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RBBP6 aggravates the progression of ovarian cancer by targeting PIK3R6

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Abstract. – OBJECTIVE: RBBP6 is identified to be a cancer-associated gene by bioinformatics analysis. This study aims to explore the role of RBBP6 in regulating proliferation and metastasis in ovarian cancer, thus providing theoretical references for ovarian cancer treatment.

PATIENTS AND METHODS: Differential expressions of RBBP6 in ovarian cancer and normal ones were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The relationship between RBBP6 and prognosis in ovarian cancer patients was analyzed. The interaction between RBBP6 and PIK3R6 was detected by bioinformatics analysis and Dual-Luciferase reporter assay. Moreover, regulatory effects of RBBP6 and PIK3R6 on proliferative and migratory potentials in A2780 and CAOV3 cells were examined by Cell Counting Kit-8 (CCK-8) and transwell assay, respectively. Finally, tumorigenicity assay was conducted in nude mice to illustrate the in vivo regulations of PBBP6 and PIK3R6 on ovarian cancer growth.

RESULTS: RBBP6 was upregulated in ovarian cancer tissues than normal ones. RBBP6 was irrelevant to age, tumor size and tumor node metastasis (TNM) staging in ovarian cancer patients, but correlated to lymphatic metastasis and distant metastasis. RBBP6 was abundantly expressed in ovarian cancer cells, and among the tested cell lines, CAOV3 and A2780 expressed the highest level of RBBP6. Knockdown of RBBP6 attenuated *in vitro* proliferative and migratory potentials in CAOV3 and A2780 cells. PIK3R6 was the target gene binding RBBP6, which was positively regulated by RBBP6. Overexpression of PIK3R6 could abolish the inhibited proliferative and migratory potentials in ovarian cancer cells with RBBP6 knockdown. In addition, the knockdown of RBBP6 slowed the *in vivo* growth of ovarian cancer in nude mice, and the alleviated cancer progression was reversed by overexpression of PIK3R6.

CONCLUSIONS: RBBP6 is highly expressed in ovarian cancer cases, which stimulates proliferative and migratory potentials by targeting PIK3R6. RBBP6 may be a novel therapeutic target for ovarian cancer.

Key Words:
RBBP6, PIK3R6, Ovarian cancer, Cancer progression.

Introduction

Although the incidence of ovarian cancer is not high, its 5-year survival is far away from satisfying[1,2]. According to the estimates in 2018, there were 14,070 deaths from ovarian cancer in the United States, accounting for 5% of all cancer deaths. Its mortality ranks fifth, following lung cancer, breast cancer, colorectal cancer, and pancreatic cancer[3,4]. Most of the patients diagnosed with advanced ovarian cancer at the first time of diagnosis and they are relatively old. Therefore, surgery is not optimal for these patients[5,6]. In addition, malignant ovarian tumors are particularly prone to develop metastases in the abdominal cavity and ascites, leading to the rapid aggravation[7,8]. It is particularly important to clarify the mechanism underlying ovarian cancer metastasis, thus developing effective therapeutic strategies[9,10].

RBBP6 is also known as P2P-R (proliferation potential protein-related) or PACT (p53 associated cellular protein-testes derived)[11,12]. It was first screened out from RBQ-1 (Rb binding Q-protein 1) in 1995 by Sakai et al[13] that RBBP6 is located on chromosome 16p11.2-p12, encoding 948 amino acids, which are extensively expressed in adult and embryo tissues. Later, experiments and bio-

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informatics analysis showed that RBQ-1 is actually a truncated form of the C-terminal segment of PACT, which is then named as RBBP6[13]. It is reported that RBBP6 is abnormally expressed in many types of tumors[11,14,15]. This study aims to explore the biological functions of RBBP6 in ovarian cancer progression by generating RBBP6 knockdown models in A2780 and CAOV3 cells, and xenograft model in nude mice.

Patients and Methods

Patients and Ovarian Cancer Samples

Fifty-eight pairs of ovarian cancer and normal tissues were surgically resected and stored at -80°C for RNA extraction. All ovarian cancer specimens were pathologically confirmed by two experienced pathologists independently. Inclusion criteria: patients with no severe diseases in other organs, those undergoing no post-operative chemotherapy and radiotherapy before operation. Tumor node metastasis (TNM) staging of ovarian cancer was determined based on the criteria proposed by The Union for International Cancer Control (UICC). This study complied with the Helsinki Declaration Clinical Practice Guidelines, and was approved by the Ethics Committee of Heze Center for Disease Control and Prevention. Signed written informed consents were obtained from all participants before the study.

Cell Lines and Reagents

Ovarian cancer cell lines (SKOV3, OVCAR3, PEO1, A2780, 3AO, CAOV3) and ovarian epithelial cell line (HOSEPiCs) purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). All cells were adherently grown.

Transfection

Cells were implanted in 6-well plates and cultured to 70-80% density. They were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and harvested at 48 h. Transfection plasmids were constructed by GenePharma (Shanghai, China).

Cell Proliferation Assay

2.5×10^3 cells were implanted in each well of a 6-well plate, where 10 μL of Cell Counting Kit-8 (CCK-8) solution was added (TaKaRa, Dalian, China). After 1-h culturing in the dark, 450 nm absorbance was measured using a microplate reader. Blank group was set by adding medium and experimental solution without cells.

Transwell Migration Assay

Transwell chambers (Millipore, Billerica, MA, USA) were inserted in each well of a 24-well plate. 2×10^4 cells suspended in 200 μL of serum-free medium was applied in the upper layer of the chamber with 500 μL of medium in the bottom. After 48-h incubation, migratory cells in the bottom were reacted with 15-min methanol, 20-min crystal violet and captured using a microscope. Migratory cells were counted in 10 randomly selected fields per sample.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used for isolating total cellular RNAs, which were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs; PrimeScript RT Reagent; TaKaRa, Dalian, China). Using the SYBR® Premix Ex Taq™ kit (TaKaRa, Dalian, China), StepOne Plus Real-time PCR system (Applied Biosystems, Foster City, CA, USA), and qRT-PCR was carried out. The relative level was calculated by $2^{-\Delta\Delta Ct}$ and normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or U6. RBBP6: forward: 5'-GGCGCTCATTTTCCAGGTCTA-3', reverse: 5'-GAGCGTGAACGTGTTGAACC-3'; GAPDH: forward: 5'-GGATTTGGTCGTATTGGG-3', reverse: 5'-GGAAGATGGTGATGGGATT-3'.

Western Blot

Cells were lysed in radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) and the concentration of isolated protein was measured by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Protein samples were separated by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), and transferred on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Afterwards, the membranes were soaked in 5% skim milk for 2 hours. Primary antibodies were applied for overnight incubation at 4°C. On the next day, horse radish peroxidase (HRP)-labeled secondary antibodies were used for 2 h incubation. Band exposure was achieved by enhanced chemilu-

Table I. Association of RBBP6 expression with clinicopathologic characteristics of ovarian cancer.

Parameters	Number of cases	RBBP6 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.710
<60	23	12	11	
≥60	35	20	15	
Tumor size				0.291
<4 cm	29	18	11	
≥4 cm	29	14	15	
TNM stage				0.213
I + II	32	20	12	
III + IV	26	12	14	
Lymph node metastasis				0.023
No	34	23	11	
Yes	24	9	15	
Distance metastasis				0.046
No	35	23	12	
Yes	23	9	14	

minescence (ECL) with GAPDH as the internal reference.

Dual-Luciferase Reporter Assay

Potential binding sites in the 3'-UTR of RBBP6 and PIK3R6 were predicted using online bioinformatics tool. Wild-type and mutant-type RBBP6 vectors were generated, which were co-transfected in cells with pcDNA-PIK3R6 or NC, respectively. Luciferase activity (Promega, Madison, WI, USA) was finally measured at 48 h.

Tumorigenicity Assay

Fifteen male nude mice with 4 weeks old were randomly implanted with A2780 cells transfected with sh-NC+NC, sh-RBBP6+NC or sh-RBBP6+pcDNA-PIK3R6, respectively by subcutaneous administration. The width and length of the xenografted tumor were weekly recorded. Six weeks later, mice were sacrificed for harvesting the tumor tissues and weighed. Tumor volume=(width^2×length)/2. The animal studies were performed in accordance with the Institutional Ethics Guidelines for Animal Experiments, which was approved by the Animal Management Committee of Heze Center for Disease Control and Prevention.

Statistical Analysis

Data were expressed as mean ± standard deviation and processed by Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA). Measurement data were compared using the Student's *t*-test, and categorical variables were analyzed by χ^2 test or Fisher's exact test. Chi-square analysis was conducted for analyzing the influences of RBBP6 on clinical indicators in ovarian cancer patients. Kaplan-Meier method was used for survival analysis, followed by log-rank test for comparing differences between curves. A significant difference was set at $p < 0.05$.

Results

Upregulation of RBBP6 in Ovarian Cancer Cases

Differential expressions of RBBP6 between ovarian cancer and normal tissues were detected by qRT-PCR. RBBP6 was upregulated in cancer tissues (Figure 1A). Identically, it was highly expressed in ovarian cancer cell lines (Figure 1B).

RBBP6 is Linked to Metastasis and Prognosis in Ovarian Cancer

According to the median level of RBBP6 in 56 cases of ovarian cancer tissues, recruited patients were classified into high and low RBBP6 expression groups, respectively. The number in each group was recorded. As data analyzed, RBBP6 level was positively correlated to incidences of lymphatic metastasis and distant metastasis in ovarian cancer patients (Table I). In addition, Kaplan-Meier curves revealed that highly expressed

RBBP6 was related to poor prognosis in ovarian cancer (Figure 1C).

Silence of RBBP6 Weakens Proliferative and Migratory Abilities in Ovarian Cancer

We tested transfection efficacy of sh-RBBP6 in A2780 and CAOV3 cells by Western blot and qRT-PCR, respectively (Figure 1D, 1E). After 48 h cell transfection, the viability in ovarian cancer cells at 24, 48, 72 and 92 h was determined by CCK-8 assay. It is shown that the knockdown of RBBP6 markedly decreased viability in ovarian cancer cells at 72 and 96 h (Figure 2A). Transwell assay uncovered the decreased migratory cell number after knockdown of RBBP6 in A2780 and CAOV3 cells (Figure 2B).

Binding Between RBBP6 and PIK3R6

Potential binding sites in the 3'-UTR of RBBP6 and PIK3R6 were predicted using online bioinformatics tool. Subsequently, Dual-Luciferase reporter assay showed that the overexpression of PIK3R6 could decrease Luciferase activity in the wild-type RBBP6 vector (Figure 3A). However, Luciferase activity in the mutant-type one was not regulated by PIK3R6, confirming the binding between RBBP6 and PIK3R6. Protein level of PIK3R6 was markedly downregulated in A2780 and CAOV3 cells transfected with sh-RBBP6 (Figure 3B). It is suggested that PIK3R6 was positively regulated by RBBP6.

Overexpression of PIK3R6 Reversed Regulatory Effects of RBBP6 on Ovarian Cancer Cell Functions

The above results have proven the interaction between RBBP6 and PIK3R6. We thereafter explored the involvement of PIK3R6 in RBBP6-regulated ovarian cancer progression. Co-transfection of pcDNA-PIK3R6 markedly upregulated protein level of PIK3R6 in A2780 and CAOV3 cells with RBBP6 knockdown (Figure 4A). Compared with ovarian cancer cells co-transfected with sh-RBBP6 and NC, viability and migratory cell number were higher in those co-transfected with sh-RBBP6 and pcDNA-PIK3R6 (Figure 4B, 4C). It is indicated that RBBP6 aggravated ovarian cancer progression by regulating PIK3R6.

RBBP6 Induced Tumorigenicity Of Ovarian Cancer In Vivo by Regulating PIK3R6

The above data have shown the regulatory effects of RBBP6 and PIK3R6 on ovarian cancer cell functions *in vitro*. Tumorigenicity assay was conducted in nude mice implanted with A2780

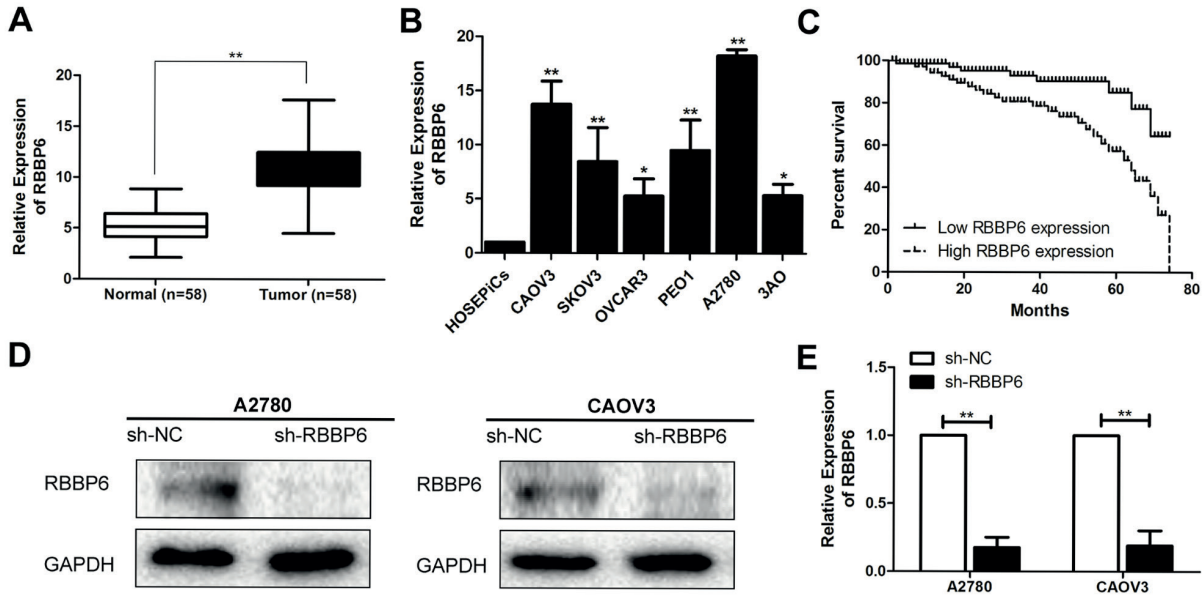

Figure 1. Upregulation of RBBP6 in ovarian cancer cases. **A,** RBBP6 levels in HCC tissues and normal ones. **B,** RBBP6 levels in ovarian cancer cells. **C,** Kaplan-Meier curves depicted based on RBBP6 levels in ovarian cancer patients. **D, E,** Transfection efficacy of sh-RBBP6 in A2780 and CAOV3 cells detected by Western blot (**D**) and qRT-PCR (**E**). Data were expressed as mean±SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

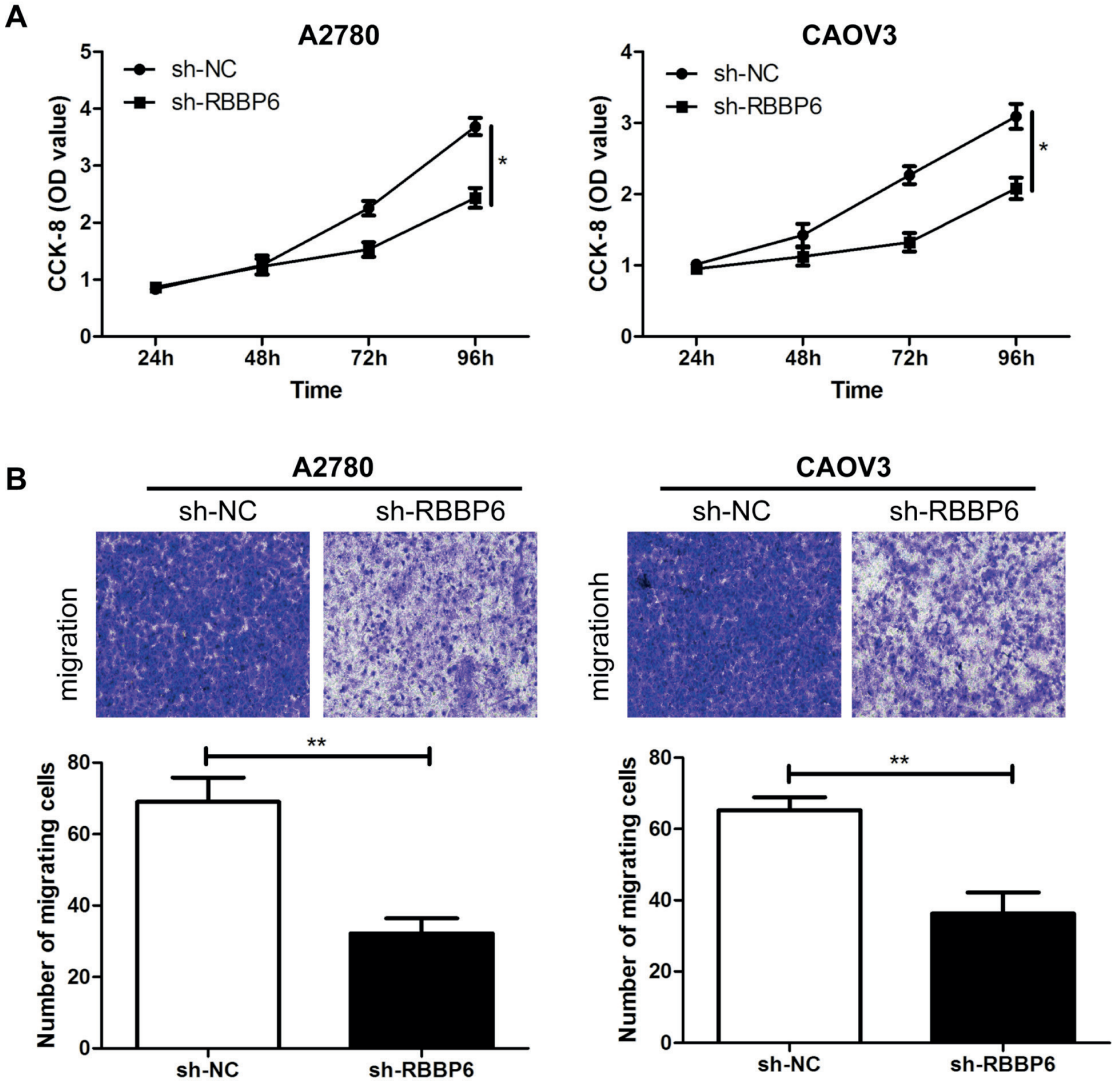

Figure 2. Silence of RBBP6 weakens proliferative and migratory abilities in ovarian cancer. **A,** Viability in A2780 and CAOV3 cells transfected with sh-NC or sh-RBBP6 at 24, 48, 72 and 96 h. **B,** Migration in A2780 and CAOV3 cells transfected with sh-NC or sh-RBBP6 (magnification 40×). Data were expressed as mean±SD. *$p < 0.05$, **$p < 0.01$.

cells transfected with sh-NC+NC, sh-RBBP6+NC or sh-RBBP6+pcDNA-PIK3R6, respectively, to explore the *in vivo* regulations. Compared the nude mice with *in vivo* knockdown of RBBP6, the tumor volume and tumor weight were lower than controls. In addition, xenografted tumors collected from those implanted with A2780 cells transfected with sh-RBBP6+pcDNA-PIK3R6 presented higher tumor volume and tumor weight compared with those with *in vivo* knockdown of RBBP6 (Figure 5A, 5B). Positively expression of RBBP6 was lower in xenografted tumors collected from sh-RBBP6+NC group than sh-NC+NC group and sh-RBBP6+pcDNA-PIK3R6, indicating the successful modeling of xenograft model (Figure 5C). Collectively, the knockdown of RBBP6 slowed down tumor growth of ovarian cancer *in vivo*.

Discussion

Recurrence and metastasis are the major reasons for treatment failure and high mortality of ovarian cancer[7,8]. Recently, targeted therapy has become the hot topic in cancer research. In 2011,

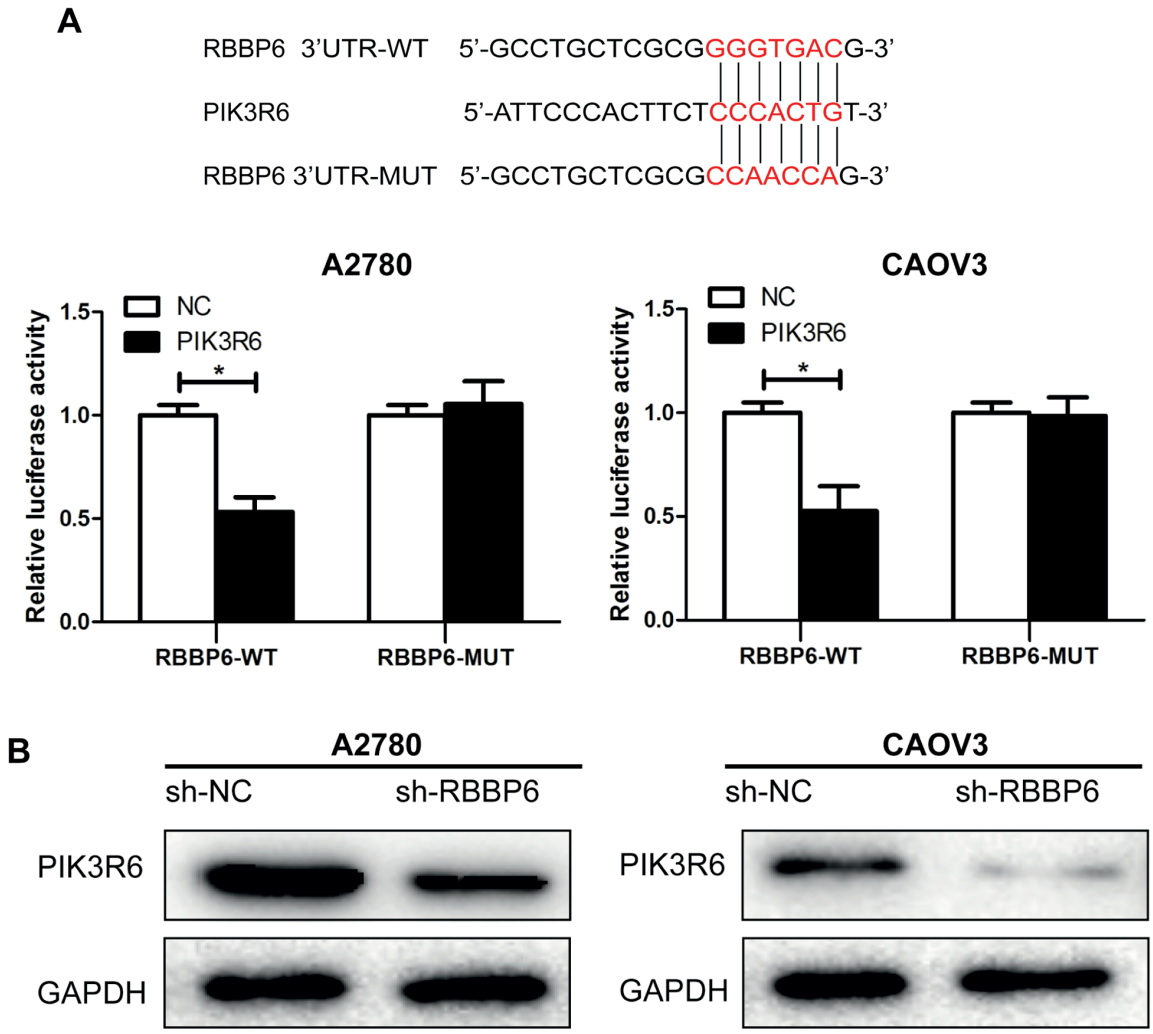

Figure 3. Binding between RBBP6 and PIK3R6. **A,** Luciferase activity in A2780 and CAOV3 cells co-transfected with NC/pcDNA-PIK3R6 and RBBP6-WT/RBBP6-MUT. **B,** Protein level of PIK3R6 in A2780 and CAOV3 cells transfected with sh-NC or sh-RBBP6. Data were expressed as mean±SD. *$p < 0.05$.

Bevacizumab, the first antiangiogenic drug has been applied for the treatment of ovarian cancer in the world. PARPi (PARP inhibitor) is approved by FDA for the treatment of epithelial ovarian cancer with BRCA1 or BRCA2 mutations[16,17]. Currently, there are many undergoing clinical trials for assessing adjuvant and relapse treatment for epithelial ovarian cancer. Most studies focus on analyzing the regulatory role of PI3K/AKT/mTOR signaling in cancer progression, which controls angiogenesis, proliferation, protein synthesis and cell survival[18]. Combined administrations of PARPi, anti-angiogenic drugs, MAPK pathway inhibitors, PI3K inhibitors, and conventional chemotherapy for cancer treatment are currently explored in the early stage[19,20]. It is urgent to analyze molecular mechanisms of ovarian cancer, thus improving clinical outcomes of affected patients[9,10].

Through bioinformatics analysis and literature review, RBBP6 is related to the progression of many types of tumors[11,14,15]. However, its biological functions in ovarian cancer are unclear. Therefore, the objective of this study was firstly to elucidate the oncogenic role of RBBP6 in in the progression of ovarian cancer, as well as its specific mechanism. Our findings showed that RBBP6 was upregulated in ovarian cancer tissues, and its level was related to metastasis and prognosis in ovarian cancer patients. We thereafter believed that RBBP6 may be an oncogene involved in ovarian cancer progression. Subse-

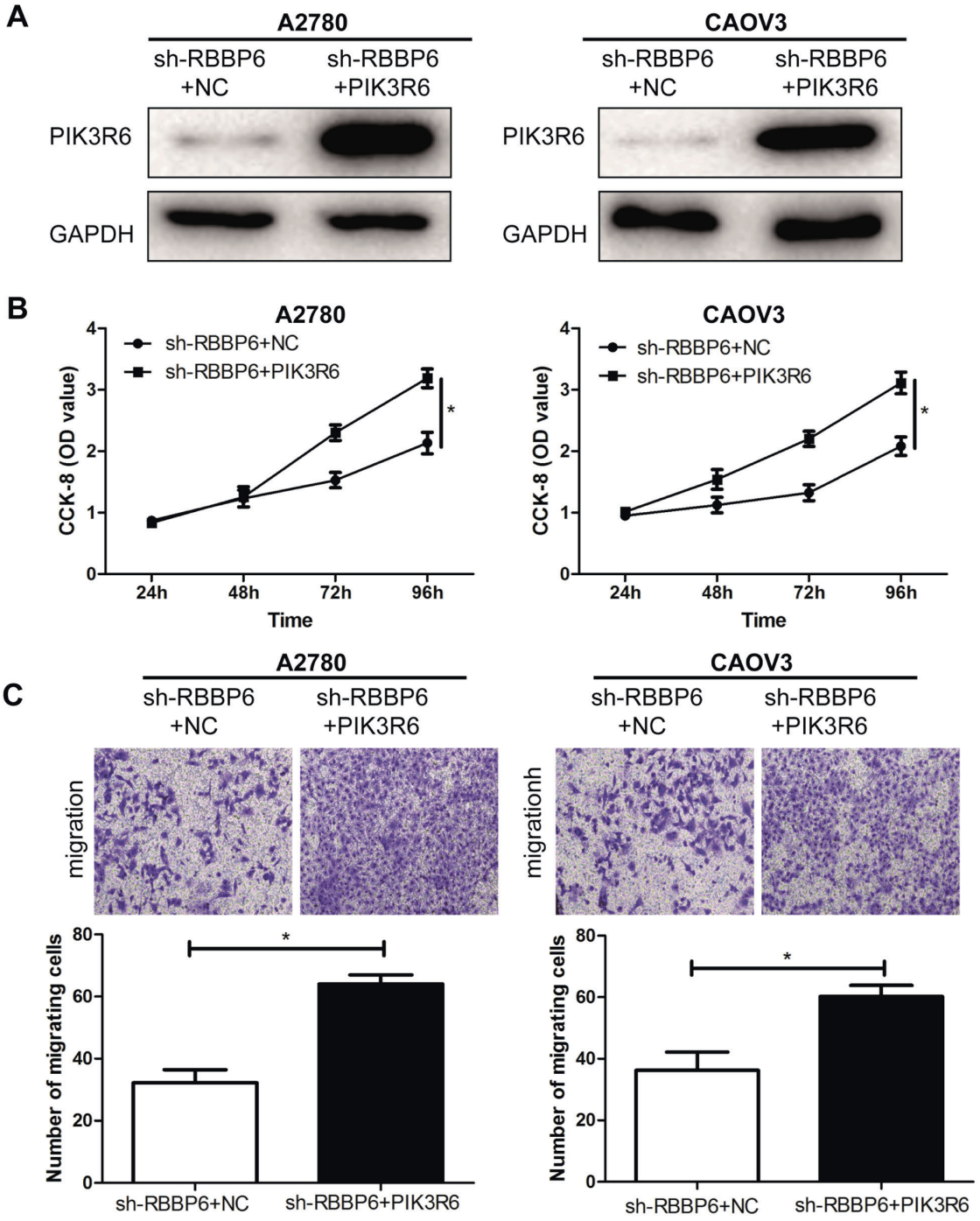

Figure 4. Overexpression of PIK3R6 reversed regulatory effects of RBBP6 on ovarian cancer cell functions. **A,** Protein level of PIK3R6 in A2780 and CAOV3 cells transfected with sh-RBBP6+NC or sh-RBBP6+pcDNA-PIK3R6. **B,** Viability in A2780 and CAOV3 cells transfected with sh-RBBP6+NC or sh-RBBP6+pcDNA-PIK3R6 at 24, 48, 72 and 96 h. **C,** Migration in A2780 and CAOV3 cells transfected with sh-RBBP6+NC or sh-RBBP6+pcDNA-PIK3R6 (magnification 40×). Data were expressed as mean±SD. *$p < 0.05$.

quently, regulatory effects of RBBP6 on *in vitro* cell functions of ovarian cancer were examined. It is revealed that RBBP6 was able to stimulate proliferative and migratory potentials in A2780 and CAOV3 cells. In addition, tumorigenicity assay was conducted in nude mice with xenograft-

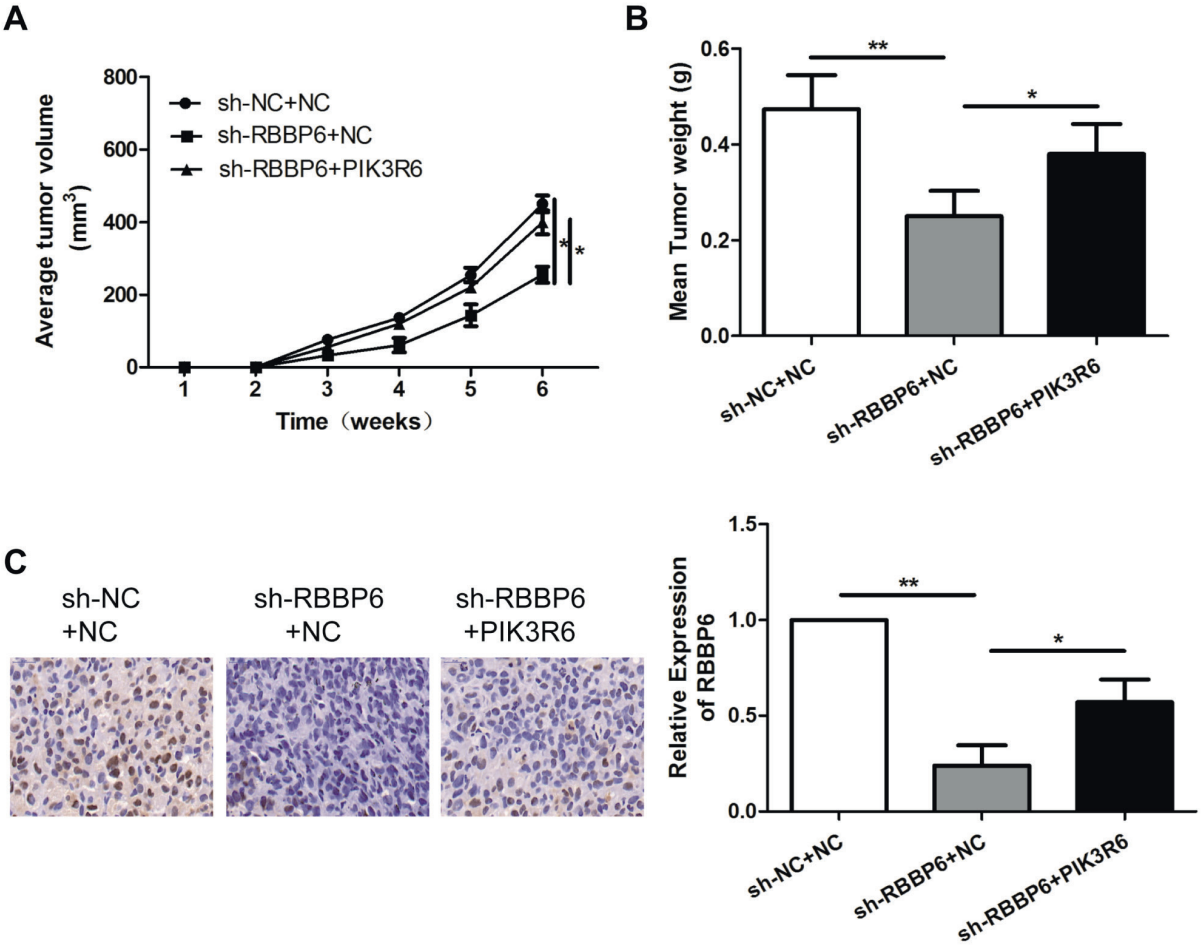

Figure 5. RBBP6 induced tumorigenicity of ovarian cancer *in vivo* by regulating PIK3R6. **A, B,** Average tumor volume (**A**) and tumor weight (**B**) in ovarian cancer tissues collected from nude mice implanted with A2780 cells transfected with sh-NC+NC, sh-RBBP6+NC or sh-RBBP6+pcDNA-PIK3R6. **C,** Positive expression of RBBP6 in ovarian cancer tissues collected from nude mice implanted with A2780 cells transfected with sh-NC+NC, sh-RBBP6+NC or sh-RBBP6+pcDNA-PIK3R6 (magnification 40×). Data were expressed as mean±SD. *$p < 0.05$, **$p < 0.01$.

ed ovarian cancer, and the knockdown of RBBP6 remarkably slowed down ovarian cancer growth *in vivo*. Therefore, as a novel oncogenic gene, RBBP6 could aggravating the malignant development of ovarian cancer.

PI3K is a family of lipid kinase proteins that integrate signals from growth factors, cytokines, and other environmental factors, and then, convert them into intracellular signals[21,22]. PI3K-induced activation of pathways is responsible for regulating cell behaviors[23,24]. Overactivation of the PI3K signaling is a common change usually detected in human tumors, which is linked to tumor cell functions and gene instability[25]. Besides, abnormally activated PI3K induces the formation of tumor microenvironment, tumor angiogenesis or recruits inflammatory factors[22,23]. In this paper, bioinformatics analysis and Dual-Luciferase reporter assay showed that the overexpression of PIK3R6 could decrease Luciferase activity in the wild-type RBBP6 vector. However, Luciferase activity in the mutant-type one was not regulated by PIK3R6, confirming the binding between RBBP6 and PIK3R6. In A2780 and CAOV3 cells transfected with sh-RBBP6, the protein level of PIK3R6 was markedly downregulated, displaying a positive regulation. By comparing the ovarian cancer cells with RBBP6 knockdown, those co-transfected with sh-RBBP6 and pcDNA-PIK3R6 displayed higher viability and migratory cell number, indicating that PIK3R6 was responsible for RBBP6-regulated proliferation and migration in ovarian cancer. As expected, RBBP6 induced tumorigenicity of ovarian cancer

in nude mice by regulating PIK3R6. Our findings proposed that the RBBP6/PIK3R6 signaling was responsible for triggering both *in vitro* and *in vivo* progression of ovarian cancer. Collectively, RBBP6/PIK3R6 axis were responsible for the malignant development of ovarian cancer, thus being a promising biomarker for diagnosis, treatment and prognosis assessment for ovarian cancer.

Conclusions

All together, these results showed that RBBP6 is highly expressed in ovarian cancer cases, which stimulates proliferative and migratory potentials by targeting PIK3R6. RBBP6 may be a novel therapeutic target for ovarian cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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