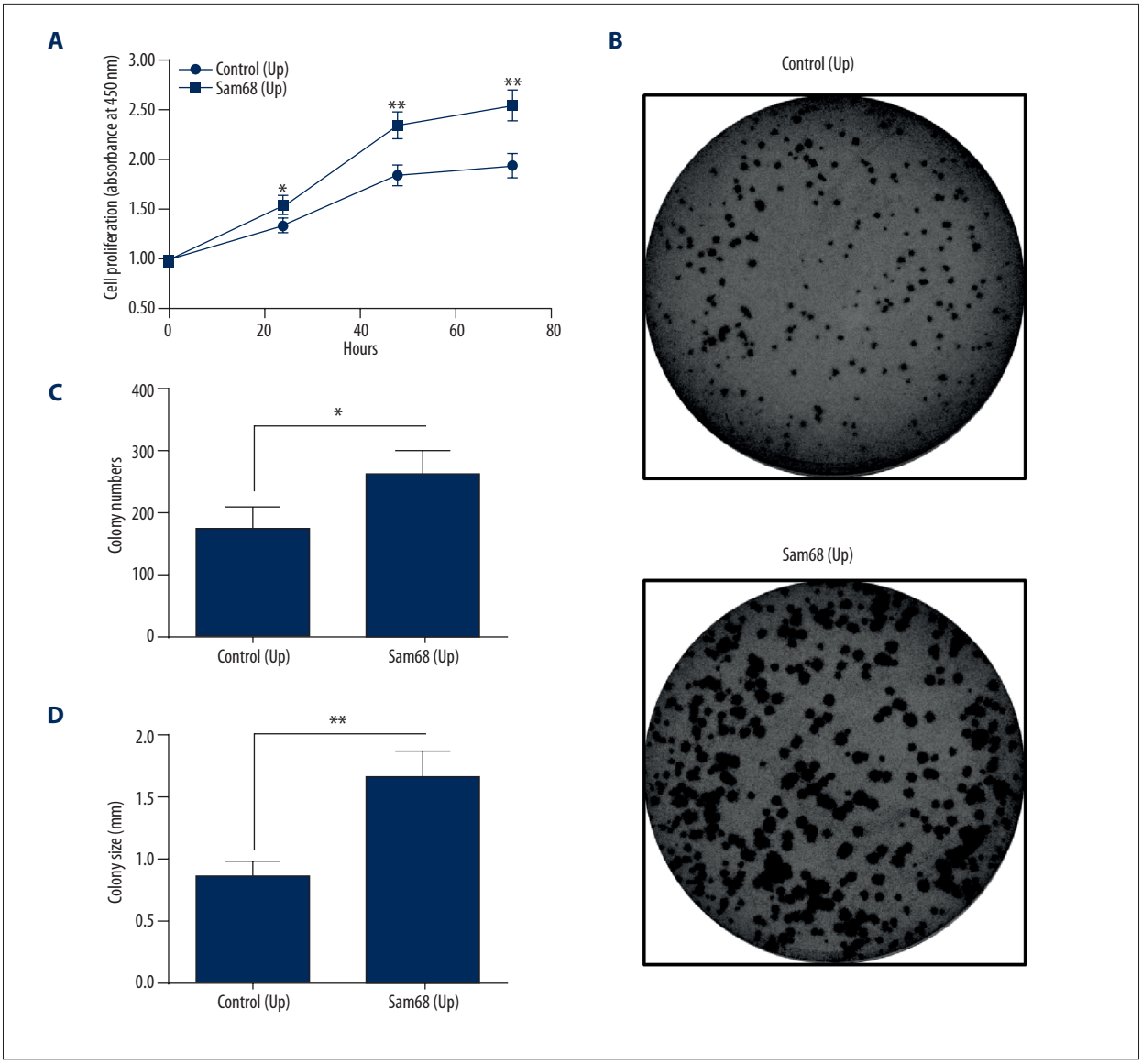

Figure 3. Sam68 upregulation promotes the proliferation of ovarian cancer OVCAR-3 cells. (**A**) Growth curve of the OVCAR-3 cells, in which Sam68 was upregulated (Sam68 [Up]) or not (Control [Up]), as was determined by CCK-8 assay; (**B**) Colony forming assay of the Sam68 (Up) or Control (Up) OVCAR-3 cells, which were incubated for 48 hours; (**C, D**) Colony counting (**C**) and colony size (**D**) of the colony which was formed by the Sam68 (Up) or Control (Up) OVCAR-3 cells. Experiments were repeated in quadruplicate independently. ANOVA or unpaired *t*-test was performed to examine the difference in the proliferation of colony formation between the Sam68 (Up) and Control (Up) OVCAR-3 cells. Data were averaged for triple independent results. Statistical significance was considered when $p<0.05$. * represented $p<0.05$ and ** represented $p<0.01$.

lymph node metastasis of EOC. Our results also demonstrated that the upregulated Sam68 promoted tumor cell proliferation. Our results indicated that the level of Sam68 expression may serve as a clinical marker for the degree of tumor growth *in vivo*. Therefore, our findings add to the understanding of the mechanism of ovarian cancer tumorigenesis in association with Sam68.

The positive regulatory role of Sam68 has also been recognized in various other types of cancer. The Sam68 upregulation correlates with, and its downregulation inhibits, proliferation and tumorigenicity of breast cancer cells [18]. Sam68 also has been found to upregulate the proliferation and the aggression of other types of cancers; Sam68 promotes cellular proliferation and predicts poor prognosis in esophageal squamous cell carcinoma [27] and prostate tumor cells [32]. In the current study, the overexpressed Sam68 promoted growth and colony formation of ovarian OVCAR-3 cells, whereas the siRNA-medicated Sam68 knockdown markedly inhibited growth and colony

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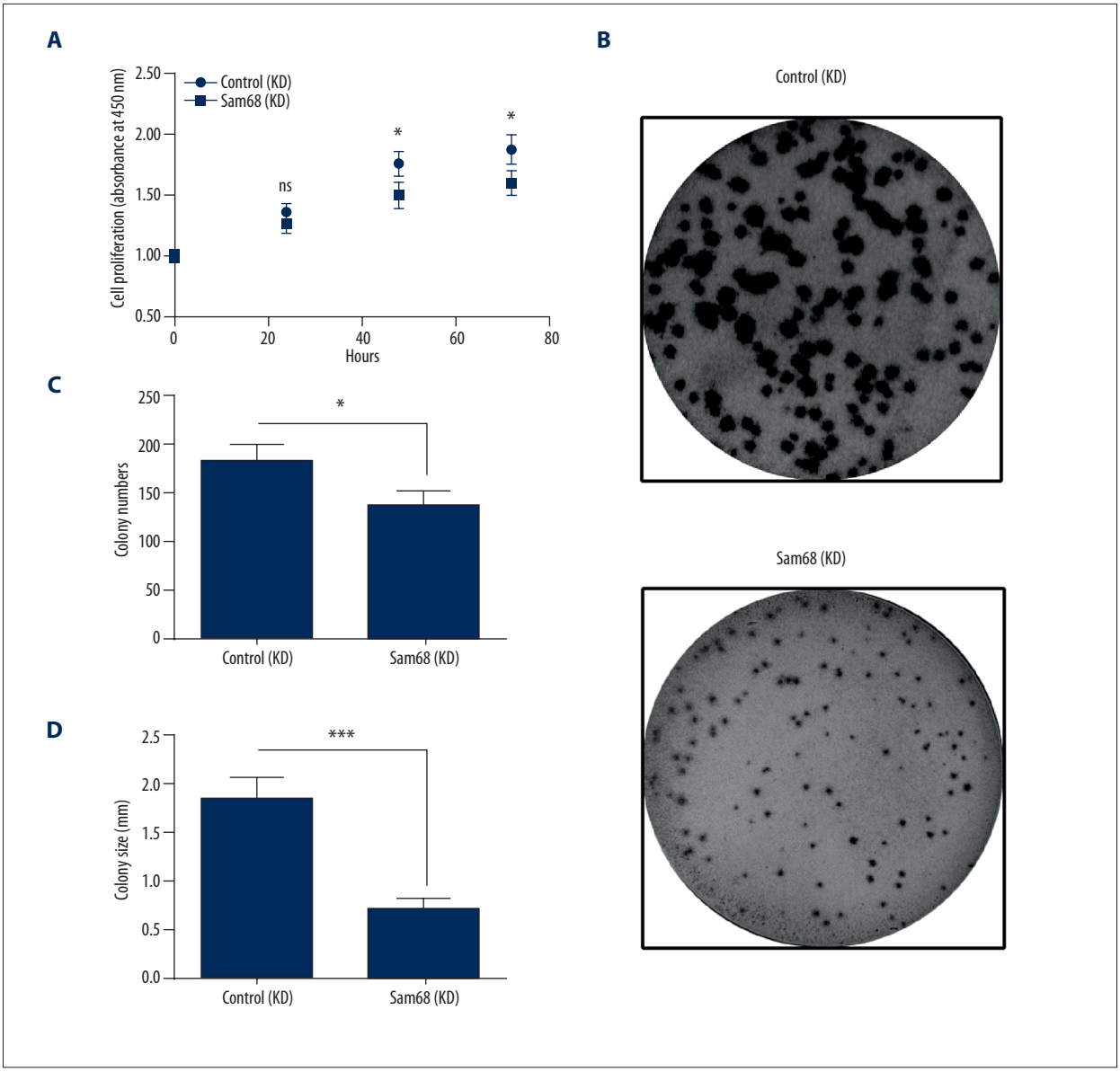

Figure 4. Sam68 knockdown inhibits the proliferation of ovarian cancer OVCAR-3 cells. (**A**) Growth curve of the OVCAR-3 cells, in which Sam68 was knocked down (Sam68 [KD]) or not (Control [KD]), as was determined by CCK-8 assay. (**B**) Colony forming assay of the Sam68 (KD) or Control (KD) OVCAR-3 cells, which were incubated for 96 hours. (**C, D**) Colony counting (**C**) and colony size (**D**) of the colony which was formed by the Sam68 (KD) or Control (KD) OVCAR-3 cells. Experiments were repeated in quadruplicate independently. ANOVA or unpaired *t*-test was performed to examine the difference in the proliferation of colony formation between the Sam68 (KD) and Control (KD) OVCAR-3 cells. Data was averaged for triple independent results. Statistical significance was considered when $p<0.05$. ns represented no significance, * represented $p<0.05$, and ** represented $p<0.01$.

formation of OVCAR-3 cells. Thus, we confirmed the positive regulation by Sam68 on the proliferation of ovarian cancer cells.

Conclusions

Sam68 is overexpressed in EOC tissues in association with cancer malignancy variables such as FIGO stage, histological grade, and lymph node metastasis, suggesting positive regulation of the proliferation of EOC cells. This research indicates that Sam68 might accelerate the cell cycle progression.

Conflict of interest

No conflict of interests.

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