KPNA2 promotes cell proliferation and tumorigenicity in epithelial ovarian carcinoma through upregulation of c-Myc and downregulation of FOXO3a

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Karyopherin alpha 2 (KPNA2), a member of the karyopherin family, has a central role in nucleocytoplasmic transport and is overexpressed in many cancers. Our previous study identified KPNA2 as significantly upregulated in epithelial ovarian carcinoma (EOC), correlating with poor survival of patients. However, the precise mechanism of this effect remains unclear. The aim of the present study was to examine the role of KPNA2 in the proliferation and tumorigenicity of EOC cells, and its clinical significance in tumor progression. Real-time quantitative RT-PCR analysis revealed high expression levels of KPNA2 in 162 out of 191 (84.8%) fresh EOC tissues, which was significantly correlated with International Federation of Gynecology and Obstetrics (FIGO) stage, differentiation, histological type, recurrence, and prognosis of EOC patients. Our results showed that upregulation of KPNA2 expression significantly increased the proliferation and tumorigenicity of EOC cells (EFO-21 and SK-OV3) in vitro and in vivo, by promoting cell growth rate, foci formation, soft agar colony formation, and tumor formation in nude mice. By contrast, knockdown of KPNA2 effectively suppressed the proliferation and tumorigenicity of these EOC cells in vitro and in vivo. Our results also indicated that the molecular mechanisms of the effect of KPNA2 in EOC included promotion of G1/S cell cycle transition through upregulation of c-Myc, enhanced transcripational activity of c-Myc, activation of Akt activity, suppression of FOXO3a activity, downregulation of cyclin-dependent kinase (CDK) inhibitor p21Cip1 and p27Kip1, and upregulation of CDK regulator cyclin D1. Our results show that KPNA2 has an important role in promoting proliferation and tumorigenicity of EOC, and may represent a novel prognostic biomarker and therapeutic target for this disease.

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Abbreviations: BrdU, 5-bromo-2-deoxyuridine; CDK, cyclin dependent kinase; EOC, epithelial ovarian carcinoma; IHC, immunohistochemical; KPNA2, Karyopherin alpha 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; qRT-PCR, real-time quantitative RT-PCR; WHO, World Health Organization

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paired normal human ovarian surface epithelial (HOSE) tissues. Immunohistochemistry revealed that overexpression of KPNA2 in EOC was correlated with poor prognosis; however, the precise mechanism underlying the correlation is unknown.

In the present study, we found that expression of KPNA2 was significantly upregulated in human EOC cell lines and tissues, leading to significant increases in the proliferation and tumorigenicity of EOC cells in vitro and in vivo. By contrast, knockdown of KPNA2 suppressed the proliferation and tumorigenicity of EOC cells in vitro and in vivo. Furthermore, we showed that KPNA2 promoted G1/S cell cycle transition, through upregulation of c-Myc; enhanced transcriptional activity of c-Myc and Akt activity, and suppression of FOXO3a; downregulated cyclin-dependent kinase (CDK) inhibitors p21Cip1 and p27Kip1; and upregulated CDK regulator cyclin D1. Taken together, our findings indicated that KPNA2 has an important role in the proliferation and tumorigenicity of human EOC and suggests that KPNA2 may be a potential target for human EOC treatment.

Results

KPNA2 is upregulated in EOC tissues and cell lines. Of the 26,863 genes detected by the Affymetrix GeneChip (Human Genome U133 Plus 2.0 Array) microarray, 19 genes were found to be upregulated in EOC cells (fold change ≥ 8), including KPNA2 (fold change = 10), compared with HOSE tissues. The hierarchical cluster analysis of these genes is shown in Figure 1a. Results from real-time quantitative RT-PCR (qRT-PCR) assays of KPNA2 expression in EOC and HOSE samples were consistent with the gene expression patterns for KPNA2 detected by the microarray experiments (Figure 1b). Western blotting analysis revealed that expression of the KPNA2 protein was markedly higher in all seven EOC cell lines compared with HOSE samples (Figures 1c and d).

**Figure 1** KPNA2 is overexpressed in EOC cells and tissue samples; and KPNA2 upregulation is associated with poor prognosis. (a) Gene expression microarray analysis showing that KPNA2 was upregulated (10-fold) compared with HOSE samples. (b) Real-time qRT-PCR analysis showing the average expression levels of KPNA2 in EOC (n = 191) and HOSE (n = 10) tissue samples. Expression levels are normalized to β-actin mRNA. Error bars represent S.E. (c) Western blot analysis of KPNA2 protein expression in two HOSE samples and seven EOC cell lines; β-actin was used as a loading control. (d) KPNA2 protein expression in two HOSE samples and seven EOC cell lines was quantitated using ImageJ software (Wayne Rashband). (e) Kaplan–Meier analysis showing that the expression of KPNA2 was significantly associated with poor overall survival in 191 EOC cases (P = 0.012, log-rank test). (f) Kaplan–Meier analysis showing that expression of KPNA2 was significantly associated with poor relapse-free survival in 191 EOC cases (P < 0.001, log-rank test)
High levels of KPNA2 expression predict poor prognosis for EOC patients. qRT-PCR assays detected high expression levels of KPNA2 in 191 EOC specimens versus 10 HOSE specimens: 3.52 ± 0.23 (mean ± S.E.) at 2^−ΔΔCt of KPNA2 (Figure 1b).

We ranked patients according to their expression levels and divided them into a high expression group (n = 95) or low expression group (n = 96) using the median expression level of KPNA2 as the cutoff point. Correlations between KPNA2 expression levels and clinicopathological characteristics of the EOC patients are summarized in Table 1. High levels of KPNA2 expression were detected in 66.3% (63/95) of stage III/IV patients and in 33.7% (32/95) of stage I/II patients. High levels of KPNA2 expression were significantly associated with advanced clinical stage EOC, histological type, differentiation, recurrence, and suboptimal cytoreductive surgery. The 5-year overall survival rates and 5-year relapse-free survival rates of patients expressing low levels of KPNA2 were significantly higher than those of patients expressing high levels of KPNA2 (68.2% versus 52.3%, P = 0.012; 62.5% versus 38.9%, P < 0.001) (Figures 1e and f). Furthermore, multivariate analysis indicated that a high level of KPNA2 expression was an independent predictor of EOC in patients, and was associated with a 1.832-fold increased risk of a poor prognosis (Table 2).

Table 1 Correlations between KPNA2 expression and clinicopathological features of patients with epithelial ovarian cancer

| KPNA2 expression | Patient and tumor characteristics | High (%) | Low (%) | P-value |
|------------------|----------------------------------|----------|---------|---------|
| Age, years       |                                  |          |         |         |
| ≤45              |                                  | 30 (31.6) | 33 (34.4) | 0.681   |
| >45              |                                  | 65 (68.4) | 63 (65.6) |         |
| Histological type|                                  |          |         |         |
| Serous           |                                  | 71 (74.7) | 48 (50.0) |         |
| Mucinous         |                                  | 21 (22.1) | 41 (42.7) |         |
| Others           |                                  | 3 (3.2)   | 7 (7.3)   | 0.002   |
| FIGO stage       |                                  |          |         |         |
| I/II             |                                  | 32 (33.7) | 60 (62.5) | <0.001  |
| III/IV           |                                  | 63 (66.3) | 36 (37.5) | <0.001  |
| Differentiation  |                                  |          |         |         |
| Well             |                                  | 23 (24.2) | 49 (51.0) |         |
| Moderate         |                                  | 26 (27.4) | 29 (30.2) |         |
| Poor             |                                  | 46 (48.4) | 18 (18.8) | <0.001  |
| Distant metastasis|                                 |          |         |         |
| Yes              |                                  | 7 (7.4)   | 4 (4.2)   | 0.351   |
| No               |                                  | 88 (92.6) | 91 (95.8) |         |
| Recurrence       |                                  |          |         |         |
| Yes              |                                  | 46 (48.4) | 26 (27.1) |         |
| No               |                                  | 49 (51.6) | 70 (72.9) | 0.002   |
| Cytoreductive surgery |                            |          |         |         |
| Optimal cytoreduction |                          | 66 (69.5) | 87 (90.6) | 0.016   |
| Suboptimal cytoreduction |                        | 19 (20.5) | 9 (9.4)   |         |

Bold P-values indicate significance (P < 0.05)

Table 2 Univariate and multivariate analysis of factors associated with overall survival

| Clinical variable | Subset | Hazard ratio (95% CI) | P-value |
|------------------|--------|-----------------------|---------|
| Univariate analysis |       |                       |         |
| Histology        | Others versus mucinous versus serious | 0.672 (0.426–1.058) | 0.086   |
| FIGO stage       | III/IV versus I/II | 3.905 (2.439–6.252) | <0.001  |
| Differentiation  | Poor versus moderate versus well | 1.701 (1.326–2.181) | <0.001  |
| Cytoreductive surgery | Suboptimal cytoreduction versus optimal cytoreduction | 3.302 (2.032–5.364) | <0.001  |
| Expression of KPNA2 | High versus low | 2.404 (1.563–3.697) | <0.001  |
| Multivariate analysis |       |                       |         |
| FIGO stage       | III/IV versus I/II | 3.570 (1.984–6.424) | <0.001  |
| Cytoreductive surgery | Suboptimal cytoreduction versus optimal cytoreduction | 1.819 (1.096–3.018) | 0.021   |
| Expression of KPNA2 | High versus low | 1.832 (1.137–2.951) | 0.013   |

Abbreviations: CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics. Bold P-values indicate significance (P < 0.05)
tumorigenicity of EOC cells in vivo, nude mice were inoculated subcutaneously with EFO-21/KPNA2 (KPNA2) cells or EFO-21/siKPNA2#1 (siKPNA2) cells (Figure 3e). The tumors that formed from EFO-21/siKPNA2#1 cells grew more slowly than those from EFO-21/KPNA2 cells (Figure 3f). This was consistent with our cell proliferation results in vitro. Furthermore, the average volume of the tumors induced by EFO-21/KPNA2 cells (279.3 ± 38.0 mm$^3$, $n = 4$) were significantly larger than those induced by vector control cells (112.8 ± 12.4 mm$^3$, $n = 4$, $P<0.01$), whereas depletion of endogenous KPNA2 in EFO-21 cells significantly inhibited tumor growth (average volume: 24.5 ± 5.3 mm$^3$ versus 108.8 ± 20.8 mm$^3$, $n = 4$, $P<0.01$) (Figure 3g).

KPNA2 expression in xenograft tumors was analyzed by western blotting; the results showed that KPNA2 expression was upregulated in tumors induced by EFO-21/KPNA2 cells, but downregulated in tumors induced by EFO-21/siKPNA2#1 cells (Figure 3h). This was consistent with our immunohistochemical (IHC) analysis, which found that tumor sections with high levels of KPNA2 also stained strongly for Ki67, whereas those corresponding to low KPNA2 expression levels only showed marginal levels of Ki67 expression (Figure 3i). Collectively, our results showed that KPNA2 is overexpressed in highly proliferative EOC cells, indicating that KPNA2 has an important role in enhancing the tumorigenicity of EOC cells both in vitro and in vivo.
Figure 3  KPNA2 is essential for EOC cell tumorigenicity in vitro and in vivo. Representative micrographs (left panel) and quantifications (right panel) of soft agar colony formation assays for the following cell lines, relative to the control: (a and b) EFO-21-vector, EFO-21-KPNA2, EFO-21-si-scramble, EFO-21-siKPNA2 #1, EFO-21-siKPNA2 #2; and (c and d) SK-OV3-vector, SK-OV3-KPNA2, SK-OV3-si-vector, SK-OV3-siKPNA2 #1, SK-OV3-siKPNA2 #2. Each bar represents the mean (± S.D.) of three independent experiments. Xenograft model in nude mice. (e) EFO-21-vector, EFO-21/KPNA2 (KPNA2), EFO-21-si-scramble, and EFO-21/siKPNA2 #1 (siKPNA2) cells were injected subcutaneously into the left and right flanks of the mice. (f) Representative pictures of tumor growth 30 days after inoculation. (g) Tumor volumes were measured on the indicated days. All data are shown as mean ± S.D. (h) Western blotting analysis of KPNA2 expression in EFO-21-vector, EFO-21/KPNA2 (KPNA2), EFO-21-si-scramble, and EFO-21/siKPNA2 #1 (siKPNA2) cells were injected subcutaneously into the left and right flanks of the mice. (i) Immunohistochemical analysis of EFO-21-vector, EFO-21/KPNA2 (KPNA2), EFO-21-si-scramble, and EFO-21/siKPNA2 #1 (siKPNA2) generated tumors 30 days after injection. Sections obtained from tumors were incubated with anti-ki67 antibody. Representative fields are shown (× 200 magnification) *P<0.05; **P<0.01
Depletion of KPNA2 induces G1/S cell cycle arrest in EOC cells. The mechanism underlying the promotion of cellular proliferation by KPNA2 was investigated by a 5-bromo-2'-deoxyuridine (BrdU) incorporation assay. As shown in Figures 4a–d, downregulation of KPNA2 in EFO-21 and SK-OV3 cell lines significantly decreased the percentage of BrdU-positive cells, significantly increased the percentage of cells at G0/G1 phase and decreased the number of cells in S-phase.

**Figure 4** Depletion of KPNA2 induces G1/S arrest of EOC cells (BrdU incorporation assay). (a and b) Representative micrographs (top panel), and (c and d) quantification (middle panel) of BrdU-incorporating cells in KPNA2-overexpressing and two KPNA2 shRNA(s)-infected cell lines, relative to the control. The cells were fixed, subjected to BrdU staining, and visualized under a fluorescence microscope. Data were obtained from three independent experiments and showed similar results. Red, BrdU; blue, DAPI.

(e and f) Flow cytometry analysis of the indicated EOC cells transfected with the KPNA2 overexpression construct or KPNA2 shRNA(s). The proportion of S-phase cells as significantly reduced in KPNA2 shRNA(s)-transfected cell lines (P < 0.05) compared with the control group; in contrast, the proportion of S-phase cells in the line transfected with the KPNA2 construct clearly increased (P < 0.05) *P < 0.05
percentage of cells at S phase of the cell cycle (Figures 4e and f). These results suggested that silencing of KPNA2 induced G1/S-phase arrest in EOC cells. Further investigations by real-time qRT-PCR and western blotting analyses revealed that the expressions of CDK inhibitors p21Cip1 and p27Kip1, were dramatically upregulated, whereas expression of the CDK regulator cyclin D1 was downregulated in KPNA2-knockdown cells at both the mRNA (Figures 5a and b) and protein levels compared with control cells (Figures 5c–f). This finding further supported our supposition that KPNA2 is involved in the regulation of EOC cell proliferation.

**Upregulation of KPNA2 enhances c-Myc transcriptional activity and suppresses FOXO3a activity.** To further investigate the molecular mechanism mediating the cell cycle arrest effect of downregulating KPNA2, the levels of cell cycle-related proteins c-Myc, AKT, and FOXO3a were examined, because the above identified altered cell cycle genes (p21Cip1, p27Kip1, and cyclin D1) are known downstream targets of c-Myc and FOXO3a. As shown in Figures 6a and b, a c-Myc reporter assay showed that KPNA2 upregulation significantly increased c-Myc transcriptional transactivating activity in both EFO-21 and SK-OV3 cell lines. By contrast, downregulation of KPNA2 attenuated c-Myc transcriptional transactivating activity. Furthermore, western blotting analysis showed that c-Myc, phosphorylated AKT, and phosphorylated FOXO3a significantly increased in KPNA2-overexpressing EOC cells and were reduced in KPNA2-knockdown EOC cells, compared with control cells (Figures 6c–f). These results suggest that the effect of KPNA2 on cell proliferation and tumorigenicity may be effected through upregulation of c-Myc transcriptional activity and activation of Akt.

**Knockdown of KPNA2 causes subcellular redistribution of c-Myc in EOC cells.** There are some recent reports that KPNA2 can interact with c-Myc; therefore, we were interested in determining whether KPNA2 mediates nuclear translocation of c-Myc. To examine the effect of KPNA2 on the subcellular distribution of c-Myc in EOC cells, we further upregulated the expression of c-Myc in KPNA2-overexpressing and -knockdown EOC cells. We confirmed this regulation by subcellular fractionation, followed by western blotting analysis. We further examined the expression of c-Myc in KPNA2-overexpressing and -knockdown EOC cells by subcellular fractionation, followed by western blotting analysis. Fractionation efficacy was validated by the detection of GAPDH and lamin B in the cytosolic and nuclear fractions, respectively. The protein level of c-Myc was normalized to that of the two marker proteins. We found that c-Myc was increased in the cytoplasmic fraction in KPNA2-knockdown EOC cells and reduced in KPNA2-overexpressing EOC cells, compared with control cells (Figures 7a–c). To confirm the role of c-Myc in KPNA2-mediated cell proliferation and tumorigenicity, we further analyzed the transcriptional activity of c-Myc in KPNA2-knockdown and -overexpressing EOC cells via transfection with a c-Myc-luciferase reporter plasmid. As expected, the luciferase activity from the c-Myc reporter was dramatically reduced in KPNA2-silenced cells and increased in KPNA2-overexpressing EOC cells compared with control cells (Figure 7d), suggesting that c-Myc has an important role in the cell proliferative and tumorigenic effect of KPNA2.

**Discussion**

We report the characterization of KPNA2 as a candidate oncogene in EOC. The pivotal finding of our study was the detection of KPNA2 upregulation in 84.8% (162/191) of primary EOC tumors that were significantly associated with poor prognosis in EOC patients. This led us to propose that KPNA2 might have an important role in EOC development and progression. This was supported by our findings: knockdown of endogenous KPNA2 inhibited the proliferation and tumorigenicity of EOC cells in vitro and in vivo, and silencing of KPNA2 with RNAi resulted in inhibition of c-Myc transactivity and Akt kinase activity, and suppression of FOXO3a activity, leading to upregulation of CDK inhibitors p21Cip1 and p27Kip1 and downregulation of CDK regulator cyclin D1. These findings provided strong evidence that upregulation of KPNA2 has an important role in promoting cell proliferation and tumorigenicity, and imply that KPNA2 may function as an oncogenic protein in the development and progression of EOC.

Nucleocytoplasmic transport mechanisms have been the target of numerous studies because of their role in key cellular processes, such as gene expression, cell cycle progression, and signal transduction. Increasing evidence suggests that these mechanisms contribute to malignant cell transformation, thereby highlighting the potential of these proteins as therapeutic targets. KPNA2 is an adaptor protein that mediates the import of signaling factors into the nucleus and the export of response molecules to the cytoplasm. Although the potential oncogenic functions of KPNA2 have been implicated in a variety of malignancies, the precise mechanism remains unclear. In the present study, we provided evidence that knockdown of KPNA2 downregulates c-Myc and decreases the transcriptional activity of c-Myc in both EFO-21 and SK-OV3 cell lines. A plausible mechanism by which KPNA2 could affect carcinogenesis is through the translocation of cancer-associated cargo proteins. It has previously been demonstrated that KPNA2 interacts with a variety of proteins that are associated with cancer, including checkpoint kinase 2, NBS1, thought to be involved in DNA repair and meiotic recombination, and the tumor-suppressor p53. KPNA2 has also been implicated in the translocation of transcription factors, including E2F1, c-Myc, PLAG1, and LOT1. Previous reports identified a potential molecular link between KPNA2 and c-Myc, involving TGF-β1 and IFN-γ. Expression of KPNA2 was specifically regulated by TGF-β1 and IFN-γ; TGF-β1 induced reversible growth arrest at the G1 phase of the cell cycle, while IFN-γ induced irreversible growth arrest in cultured keratinocytes and promoted aberrant terminal differentiation.

In addition, KPNA2 is a target of c-Myc; the expression and nuclear localization of c-Myc is rapidly downregulated when keratinocytes are treated with TGF-β1. In addition, the transcription factor c-Myc downregulates p21Cip1 and p27Kip1, and upregulates cyclin D1 at the transcriptional level. The results of the present study...
are consistent with these reports. We found that c-Myc was reduced in the nuclear fraction in KPNA2-knockdown EOC cells and increased in KPNA2-overexpressing EOC cells, which led us to hypothesize that KPNA2 transports c-Myc into the nucleus where it accumulates, thereby inhibiting transcription of p21Cip1 and p27Kip1, and inducing G1/S-phase arrest.
KPNA2 promotes proliferation and tumorigenicity of EOC
L. Huang et al.

Figure 6 Downregulation of KPNA2 decreases transcriptional activity of c-Myc and the activity of Akt, and increases the activity of FOXO3a. (a and b) c-Myc reporter activity in EOC cells transduced with vector KPNA2; si-scramble; siKPNA2 #1; and siKPNA2 #2, relative to the control. (c and d) Western blotting analysis of phosphorylated Akt (p-Akt), total Akt, phosphorylated FOXO3a (p-FOXO3a), total FOXO3a, and c-Myc proteins in the indicated EOC cells. (e and f) Expression levels were quantitated using ImageJ software (Wayne Rashband; bottom); β-actin was used as a loading control. Error bars represent the S.D. from three independent experiments. *P < 0.05 compared with the control.

arrest. However, further studies are needed to establish what role KPNA2 has in the translocation and regulation of the critical transcriptional factor c-Myc.

FOXO3a is a critical tumor suppressor that transcriptionally regulates multiple proteins, including p21Cip1, p27Kip1, and cyclin D1. There is considerable evidence that Akt kinase, which acts upstream of FOXO3a, has important roles in cell cycle control. In the current study, we found that KPNA2 knockdown decreased the level of phosphorylated Akt and phosphorylated FOXO3a, indicating that downregulation of KPNA2 may arrest EOC cells at the G1 phase through the Akt/FOXO3a pathway. A study by Brysk et al. reported that the MRE11–RAD50–NBS1 complex serves as a sensor and a mediator in cell cycle checkpoint signaling. NBS1 functions as a tumor suppressor by preserving genome integrity in the nucleus, and may also have an oncogenic role in the cytoplasm associated with the PI3-kinase/Akt-activation pathway. Experimental evidence has demonstrated that NBS1 is translocated into the nucleus by importin KPNA2, which mediates NBS1 subcellular localization and its functions in tumorigenesis, as part of the MRE11–RAD50–NBS1 complex. However, we did not find changes in NBS1 expression or location in KPNA2-knockdown and -overexpressing EOC cells. We speculate that KPNA2 may contribute to other mechanisms in Akt activation, which require further investigation.

In summary, we have clearly shown that KPNA2 is overexpressed in EOC, and that its upregulation is associated with poor prognosis. Furthermore, knockdown of KPNA2 inhibited the proliferation and tumorigenicity of EOC cells by inhibiting c-Myc transactivation activity and by enhancing FOXO3a activity, revealing a new mode of action in the
molecular mechanisms underlying tumorigenesis of EOC. Our characterization of the oncogenic functions and mechanisms of KPNA2 will not only increase our understanding of EOC development and progression but also presents a novel therapeutic target for EOC treatment.

**Materials and Methods**

**Cell culture.** Ovarian cancer cell lines (OVSAHO, OV56, OV90, COV644, CaO3, COVAR4, SKOV3, and EFO21) were grown in DMEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin–streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂.

**Plasmids.** A KPNA2 expression construct was generated by subcloning the PCR-amplified full-length human KPNA2 cDNA into the pMSCV plasmid. For depletion of KPNA2, two human shRNAs sequences were cloned into the pSuper-retro-puro plasmid to generate pSuper-retro-KPNA2-RNAi(s) (Langri, GangZhou, China) with the following sequences: RNAi#1, 5'-ATTACAGTGCCCGCTGGTTG-3' and RNAi#2, 5'-TTAACGAAAGCTTATACAC-3'. Stable cell lines expressing KPNA2 or KPNA2 shRNAs were selected for 10 days with 0.5 mg/ml puromycin. A c-Myc expression construct was generated by subcloning the PCR-amplified full-length human c-Myc cDNA into the pMSCV plasmid. Transfection of plasmids was carried out using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer’s instructions.

**Patients and tissue specimens.** Snap-frozen tissues were obtained from 191 EOC patients between January 2001 and October 2007, and preserved at −80 °C. All patients had undergone oophorosalpingectomy, or surgical debulking, before chemotherapy at the Cancer Center, Sun Yat-Sen University, Guangzhou, PR China. EOC tissues were dissected from the resected tumors; HOS specimens were obtained from the ovarian surface epithelium of the normal-appearing ovary removed from patients, where only one ovary had been classed as EOC (stage IA), based on the principles of surgical management of cancer. This was confirmed by histopathological review. EOC patients were staged according to the FIGO 1994 classification guidelines. An experienced pathologist performed the grading and histopathological subtyping of specimens, based on World Health Organization (WHO) criteria. Clinical information of the samples is summarized in Table 1. The Ethical Committee of the Cancer Center, Sun Yat-Sen University, approved this study.

**RNA extraction and real-time qRT-PCR.** Total RNA from cultured cells and fresh frozen EOC tissues was extracted using Trizol reagent (Invitrogen), according to the manufacturer’s instructions. Reverse transcriptase reactions using MMLV reverse transcriptase reagents (Promega Corporation, Madison, WI, USA) were performed following the manufacturer’s protocol. Real-time qRT-PCR was performed using Platinum SYBR Green qPCR SuperMix-UDG reagents (Invitrogen) in an Applied Biosystems PRISM 7900HT, according to the manufacturer’s protocol. The following primers were selected: KPNA2, forward 5'-ATTSCAGGATGTGGCTTACG-3' and reverse 5'-CTGCTCAАCAGCТСА-3'; β-actin, forward 5'-TG6CACCACACAACTGA-3' and reverse 5'-CATCAGGGTCTCACTAGCTG-3'.
5'-CTAAGTCTATGTCGGCTAGAGCA-3'
and reverse 5'-AGGGTGTAGCCCGGTTAGC-3'; Kif7, forward 5'-CATCAAGGAAAACGCTCAAC-3' and reverse 5'-GTTGACTTGGCTGATGAC-3'; p21, forward 5'-GATGCTGCTTACTGCTTCT-3' and reverse 5'-GCGCGAAGAGTCTTCTCG-3'; and p27, forward 5'-GCGCGAAGAGTCTTCTCG-3'.

Western blotting. The western blotting analysis was performed according to standard methods, as previously described, using anti-KPNA2 (Abcam, Cambridge, UK), anti-total Akt, anti-total FOXO3a, and anti-FOXO3a (Ser253), anti-ki67, anti-p21Cip1, anti-p27Kip1, anti-cyclinD1, and anti-c-Myc antibodies (Cell Signaling Technology, Danvers, MA, USA). The polyvinylidene fluoride membranes were stripped and re-blotted with an anti-β-actin monoclonal antibody (Sigma, St. Louis, MO, USA) as a loading control.

Subcellular fractionation. KPNA2-knockdown and -overexpressing SK-OV3 cells transfected with pMSCV-c-Myc plasmid were subjected to subcellular fractionation using the cytoplasmic and nuclear protein extraction kit P0028 (Beyotime Biotechnology, Shang Hai, China), according to the manufacturer's instructions. The efficacy of fractionation was determined via western blotting using anti-GAPDH antibodies (Sigma) as the cytosolic control and anti-Lamin B antibodies (Cell Signaling Technology) as the nuclear control protein. The experiment was performed in triplicate.

MTT assays. Cells (2000 cells/well) were seeded in 96-well plates and stained at specified time points with 100 ml sterile MTT dye (0.5 mg/ml, Sigma) for 4 h at 37 °C. The culture medium was removed and 150 ml of dimethyl sulfoxide (Sigma) was added. The absorbance was measured at 570 nm, with 655 nm as the reference wavelength. All experiments were performed in triplicate.

Anchorage-independent growth ability assay. Cells (500 cells/plate) were trypsinized and suspended in 2ml complete medium plus 0.3% agar (Sigma). The agar–cell mixture was plated on top of a bottom layer with 1% complete medium agar mixture. After 10 days, viable colonies that contained more than 50 cells or were larger than 0.1 mm in diameter were counted. Colony sizes were measured using an ocular micrometer. The experiment was performed in triplicate for each cell line.

Colony formation assays. Cells were plated on 6-well (500 cells/plate) plates and cultured for 10 days. The colonies were fixed with 10% formaldehyde for 5 min and then stained with 1.0% crystal violet for 30 s. Colonies larger than 50 cells or were larger than 0.1 mm in diameter were counted. Colony sizes were measured using calipers, and tumor volumes were calculated (V = 0.5 × L × W²). The mice were killed and the tumors were excised, fixed in 10% formalin, and embedded in paraffin blocks for IHC study.

Statistical analysis. The x2 test was used for correlation analysis between clinicopathological features of patients with EOC and KPNA2 expression profiles. Survival curves were plotted by the Kaplan–Meier method and compared using the log-rank test. Survival data were evaluated by univariate and multivariate Cox regression analyses. Student’s t-test (two-tailed) was used to evaluate significant differences between pairs of experimental data where appropriate. SPSS version 16.0 statistical software package (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses. Statistical significance was set at P < 0.05.

Conflict of Interest

The authors declare no conflict of interest.

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KPNA2 promotes proliferation and tumorigenicity of EOC
L Huang et al

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