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ORIGINAL RESEARCH

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CKS1B promotes the growth of ovarian cancer cells by regulating the expression of PD-L1

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Abstract

Ovarian cancer has high rates of morbidity and mortality and a poor prognosis. A growing body of research suggests that CDC28 protein kinase regulatory subunit 1B (CKS1B) influences the progression of numerous carcinomas. However, the specific mechanism of CKS1B in ovarian cancer has yet to be elucidated. Here, this study aimed to investigate the involvement of CKS1B in the progression of ovarian cancer. Initially, we collected data from the Cancer Genome Atlas (TCGA) website and ovarian cancer tissues showed elevated CKS1B expression compared to healthy tissues, indicating a poorer prognosis. Immunohistochemistry (IHC) was used to detect CKS1B and programmed cell death 1-ligand 1 (PD-L1) levels and demonstrated that CKS1B is positively related to PD-L1 expression. Then, short-interfering (si)-CKS1B or si-negative control (si-NC) constructs were transfected into SKOV3 and OVCAR3 cells. Subsequently, cell viability was measured by cell counting kit-8 (CCK8) and colony formation assays, and the extent of apoptosis was assessed using flow cytometry. We found that CKS1B silencing inhibited cell growth and promoted cell apoptosis in ovarian cancer. In addition, the expression levels of CKS1B, B-cell lymphoma-2 (BCL2)-associated X (Bax), cleaved-caspase3 and PD-L1 were determined by western blotting. Furthermore, to investigate whether the inhibitory effects of si-CKS1B on ovarian cancer is related to the levels of PD-L1, we added PD-L1 to si-CKS1B transfected cells and found that cells that had been supplemented with PD-L1 exhibited an increased cell growth rate and a reduced apoptosis rate than si-CKS1B cells. Collectively, our data we demonstrated high levels of CKS1B in ovarian cancer cells, and found that the knockdown of *CKS1B* could alleviate the development of ovarian tumors by dysregulating the level of PD-L1 expression.

Keywords

CKS1B; PD-L1; Ovarian cancer; Proliferation

1. Introduction

Ovarian cancer is the third most common malignancy in the female reproductive system [1–3]. Ovarian cancer is the fifth leading cause of death among gynecological cancers, as reported by the American Cancer Society [4]. Despite recent advances in treatments like surgery, chemotherapy and radiation therapy, the 5-year survival rate for ovarian cancer patients has remained stable at around 35% [4, 5]. Unfortunately, the initial signs of ovarian cancer are inconspicuous, and the scarcity of exceptionally sensitive and precise screening techniques is attributed to the distinct size and positioning of ovarian tissue [6, 7]. Consequently, most patients receive a cancer diagnosis at an advanced stage, often with abdominal metastases [7, 8].

Recent studies have revealed that immunosuppression creates an opportunity for cancer progression, and certain immunosuppressive factors can play a crucial role in this process [9]. Previous research discovered that PD-L1 played a role in controlling the advancement of various tumors. For instance, in melanoma, the level of PD-L1 within the tumor was established as a prognostic marker. Conversely, in hepatocellular carcinoma, treatment with a PD-L1 antibody reduces the escape of liver tumor cells. Moreover, PD-L1 serves as a prognostic indicator for poor outcomes in soft-tissue sarcomas, while the high expression levels of PD-L1 in inflammatory breast cancers is suggestive of a weaker response to chemotherapy. In comparison to adjacent normal tissues, the levels of PD-L1 is elevated in ovarian tumor tissues; furthermore, PD-L1 is recognized for promoting ovarian cancer cells progression through activation the protein kinase B (AKT)-mammalian target of rapamycin complex 1 (mTORC) signaling [10]. Additionally, PD-L1 is known to induce angiogenesis, further facilitating the *vitro* and *in vivo* migration and invasion of ovarian cancer cells [11].

CKS family member CDC28 protein kinase regulatory subunit 1B (CKS1B), which binds to the catalytic subunit of

cyclin-dependent kinase (CDK), is essential for controlling key processes in the cell cycle. The emergence and dissemination of multiple malignant tumors, including pancreatic cancer, papillary thyroid carcinoma, colorectal cancer and gastric cancer, have been associated with the overexpression of CKS1B. The inhibition of CKS1B has been shown to attenuate cell proliferation and migration in colorectal carcinoma (31717435) while suppressing CKS1B has been demonstrated to inhibit gastric cancer cell proliferation (29283424). Reducing CKS1B in pancreatic cancer cells has been demonstrated to markedly lower cell viability and invasion through the modulation of PD-L1 expression [12]. Moreover, in papillary thyroid carcinoma cells, CKS1B has been found to promote proliferation and invasion by activating the signal transducer and activator of transcription 3 (STAT3)/PD-L1 and phosphorylated protein kinase B (Akt) signaling pathways [13]. Although CKS1B has been researched in various cancer types, its function in ovarian cancer remains unexplored.

2. Methods

2.1 Cell culture

The ovarian cancer lines SKOV3 (HTB-77) and OVCAR3 (HTB-161) were acquired from the American Type Culture Collection (ATCC) and culture in DMEM (Gibco, 12800017, New York, NY, USA) with 10% fetal bovine serum (Gibco, 10099-141, New York, NY, USA).

2.2 Dysregulation of the *CKS1B* gene by siRNA

The *CKS1B* gene was knocked down with an siRNA transfection kit (SR300836-OR, OriGene, Beijing, China) and the following si-RNA sequences: si-CKS1B #1: 5′-GCGCTGAGAGAGTTGAATATT-3′; si-CKS1B #2: 5′-GCTGAGAGAGTTGAATATTGC-3′; si-NC: 5′-CTGGACTGGTATTTGGACCAG-3′. SKOV3 and OVCAR3 cells (1×10^4/well in 6-well plates) were transfected with the siRNAs by adding 10 μL of Lipofectamine RNAiMAX complex (Invitrogen, Carlsbad, CA, USA) with siRNA (35 pmol) to the culture mix for 6 h; subsequently, this was replaced with normal culture medium for a further 24 h. A well-contained Lipofectamine RNAiMAX and siRNA and the ratio were 1.5 μL:5 pmol per well.

2.3 PD-L1 overexpression

SKOV3 and OVCAR3 cells (1×10^4/well, 6 well plate) with PD-L1 RNA (40 pmol) and Lipofectamine RNAiMAX (10 μL) complex (Invitrogen, Carlsbad, CA, USA) for 6 h. After that, the transfected cells were identified by Polymerase Chain Reaction (PCR) and western blotting and cultured further for *in vivo* experiments.

2.4 TCGA data analysis

We obtained CKS1B mRNA expression data from both cancer and healthy tissues using The Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov/). Following data download, we performed data analysis with the R programming language.

2.5 Western blotting

Western blotting was used to determine CKS1B, Bax, cleaved caspase 3, PD-L1 and β-actin in SKOV3 and OVCAR3 cells. Following extraction with RIPA buffer (P0013K, Beyotime, Shanghai, China), cellular debris was removed by centrifugation (Thermo Fisher, 75004061) at 12,000 rpm for 10 minutes at 4 °C. Total protein levels in each sample were assessed using a Bicinchoninic Acid (BCA) kit (P0009, Beyotime, Shanghai, China). Prior to transfer onto polyvinylidene fluoride (PVDF) membranes, denatured proteins (10 μg each) were separated by electrophoresis based on their molecular weight using LMAI Bio's LM1136 system. After blocking with 5% non-fat dried milk for one hour at room temperature, membranes were incubated overnight at 4 °C with the relevant primary antibodies. The next day, secondary antibodies were applied for one hour at 37 °C. Specific protein bands were subsequently detected using the Emitter-coupled logic (ECL) method (GE Healthcare, Piscataway, NJ, USA). The following primary antibodies were acquired from Abcam: CKS1B (#ab72639, 1:1000), Bax (#ab32503, 1:1000), cleaved caspase 3 (#ab32042, 1:1000), PD-L1 (#ab205921, 1:1000), and β-actin (#ab8226, 1:1000). We used anti-mouse (#4410, 1:10,000) and anti-rabbit (#4414, 1:10,000) peroxidase-conjugated secondary antibodies from Cell Signaling Technologies, and the experiment was performed in triplicate.

2.6 Immunohistochemistry (IHC)

Immunohistochemistry was performed using a protocol described previously. Thirty ovarian cancer specimens (cancerous and para-cancerous tissues) were randomly collected. These tissues were paraffin-embedded, cut into sequential sections (4 μm-thick) and mounted. Next, the sections were dewaxed, rehydrated in a series of alcohols, washed in Phosphate-buffered saline (PBS), and an antigen retrieval protocol was applied. Subsequently, sections were exposed to a primary antibody overnight at 4 °C. Next morning, the slices were rinsed in PBS and then exposed to a horseradish peroxidase-labeled secondary antibody. Finally, positive staining was visualized with 3,3'-Diaminobenzidine (DAB) as a chromogen. Two experts independently assessed the IHC staining intensity and distribution, and the mean results were recorded. Staining intensity was rated from 1 to 4 and staining distribution was defined as the proportion (%) of positive cells counted by counting in four randomly chosen high-power microscope fields.

2.7 CCK8 assays

SKOV3 and OVCAR3 cells (3×10^4/well) were transferred to 96-well plates for culture. After 24 hours, each well received 10 μL CCK8 solution for an additional 4 hours incubation. Then, we measured the Optical Density (OD) value for each sample at 450 nm. All experiments were conducted three times.

2.8 Colony formation assays

SKOV3 and OVCAR3 cells (3×10^4/well) were incubated in DMEM containing 10% fetal bovine serum (FBS). Fresh media and medication were added every three days for three weeks until colonization. Then, cells were stained using 1% crystal violet (C0121, Beyotime, Shanghai, China), washed with PBS (Gibco, 11965092, New York, NY, USA), and fixation with 4% paraformaldehyde (P0099, Beyotime, Shanghai, China) at 37 °C for 15–20 minutes to analyze the colonies. Finally, images were acquired using ImageJ software (Version 1.53k, Wayne Rasband, Bethesda, MD, USA). All experiments were performed in triplicate.

2.9 Flow cytometry

An Apoptosis Detection Kit (eBioscience, 88-8005-72, San Diego, CA, USA) was utilized to measure the number of apoptotic cells. Each cell type was transferred onto 6-well plate (3×10^5 cells/plate) and grown for 24 hours. Cells were then suspended in binding buffer and incubated with Annexin V-FITC for 15 minutes. PI was then added to the cells and a FACS-Calibur flow cytometer (2350 Qume Drive, BD Biosciences, San Jose, CA, USA) was used to analyze the cells. All experiments were performed in triplicate.

2.10 Statistical analysis

Statistical analysis was conducted with SPSS version 22.0 (IBM, Armonk, NY, USA). The student's *t*-test and analysis of variance (ANOVA) were used to identify significant differences between groups. The results of each experiment are presented as means and standard deviations (SD), and statistically significant was defined as $p < 0.05$.

3. Results

3.1 High levels of CKS1B expression were detected in ovarian cancer tissues, showing a positive correlation with PD-L1 expression

We collected data from 426 ovarian cancer patients and 88 healthy individuals from the TCGA database and assessed the CKS1B mRNA levels in both healthy normal tissues and ovarian cancer tissues. *CKS1B* mRNA level in ovarian cancer tissues was markedly elevated compared to healthy tissues ($p < 0.05$) (Fig. 1A). Tissues were categorized into low and high expression groups based on the median CKS1B expression level, aiming to explore the association between expression and prognosis. Analysis showed that high *CKS1B* expression in ovarian cancer were substantially associated with a worse overall survival (OS) than low expression levels of *CKS1B* ($p < 0.01$) (Fig. 1B).

In total 30 specimens of ovarian cancer (cancerous and para-cancerous tissues) were assessed by IHC (Fig. 1C). We observed significantly elevated levels of CKS1B and PD-L1 expression in tumor tissues. In contrast, we only detected weak or negative immunostaining for CKS1B and PD-L1 in normal tissues (Fig. 1D). Furthermore, correlation analysis indicated that CKS1B expression was positively correlated with PD-L1 level in ovarian cancer (Fig. 1E).

3.2 The knockdown of CKS1B inhibited the growth of ovarian cancer cells

To understand the importance of *CKS1B* in ovarian cancer, we used siRNAs against *CKS1B* for gene silencing experiments in SKOV3 and OVCAR3 cells. Following treatment, CKS1B protein were evaluated by western blotting, which showed that CKS1B protein was markedly reduced in the si-CKS1B#1 and si-CKS1B#2 groups than that in the control or si-NC groups (Fig. 2A). Furthermore, we investigated the impact of CKS1B on cell viability by performing CCK8 assays (Fig. 2B) and colony formation assays (Fig. 2C). The cell viability and the numbers of proliferating si-CKS1B cells were significantly reduced (Fig. 2B,C). Collectively, these findings showed that CKS1B silencing substantially inhibited the growth of ovarian cancer.

3.3 The knockdown of CKS1B promoted apoptosis in ovarian cancer cells

Next, we used flow cytometry (Fig. 3A) and western blotting (Fig. 3B) to identify the levels of CKS1B in two ovarian cancer cell lines, SKOV3 and OVCAR3. The transfection of si-CKS1B significantly enhanced apoptosis rates in both SKOV3 and OVCAR3 cells (Fig. 3A), and Bax and cleaved-caspase 3 expression were markedly elevated than control (Fig. 3B). These results showed that CKS1B silencing promoting cellular apoptosis.

3.4 The knockdown of CKS1B inhibited the growth of ovarian cancer by regulating PD-L1 expression

To further investigate the mechanism of CKS1B on ovarian cancer, we next detected PD-L1 expression in si-CKS1B-treated SKOV3 and OVCAR3 cells by western blotting. Analysis indicated that si-CKS1B down-regulated PD-L1 (Fig. 4A). Moreover, we observed increased cell viability and a decrease in the quantity of apoptotic cells in the si-CKS1B cells supplemented with PD-L1. In contrast, the si-CKS1B group showed reduced cell growth and an increased rate of apoptosis (Fig. 4B,C). These results showed that si-CKS1B inhibited the growth of ovarian cancer by dysregulating PD-L1.

4. Discussion

Ovarian cancer is becoming increasingly prevalent each year and has a high mortality rate. CKS1B has been previously identified as a crucial target in tumorigenesis, including pancreatic cancer [12], glioma [14] and myeloma [15]. However, the association between CKS1B and ovarian cancer has yet to be elucidated. In this study, we discovered a substantial elevation in CKS1B within ovarian cancer tissues compared to in normal tissues, and that patients with increased CKS1B levels have a poorer prognosis, thus indicating that CKS1B could be used to monitor the development of ovarian cancer. Furthermore, ICH experiments revealed a positive correlation between elevated CKS1B and PD-L1 expression. This study represents the first investigation of CKS1B's potential functional role in ovarian cancer progression.

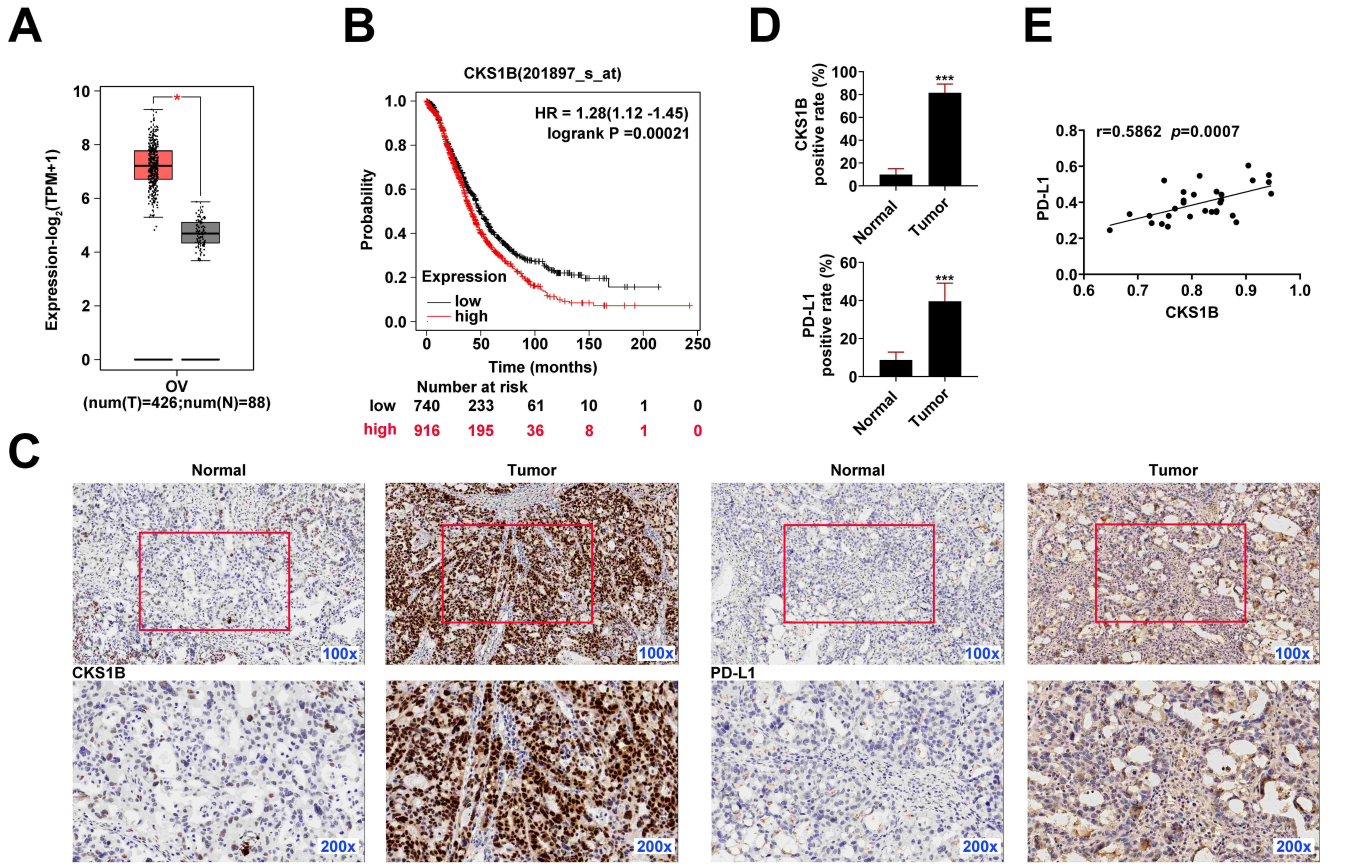

FIGURE 1. High levels of CKS1B expression were detected in ovarian cancer tissues, showing a positive correlation with PD-L1 expression. (A) Up-regulation of *CKS1B* mRNA in ovarian cancer tissues and normal tissues. (B) Survival curves showing the prognostic value of *CKS1B* expression in ovarian cancer. (C) IHC was used to assess ovarian cancer and para-cancerous tissues (100× and 200× magnification). (D) The expression of CKS1B and PD-L1 in ovarian cancer and para-cancerous tissues. (E) Correlation analysis for the relationship between CKS1B and PD-L1. Data are expressed as mean ± SD. *$p < 0.05$, ***$p < 0.001$ *vs.* normal tissues. CKS1B: CDC28 protein kinase regulatory subunit 1B; PD-L1: programmed cell death 1-ligand 1; OV: ovarian; HR: hazard ratio.

In this study, we infected SKOV3 and OVCAR3 cells with si-NC, si-CKS1B#1 and si-CKS1B#2 and found that cell viability and the number of cells forming colonies were inhibited in ovarian cancer cells when treated with si-CKS1B. Moreover, flow cytometry revealed a higher rate of apoptosis ovarian cancer cells that had been treated with si-CKS1B; moreover, the levels of Bax and cleaved caspase 3 were higher. Similarly, Liu *et al.* [16] reported that the silencing of CKS1B markedly suppressed the survival and viability of hepatocellular carcinoma cells, thus suppressing the development and metastasis of hepatocellular carcinoma. In lung adenocarcinoma, CKS1B serves as a vital regulator involved in promoting the proliferation of tumors [17]. In another study, Wang *et al.* [13] reported that the silencing of CKS1B inhibited cell growth and invasion in papillary thyroid carcinoma (PTC). In addition, these authors reported that CKS1B and PD-L1 were substantially elevated in papillary thyroid carcinoma cells, thus indicating that *CKS1B* is an oncogene and promotes tumorigenesis by up-regulating PD-L1 [13].

Recently, the development of ovarian cancer has been linked with PD-L1. Researchers have found that PD-L1 is up-regulated in patients with ovarian cancer and that the application of PD-1/PD-L1 inhibitors can effectively alleviate the progress of ovarian cancer [18, 19]. These studies revealed that PD-1/PD-L1 has significant potential as a therapeutic target for ovarian cancer. Moreover, research has shown that CKS1B increases the level of PD-L1, thereby enhancing the development of lung cancer [20]. Furthermore, the positive interaction between CKS1B and PD-L1 has also been reported in multiple cancers such as lung cancer [20] and papillary thyroid carcinoma [13]. In our study, we also found that the supplementation of PD-L1 to ovarian cancer cells suppressed the anti-tumor effect of si-CKS1B, manifesting with increased cell viability and a decrease of apoptotic cells, thus revealing that the activation of PD-L1 by CKS1B could facilitate the advancement of ovarian cancer.

This study had some limitations that need to be considered. First, is not known if other signaling pathways also played a role in the anti-tumor process arising from the knockdown of CKS1B, although PD-L1 was confirmed to be dysregulated by CKS1B silencing. Furthermore, other molecules, similar to PD-L1, may also be involved in the regulation of CKS1B, tumor proliferation and apoptosis.

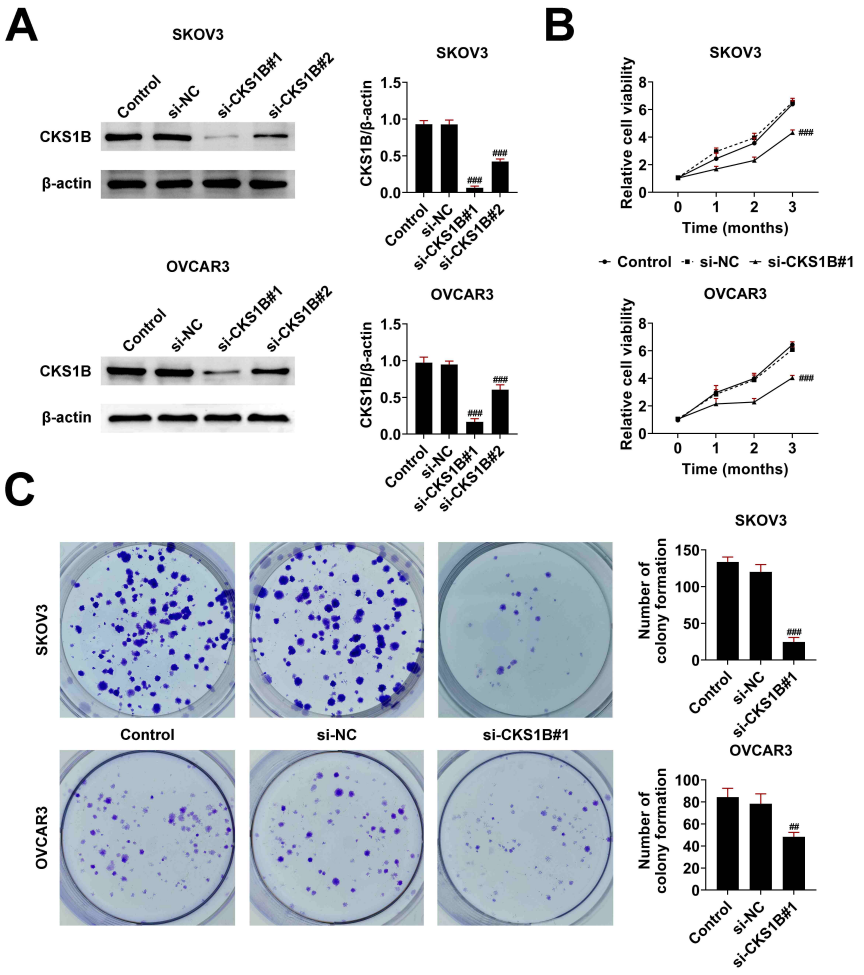

FIGURE 2. The knockdown of *CKS1B* inhibited the growth of ovarian cancer cells. (A) The expression of CKS1B was measured by western blotting in SKOV3 and OVCAR3 cells transfected with si-CKS1B, si-NC or control siRNAs. (B) Cell availability was assessed in SKOV3 and OVCAR3 cells by CCK8 assays. (C) Proliferative ability was evaluated by colony formation assays. Data are expressed as mean ± SD. ##$p < 0.01$, ###$p < 0.001$ *vs.* control or si-NC. CKS1B: CDC28 protein kinase regulatory subunit 1B; si-NC: si-negative control.

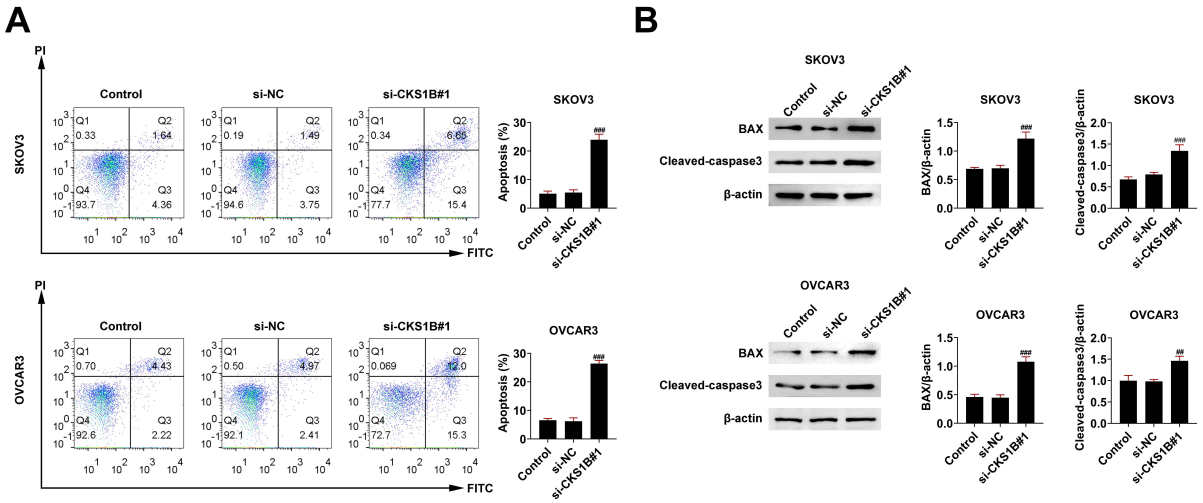

FIGURE 3. The knockdown of CKS1B promoted apoptosis in ovarian cancer cells. (A) The apoptosis rate of SKOV3 and OVCAR3 cells as determined by flow cytometry. (B) Western blotting was used to determine the protein levels of Bax and cleaved caspase 3 in SKOV3 and OVCAR3 cells. Data are expressed as mean ± SD. ##$p < 0.01$, ###$p < 0.001$ *vs.* control or si-NC. CKS1B: CDC28 protein kinase regulatory subunit 1B; si-NC: si-negative control; BAX: BCL2-associated X.

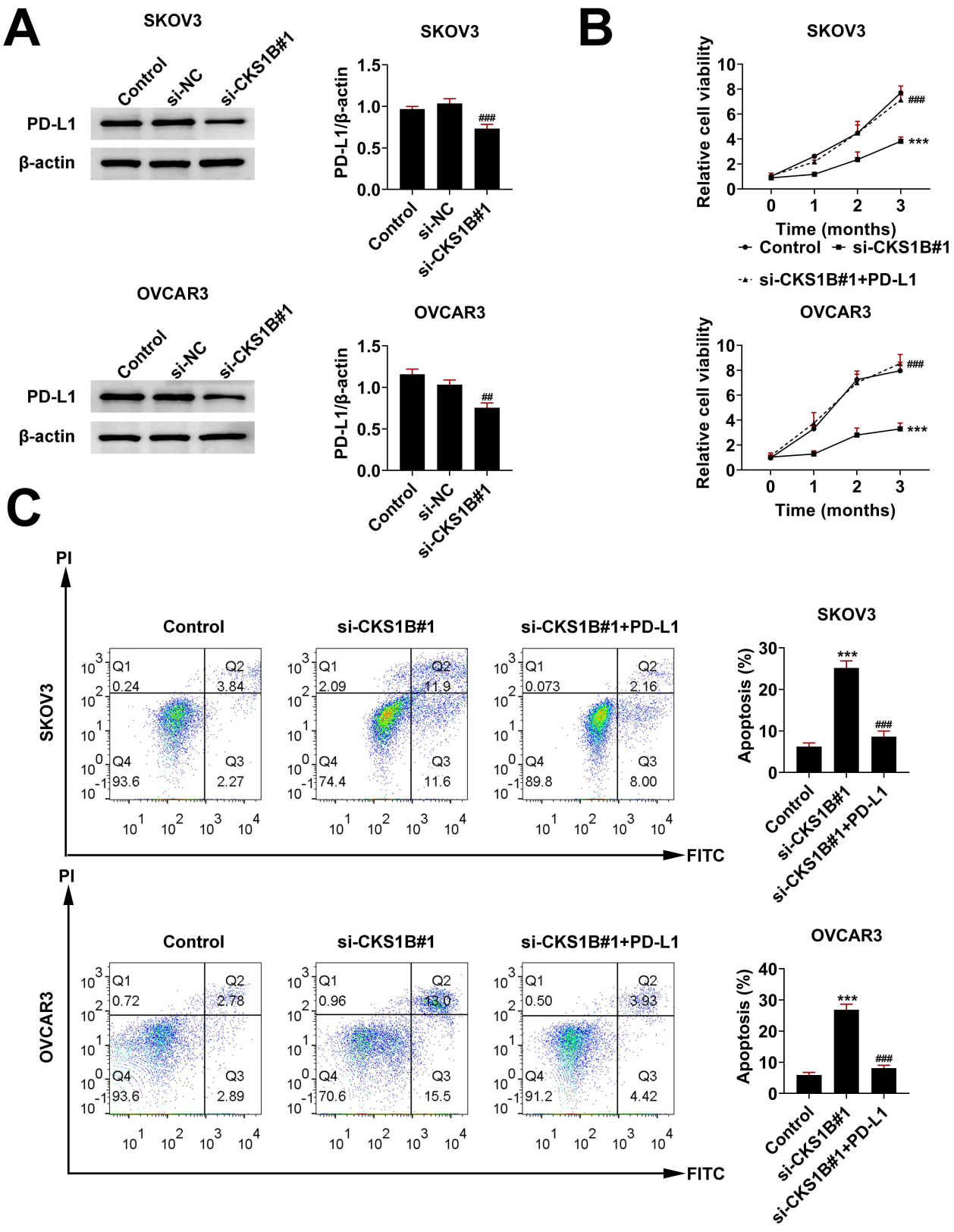

FIGURE 4. The knockdown of CKS1B inhibited the growth of ovarian cancer by regulating PD-L1 expression. Ovarian cancer cells were transfected with si-CKS1B with or without PD-L1 supplementation. (A) The expression of PD-L1 was determined by western blotting. (B) Cell availability was assessed in SKOV3 and OVCAR3 cells by CCK8 assays. (C) The apoptosis rate of SKOV3 and OVCAR3 cells were measured by flow cytometry. Data are expressed as mean ± SD. ***$p <$ 0.001 *vs.* control. ##$p < 0.01$, ###$p < 0.001$ *vs.* si-CKS1B. CKS1B: CDC28 protein kinase regulatory subunit 1B; PD-L1: programmed cell death 1-ligand 1; si-NC: si-negative control.

5. Conclusions

In conclusion, our findings first demonstrated that CKS1B were markedly elevated in patients with ovarian cancer and that CKS1B knockdown inhibited tumor proliferation and improved the rate of apoptosis in cancer cells, inhibiting ovarian cancer advancement. In addition, our findings show that the pro-tumor effects of CKS1B on ovarian cancer may act by upregulating PD-L1. Finally, we demonstrated that CKS1B silencing alleviated the progression of ovarian cancer by suppressing PD-L1 expression. These findings provide enhanced knowledge of the roles of CKS1B/PD-L1 in the development of ovarian cancer.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

YYZ, YJS—designed the study and carried them out; prepare the manuscript for publication and reviewed the draft of the manuscript. YYZ, YJS, GHD—supervised the data collection, analyzed the data, interpreted the data. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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