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p27^{Kip1} and cytoplasmic pSer10p27 are promising biomarkers for predicting prognosis and chemotherapy response in ovarian cancer

Mengna Zhu*, Si Sun*, Lin Huang, Lingling Gao, Mengqing Chen, Jing Cai, Zehua Wang and Minggang Peng

Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

*These authors contributed equally to this work

Summary. Purpose. The biological function of p27^{Kip1} largely depends on its subcellular localization and phosphorylation status. Different subcellular localizations and phosphorylation statuses of p27^{Kip1} may represent distinct clinical values, which are unclear in ovarian cancer. This study aimed to elucidate different subcellular localizations of p27^{Kip1} and pSer10p27 in predicting prognosis and chemotherapy response in ovarian cancer.

Methods. Meta-analyses were executed to evaluate the association of p27^{Kip1} and phosphorylated p27^{Kip1} with the prognosis of ovarian cancer patients. The expression levels and patterns of p27^{Kip1} and pSer10p27 were evaluated by immunohistochemistry. The correlations between different p27^{Kip1} states, clinicopathological features, and prognosis were analyzed. p27^{Kip1} and pSer10p27 expression levels in cisplatin-sensitive and cisplatin-resistant ovarian cancer cell lines were detected using WB. KEGG analysis and WB were performed to evaluate the pathways in which p27^{Kip1} was involved.

Results Meta-analyses showed that p27^{Kip1} was associated with significantly better overall survival (OS) in ovarian cancer (HR=2.14; 95% CI [1.71 - 2.68]) and pSer10p27 was associated with significantly poor OS in mixed solid tumors (HR=2.56; 95% CI [1.76 - 3.73]). In our cohort of ovarian cancer patients, low total p27^{Kip1} remained independent risk factors of OS (HR=2.097; 95% CI [1.121 - 3.922], *P*=0.021) and PFS (HR=2.483; 95% CI [1.364 - 4.518], *P*=0.003), while low cytoplasmic pSer10p27 had independent protective effects in terms of OS (HR=0.472; 95% CI [0.248 - 0.898], *P*=0.022) and PFS (HR=0.488; 95% CI [0.261 - 0.910], *P*=0.024). Patients with low total p27^{Kip1}/pSer10p27 and low nuclear p27^{Kip1} had worse chemotherapy responses, while patients with low cytoplasmic pSer10p27 expression had better chemotherapy responses. The protein levels of p27^{Kip1} and pSer10p27 were significantly reduced in the cisplatin-resistant cell lines SKOV3-cDDP and A2780-cDDP, and the level of p27^{Kip1}/pSer10p27 was subjective to Akt activation.

Conclusions The present study demonstrates that p27^{Kip1} and cytoplasmic pSer10p27 are promising biomarkers for predicting prognosis and chemotherapy response in ovarian cancer.

Key words: p27^{Kip1}, pSer10p27, Ovarian cancer, Chemotherapy response, Prognosis

Introduction

Ovarian cancer is one of the most lethal malignancies of the female reproductive system worldwide (Duan et al., 2023). Due to the lack of early diagnosis methods, more than 70% of patients reach advanced stages by the time of diagnosis (Dochez et al., 2019). Although ovarian cancer patients can benefit from a combination of tumor reduction and platinum-based adjuvant chemotherapy after diagnosis, relapse occurs in 70-80% of patients with International Federation of Gynecology and Obstetrics stage III to IV disease (FIGO III-IV) within five years (Pignata et al., 2019). Due to the emergence of platinum resistance, adjuvant treatment options, including PARP inhibitor Olaparib, have been limited (Pignata et al., 2019). Enriching platinum-based chemotherapy-associated biomarkers may provide evidence for developing novel treatment strategies by identifying novel patient selection markers and therapeutic targets.

Corresponding Author: Minggang Peng and Zehua Wang, Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, PR China. e-mail: mgpengwhuh@hust.edu.cn and zehuawang@hust.edu.cn

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p27^{Kip1} protein, a cyclin-dependent kinase inhibitor, has long been known as a tumor suppressor (Polyak, 2006; Bury et al., 2021). However, in recent years, it was found that p27^{Kip1} plays a pivotal dual role in tumorigenesis (Yoon et al., 2019). The p27^{Kip1} protein structure has a CDK-binding domain, which allows it to bind and inhibit CDK or CDK-cyclin complex in the nucleus, arresting the cell cycle (Blain et al., 2003; Oboshi et al., 2020). p27^{Kip1} can functionally disrupt cancer through excessive proteolysis, reduced translation, or C-terminal phosphorylation (Chu et al., 2008; Larrea et al., 2009). However, different from previous findings, some studies have shown that p27^{Kip1} is not completely localized to the nucleus; hence, it has distinct functions in the field (Lian et al., 2019). Phosphorylation of p27^{Kip1} promotes its nuclear-cytoplasmic trans-localization, which deprives the nuclear function of p27^{Kip1} as a CDK inhibitor. Miscellaneous phosphorylation sites of p27^{Kip1} have been reported, and Ser10, Thr157, Thr187, and Thr198 were the most extensively studied (Ishida et al., 2000; Abbastabar et al., 2018; Bencivenga et al., 2021). Among these phosphorylation sites, Ser10 phosphorylation of p27^{Kip1} determines protein stability and subcellular localization (Ishida et al., 2000; Xiao et al., 2023). However, whether the different localization of p27^{Kip1} and pSer10p27 are associated with the prognosis and response to chemotherapy in ovarian cancer is unknown.

In the present study, we investigated the correlation of different localizations of p27^{Kip1} and pSer10p27 with chemotherapy response and prognosis in ovarian cancer. We found that total p27^{Kip1} and cytoplasmic pSer10p27 could better predict chemotherapy response and were independent prognostic factors in ovarian cancer. These results provide a new basis for the prediction of chemotherapy response and prognosis in ovarian cancer.

Materials and methods

Public data mining

The association of *CDKN1B* with overall survival (OS) and progression-free survival (PFS) in ovarian cancer was validated at the transcriptome level using the Kaplan-Meier (KM) plotter with the JetSet probe set (https://kmplot.com/analysis/). Log-rank test and univariate Cox proportional proportional hazard regression generated KM curves, *P*-values, and hazard ratios (HRs) with 95% confidence intervals (CIs). The GEPIA database (http://gepia.cancer-pku.cn/) was used to analyze the expression distribution of *CDKN1B* in ovarian serous cystadenocarcinoma (OV) and normal tissues. The genomic alterations of *CDKN1B*, including copy number variations (CNVs) and mutations, were detected in 585 ovarian cancer samples from the TCGA Pan-Cancer Atlas project, and the association between genomic alterations in *CDKN1B* and survival of ovarian cancer patients was provided through the cBioPortal database (https://www.cbioportal.org/). The median expression of *CDKN1B* was used as the cut-off value to distinguish between high and low expression of *CDKN1B* in the TCGA-OV database (https://portal.gdc.cancer.gov/). The two groups of differentially expressed genes (DEGs) were further used to perform the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and the Gene Ontology (GO) enrichment analysis through the "clusterProfiler" package in R 4.2.3 software.

Meta-analysis

An electronic search of PubMed, Embase, and Web of Science from January 1990 to May 2021 was conducted with the following keywords: 'ovarian cancer,' 'ovarian carcinoma' or 'ovarian neoplasm' and 'p27^{Kip1}' or 'phosphorylation of p27^{Kip1}'. The search strategies were supplemented by reviewing similar articles and checking references in the retrieved literature. In addition, we searched the reference lists of all selected publications. Data were extracted from the access database, which was independently reviewed by two authors to determine which studies met the following criteria: 1) assessed the relevance of p27^{Kip1} protein levels or phosphorylated p27^{Kip1} protein levels to patient prognosis by IHC staining; 2) published as full text in English. We excluded reviews, non-original articles, and studies on ovarian cancer cell lines or animal models. In survival predictive analysis, we assessed the prognostic impact of p27^{Kip1} protein levels or phosphorylated p27^{Kip1} protein levels by HR. HRs and 95% CIs were extracted. If these parameters were not available in the study, we used Engauge Digitizer 4.1 software to extract specific survival according to the Kaplan-Meier curves and calculated HRs by the method described by Tierney et al. (2007). Upon cohort overlapping, only the largest cohort was included in the analysis. The meta-analysis was performed using the "meta" package in R 4.2.3 software. Considering possible heterogeneities among studies, we calculated the overall HR, I^2, and *P*-value using a random effects model.

Cell culture and drugs

Ovarian cancer cell lines SKOV3 (adenocarcinoma) were purchased from the American Type Culture Collection (ATCC, USA), and A2780 (adenocarcinoma) was purchased from the BLUEFBIO company. Parental cells were treated with increasing concentrations of cisplatin for 12 cycles to obtain the cisplatin-resistant cell lines SKOV3-cDDP and A2780-cDDP (Sun et al., 2018). All cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum. Cisplatin-resistant cell lines were given 0.5 μg/ml cisplatin to maintain resistance. All cells were grown in a humidified incubator at 37°C 5% CO_2. Cisplatin was purchased from the Department of Pharmacy, Wuhan Union Hospital. MK2206 was purchased from Selleck

(S1078, USA).

Cell viability assay

Cellular viability was assessed via MTT assays. MTT reagents were purchased from Aladdin, Shanghai, China. Ovarian cancer cells were seeded into 96-well plates at a density of 5×10^3 cells per well and incubated until the next day. Then cisplatin at different concentrations (0.005 μM, 0.05 μM, 0.5 μM, 5 μM, 10 μM, 20 μM, 40 μM, or 80 μM) were added to the cells for 72 hours. Subsequently, 20 μL of MTT solution (5 mg/mL) was added to each well and incubated at 37°C for 4 hours. Next, 150 μL dimethyl sulfoxide was added to each well, and the plate was shaken in the dark until the crystals were completely dissolved. Finally, the absorbance at 570 nm was measured using a microplate reader (SpectraMax, Sunnyvale, CA, USA). The IC_{50} values of drugs in different cell lines were analyzed with GraphPad Prism 9.4 (USA) software.

Western blot (WB) analysis

WB was performed to detect p27^{Kip1}, pSer10p27, Akt, p-Akt, and β-actin protein levels in ovarian cancer cell lines. The total protein extraction process and WB were performed as previously described (Wen et al., 2021). The primary antibodies used were anti-p27^{Kip1} antibody (Proteintech; 25614-1-AP; 1:1000 dilution), anti-pSer10p27 antibody (Abcam; ab62364; 1:1000 dilution), anti-Akt (CST; 2920S; 1:1000 dilution), anti-p-Akt (Ser473) antibody (CST; 4060S; 1:1000 dilution), and anti-β-actin antibody (ABclonal; AC026; 1:10000 dilution). The experiments were performed in three biological replicates.

RNA extraction and quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was used to detect CDKN1B mRNA levels in ovarian cancer cell lines. Briefly, total RNA was extracted from cisplatin-resistant ovarian cancer cell lines and parental ovarian cancer cell lines separately using TRIzol reagent (Takara, Japan). A total of 1 μg of total RNA was reverse-transcribed to first-strand cDNA by the HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, China). Subsequently, qRT-PCR was performed on a Step-One Plus Real-Time PCR system using SYBR Green PCR Master Mix (Vazyme, China). The *GADPH* gene was selected as a reference gene. The relative expression of each mRNA was normalized using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). The primers used in this study were GAPDH (Forward): 5'-TCCCATCACCATCTTCCAG-3', GAPDH (Reverse): 5'-ATGAGTCCTTCCACGATACC-3', CDKN1B (Forward): 5'-AACGTGCGAGTGTCTAACGG-3', CDKN1B (Reverse): 5'- CCCTCTAGGGGTTTGTG ATTCT-3'. The experiments were performed in three biological replicates.

Clinical samples and characteristics

The clinical tissue microarrays consisted of 51 ovarian cancer patients' tissue samples from Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. All patients underwent surgery and received standard platinum-based chemotherapy. Clinical and pathological data were collected retrospectively. A waiver of informed consent has been applied. Patient characteristics included age, histological type, International Federation of Gynecology and Obstetrics (FIGO) stage, treatment regimen, chemotherapy response, and follow-up information. Chemotherapy resistance was defined as relapse within six months after completing chemotherapy or progression during the primary chemotherapy. Relapses were diagnosed on clinical symptoms, radiological evidence, and biochemical abnormalities, such as elevated CA125. OS was defined as from diagnosis to death or date of last follow-up, and PFS was defined as from surgery to relapse or date of last follow-up. Diagnoses of all patients were confirmed pathologically. This study was approved by the Independent Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (20210567).

Immunohistochemistry (IHC)

IHC staining of tissue microarrays was performed according to the previously described protocol (Li et al., 2021). Primary antibodies were anti-p27^{Kip1} antibody (Proteintech; 25614-1-AP; 1:50 dilution) and anti-pSer10p27 antibody (Abcam; ab62364; 1:100 dilution). IHC staining scores for p27^{Kip1} and pSer10p27 were evaluated according to total expression (total positive staining was evaluated without considering localization), nuclear expression (only nuclear staining was evaluated), and cytoplasmic expression (only cytoplasmic staining was evaluated), respectively. Total and cytoplasmic IHC scores were assessed according to the following formula: staining intensity (0, negative; 1, weak; 2, mild; 3, strong) and staining area (0, 0%; 1, <25%; 2, 25% - 50%; 3, 50% - 75%; 4, >75%). Nuclear IHC score was assessed according to the following formula: percentage of positive tumor cells (0, 0%; 1, <25%; 2, 25% - 50%; 3, 50% - 75%; 4, >75%) and staining intensity (0, negative; 1, weak; 2, mild; 3, strong).

Statistical analysis

Data was analyzed using GraphPad Prism 9.4 and SPSS 27.0 software. The χ^2 and Fisher's exact tests were used to analyze the relationship between IHC scores of p27^{Kip1} and pSer10p27 and clinicopathological characteristics in ovarian cancer. Spearman's correlation test was used to analyze the correlation between p27^{Kip1} and pSer10p27, and the Mann-Whitney test was used to

compare the difference between the two groups. Receiver operating characteristic (ROC) curves were plotted to analyze the value of total, nuclear, and cytoplasmic $p27^{Kip1}$ and pSer10p27 in predicting chemotherapeutic efficacy. Logistic and Cox regression were used to analyze the risk of chemotherapy response and survival. Chemotherapeutic efficacy was evaluated using an odds ratio (OR). Survival curves were plotted and analyzed using the Kaplan-Meier method and log-rank test. Differences were considered statistically significant at $P<0.05$.

Results

Meta-analysis of the correlation between p27Kip1 and pSer10p27 and prognosis in ovarian cancer.

First, the correlation of $p27^{Kip1}$ protein with PFS and OS in ovarian cancer patients was evaluated. A total of 650 studies were identified through a literature search after duplicate removal (N=12) and study exclusion after initial review of titles and abstracts (N=589). After the data check, nine studies with 805 patients were included in the meta-analysis (Masciullo et al., 1999, 2000; Sui et al., 2001; Bali et al., 2004; Psyrri et al., 2005; Schmider-Ross et al., 2006; Fei et al., 2009; Hashimoto et al., 2011; Lu et al., 2011). A low $p27^{Kip1}$ protein level was associated with poor OS (HR=2.14; 95% CI [1.71 - 2.68]) and PFS (HR=1.61; 95% CI [0.93 - 2.78]) in ovarian cancer. (Figs. 1A, 2A). In addition, *CDKN1B* (encoding $p27^{Kip1}$ protein) mRNA levels were negatively correlated with OS (HR=1.23; 95% CI [1.07 - 1.41]) and PFS (HR=1.28; 95% CI [1.12 - 1.45]) in ovarian cancer using the KM-Plotter database analysis (Fig. 3A,B). According to the expression analysis from the GEPIA database, *CDKN1B* was significantly reduced in ovarian cancer tissues compared with paired normal tissues (Fig. 3C). According to genomic alterations of *CDKN1B* including CNVs and mutations in 585 ovarian cancer samples from the cBioportal database (Fig. 3D), *CDKN1B* was relatively conserved and had a low frequency of alteration (5%), which did not show statistically significant associations with PFS and OS in the cohort (Fig. 3E,F). Therefore, we assumed that the correlation between $p27^{Kip1}$ and the prognosis of ovarian cancer patients might be mainly due to the change in protein levels and phosphorylation status. Furthermore, we performed another meta-analysis, including five studies with 428 patients (He et al., 2011, 2012; Wang et al., 2014a,b; Wu et al., 2021), to analyze the prognostic effects of phosphorylation modifications of $p27^{Kip1}$ that might affect their protein level changes in tumors, and found that high Ser10 phosphorylation modification of $p27^{Kip1}$ was associated with poor OS (HR=2.56; 95% CI [1.76 - 3.73]) in ovarian cancer (Fig. 1B).

Total p27Kip1 and pSer10p27 correlated with chemotherapy response in ovarian cancer.

To further validate the results of the meta-analysis and bioinformatic analysis, we used immunohistochemistry (IHC) to assess the total positive staining of $p27^{Kip1}$ and pSer10p27 in the nucleus and cytoplasm in ovarian cancer tissue microarrays (Fig. 4A,B). Both total $p27^{Kip1}$ (46/51) and total pSer10p27 (34/51) can be robustly detected in most specimens and total pSer10p27 positively correlated with total $p27^{Kip1}$ as expected (Spearman r=0.323, P=0.021) (Fig. 4C). The clinical significance of total $p27^{Kip1}$ and total pSer10p27 in ovarian cancer were subsequently analyzed. The total $p27^{Kip1}$ expression level was significantly correlated with chemotherapy response ($P<0.001$), and the total pSer10p27 expression level was correlated with chemotherapy response (P=0.001) (Fig. 4D, Table 1). Univariate Cox regression analysis and Kaplan-Meier survival analysis showed that low total $p27^{Kip1}$

Table 1. Association between total of $p27^{Kip1}$ and pSer10p27 to clinical pathological features in ovarian cancer by IHC-score stratification[a].

Clinicopathological features	N	$p27^{Kip1}$ (Total[b])			N	pSer10p27 (Total)		
		Low	High	*P*		Low	High	*P*
Age (years)[c]				0.572				0.470
<50	20	10	10		20	4	16	
≥50	31	13	18		31	9	22	
Histology				0.965				0.417
High-grade serous	42	19	23		42	12	30	
Others	9	4	5		9	1	8	
FIGO stage[d]				0.037				0.309
I-II	14	3	11		14	5	9	
III-IV	37	20	17		37	8	29	
Chemo response				<0.001				0.027
Sensitive	37	11	26		37	6	31	
Resistant	14	12	2		14	7	7	

[a], The median IHC score was chosen as the cut-off for total $p27^{Kip1}$ and total pSer10p27; [b], total positive staining was evaluated without considering localization; [c], Age at surgery; [d], International Federation of Gynecology and Obstetrics.

Table 2. Univariate Cox regression survival analysis.

	PFS			OS		
	HR	95% CI	*P*	HR	95% CI	*P*
Age (< 50 vs. ≥ 50y)	1.738	0.969-3.116	0.064	1.281	0.705-2.329	0.416
Histology (high grade serous vs. others)	0.503	0.239-1.062	0.072	0.386	0.179-0.830	0.015
FIGO stage (I-II vs. III-IV)	0.470	0.237-0.932	0.031	0.594	0.291-1.214	0.153
p27^{Kip1} (Total) expression (low vs. high)	2.654	1.472-4.784	0.001	2.323	1.269-4.255	0.006
p27^{Kip1} (Nuc[a]) expression (low vs. high)	1.563	0.881-2.772	0.127	1.933	1.063-3.515	0.031
p27^{Kip1} (Cyt[b]) expression (low vs. high)	1.129	0.632-2.017	0.682	1.252	0.686-2.285	0.464
pSer10p27 (Total) expression (low vs. high)	0.838	0.407-1.728	0.633	0.787	0.375-1.649	0.525
pSer10p27 (Nuc) expression (low vs. high)	1.507	0.829-2.739	0.179	1.533	0.819-2.869	0.182
pSer10p27 (Nuc) expression (low vs. high)	0.509	0.286-0.904	0.021	0.473	0.258-0.865	0.015

[a], only nuclear staining was evaluated; [b], only cytoplasmic staining was evaluated.

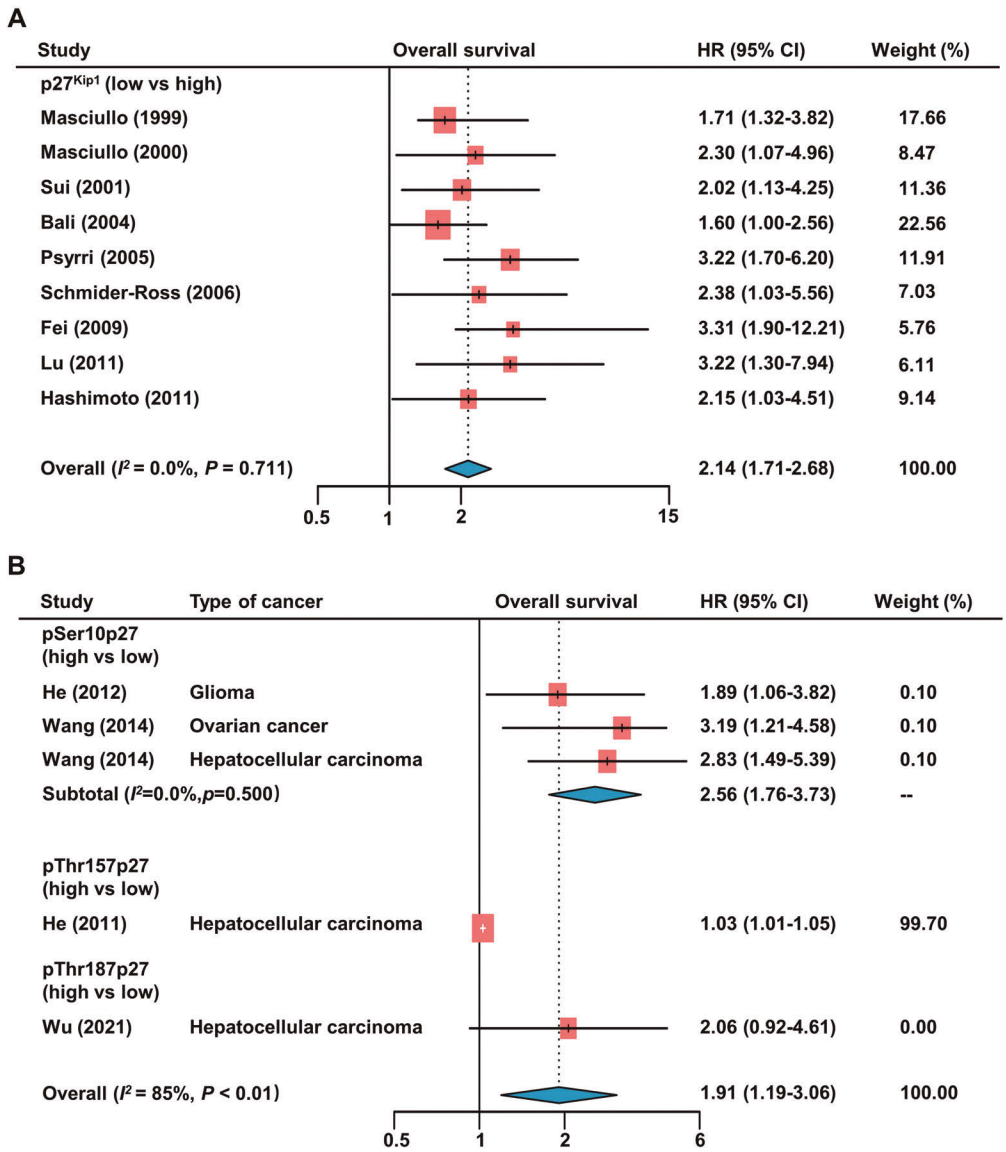


Fig. 1. The prognosis of p27^{Kip1} and phosphorylated p27^{Kip1} in ovarian cancer. **A.** Meta-analysis of p27^{Kip1} associated with overall survival in ovarian cancer. **B.** Meta-analysis of phosphorylated p27^{Kip1} associated with overall survival in different cancers.

expression was associated with poor OS (HR=2.323; 95% CI [1.269 - 4.255], *P*=0.006) and PFS (HR=2.654; 95% CI [1.472 - 4.784], *P*=0.001) (Fig. 4E,F, Table 2). These results suggest that total $p27^{Kip1}$ expression correlates with chemotherapy response and prognosis.

Expression patterns of $p27^{Kip1}$ and pSer10p27 predict chemotherapy response and prognosis in ovarian cancer

Since previous studies that reported the correlation between $p27^{Kip1}$ and patient prognosis did not specify the subcellular location of $p27^{Kip1}$, we analyzed different subcellular locations of $p27^{Kip1}$ and pSer10p27 and their underlying clinical values. Four expression patterns of $p27^{Kip1}$ and pSer10p27 were identified: nuclear and cytoplasmic dominant (NCD), nuclear dominant (ND), cytoplasmic dominant (CD), and nuclear and cytoplasmic weak-to-none (NCWN) (Fig. 5A,B). We also analyzed the compositional proportion of the four expression patterns of $p27^{Kip1}$ and pSer10p27 (Fig. 5C). The patients with NCD, ND, CD, and NCWN expression patterns of $p27^{Kip1}$ and pSer10p27 accounted for 33.33%, 13.73%, 43.14%, 9.80% and 13.73%, 17.65%, 35.29%, 33.33%, respectively. Around 1/3 to 1/2 of patients presented a CD pattern of $p27^{Kip1}$ or pSer10p27 and 47.06% of $p27^{Kip1}$-positive patients were accompanied with positive pSer10p27 staining. The inexistence of the pSer10p27 positive/$p27^{Kip1}$ negative pattern suggested the robustness of the staining.

We then explored the correlation of nuclear and cytoplasmic $p27^{Kip1}$/pSer10p27 with clinical features. Nuclear $p27^{Kip1}$ (*P*=0.020), nuclear pSer10p27 (*P*=0.010), and cytoplasmic pSer10p27 (*P*<0.001) were significantly correlated with chemotherapy response (Fig. 5D,E, Table 3). Logistic regression analysis revealed that patients with low expression of total $p27^{Kip1}$ (OR=14.182; 95% CI [2.711 - 74.188], *P*=0.002), nuclear $p27^{Kip1}$ (OR=4.615; 95% CI [1.207 - 17.656], *P*=0.025) total pSer10p27 (OR=5.167; 95% CI [1.320 - 20.220], *P*=0.018), and nuclear pSer10p27 (OR=11.050; 95% CI [1.308 – 93.380], *P*=0.027) had

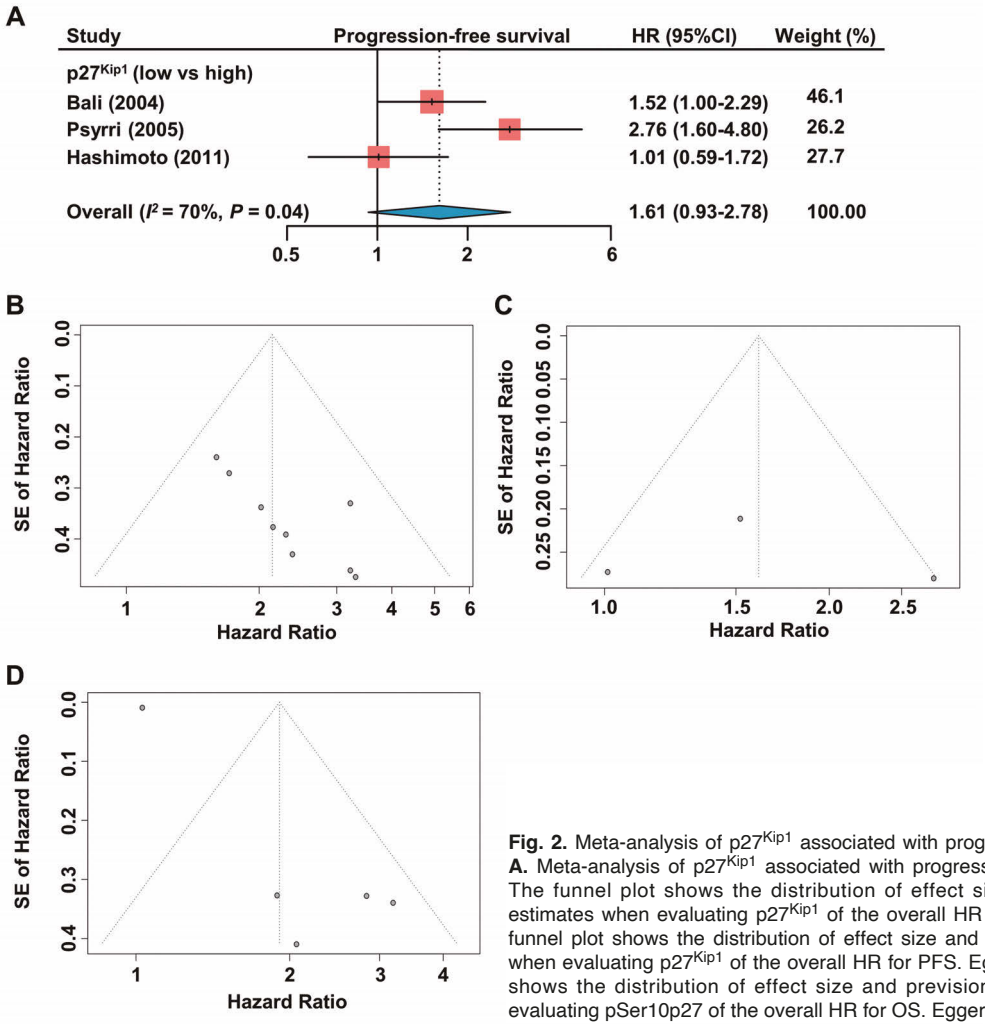
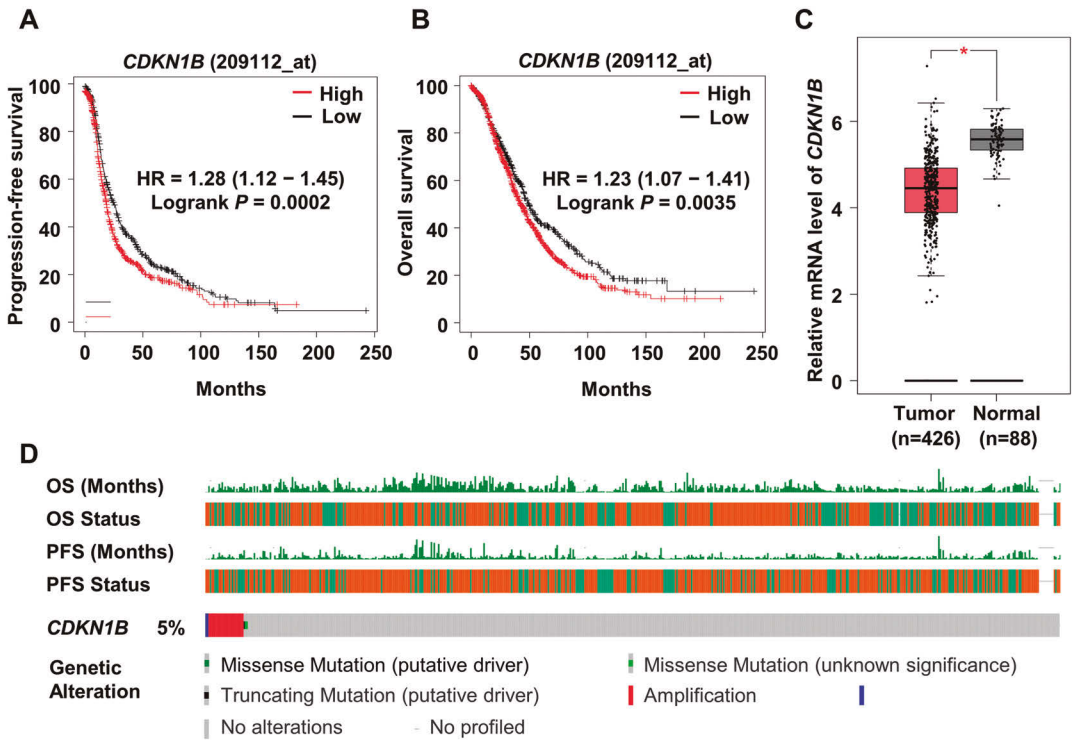


Fig. 2. Meta-analysis of $p27^{Kip1}$ associated with progression-free survival in ovarian cancer. **A.** Meta-analysis of $p27^{Kip1}$ associated with progression-free survival in ovarian cancer. **B.** The funnel plot shows the distribution of effect size and prevision of individual study estimates when evaluating $p27^{Kip1}$ of the overall HR for OS. Egger's test: *P*=0.011. **C.** The funnel plot shows the distribution of effect size and prevision of individual study estimates when evaluating $p27^{Kip1}$ of the overall HR for PFS. Egger's test: *P*=0.879. **D.** The funnel plot shows the distribution of effect size and prevision of individual study estimates when evaluating pSer10p27 of the overall HR for OS. Egger's test: *P*=0.010.

Table 3. Association between p27Kip1 and pSer10p27 to clinical pathological features in the nuclear and cytoplasm of ovarian cancer by IHC-score stratification.

Clinicopathological features	N	p27Kip1 (Nuc)			p27Kip1 (Cyt)			pSer10p27 (Nuc)			pSer10p27 (Cyt)		
		Low	High	*P*	Low	High	*P*	Low	High	*P*	Low	High	*P*
Age (years)				0.557			0.108			0.311			0.050
<50	20	8	12		7	13		5	15		6	14	
≥50	31	15	16		18	13		12	19		18	13	
Histology				0.159			0.726			0.699			0.718
High grade serous	42	21	21		20	22		15	27		19	23	
Others	9	2	7		5	4		2	7		5	4	
FIGO stage				0.145			0.072			1.000			0.375
I-II	14	4	10		4	10		5	9		8	6	
III-IV	37	19	18		21	16		12	25		16	21	
Chemo response				0.020			0.072			0.010			<0.001
Sensitive	37	13	24		21	16		20	17		23	14	
Resistant	14	10	4		4	10		13	1		1	13	



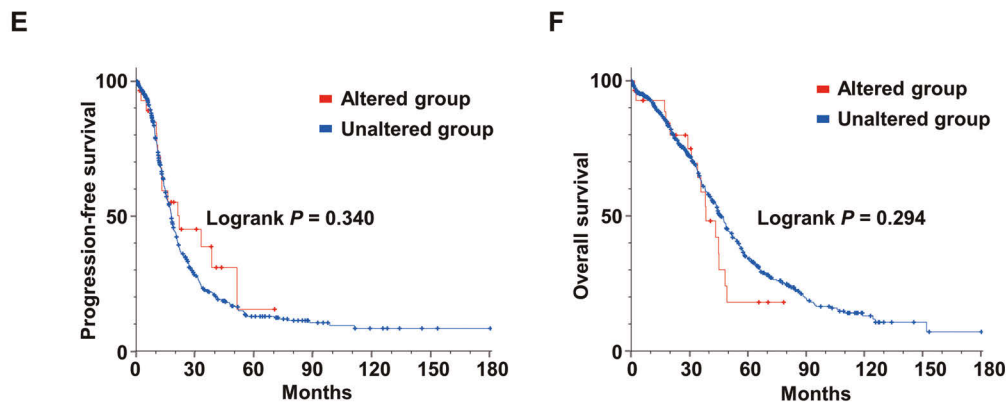


Fig. 3. The prognosis of *CDKN1B* in ovarian cancer. **A, B.** Survival curves of *CDKN1B* for progression-free survival and overall survival in ovarian cancer patients (KM-plotter). **C.** The boxplot of *CDKN1B* expression in tumor and normal tissues of ovarian cancer. **D.** An overview of genomic alterations of *CDKN1B* including CNVs and mutations in ovarian cancer patients from the cBioPortal database. **E, F.** Relationship between *CDKN1B* genomic alterations and prognosis in ovarian cancer patients.

Table 4. Association of different parameters and chemotherapy resistance in ovarian cancer.

	Chemotherapy resistance		
	OR	95% CI	*P*
Age (<50 vs. ≥50y)	2.778	0.786-9.819	0.113
Histology (high-grade serous vs. others)	0.710	0.151-3.334	0.664
FIGO stage (I-II vs. III-IV)	0.142	0.017-1.211	0.074
p27^{Kip1} (Total) expression (low vs. high)	14.182	2.711-74.188	0.002
p27^{Kip1} (Nuc) expression (low vs. high)	4.615	1.207-17.656	0.025
p27^{Kip1} (Cyt) expression (low vs. high)	0.305	0.081-1.152	0.080
pSer10p27 (Total) expression (low vs. high)	5.167	1.320-20.220	0.018
pSer10p27 (Nuc) expression (low vs. high)	11.050	1.308-93.380	0.027
pSer10p27 (Cyt) expression (low vs. high)	0.047	0.006-0.398	0.005

FIGO, International Federation of Gynecology and Obstetrics.

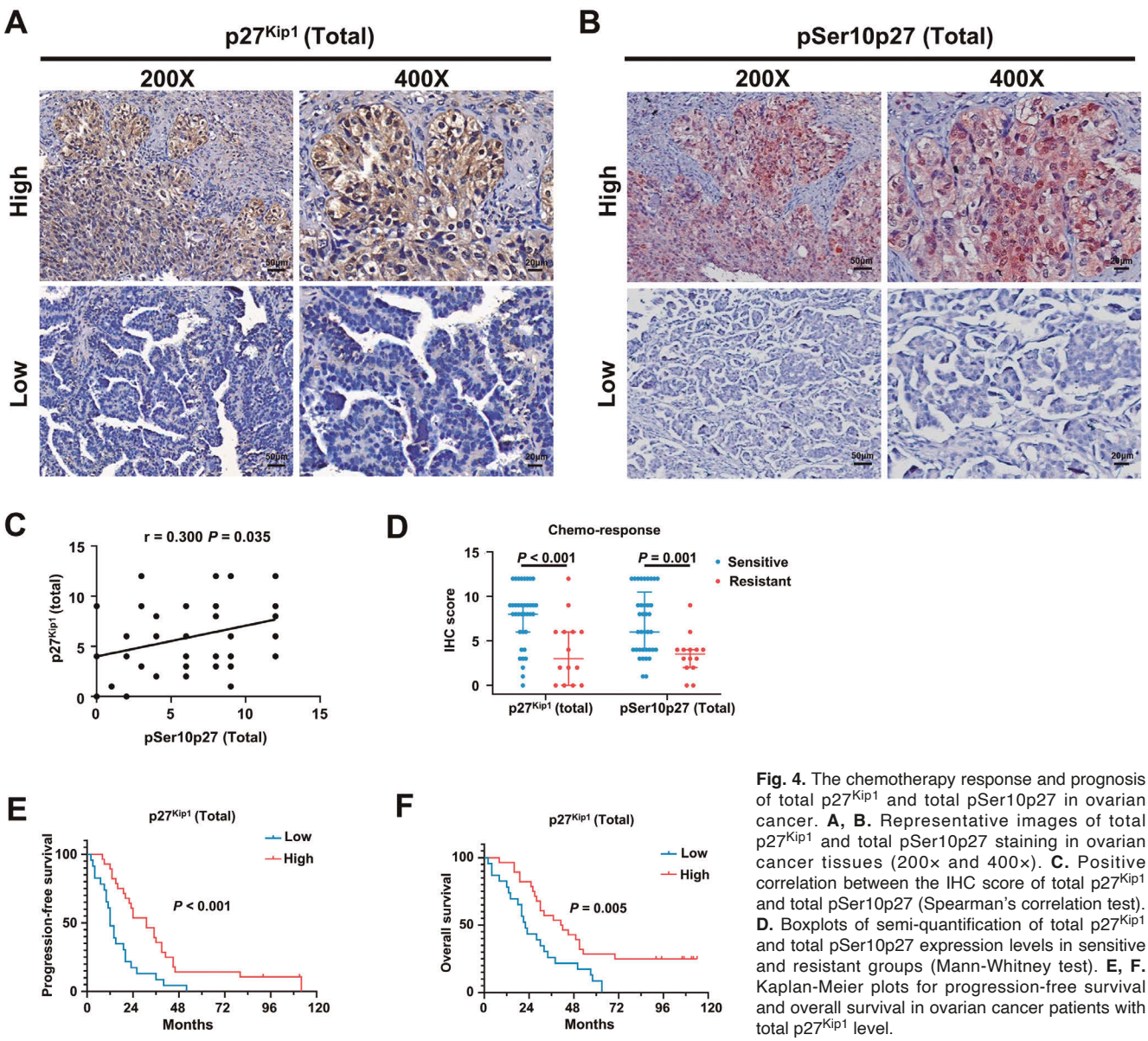


Fig. 4. The chemotherapy response and prognosis of total p27^{Kip1} and total pSer10p27 in ovarian cancer. **A, B.** Representative images of total p27^{Kip1} and total pSer10p27 staining in ovarian cancer tissues (200× and 400×). **C.** Positive correlation between the IHC score of total p27^{Kip1} and total pSer10p27 (Spearman's correlation test). **D.** Boxplots of semi-quantification of total p27^{Kip1} and total pSer10p27 expression levels in sensitive and resistant groups (Mann-Whitney test). **E, F.** Kaplan-Meier plots for progression-free survival and overall survival in ovarian cancer patients with total p27^{Kip1} level.

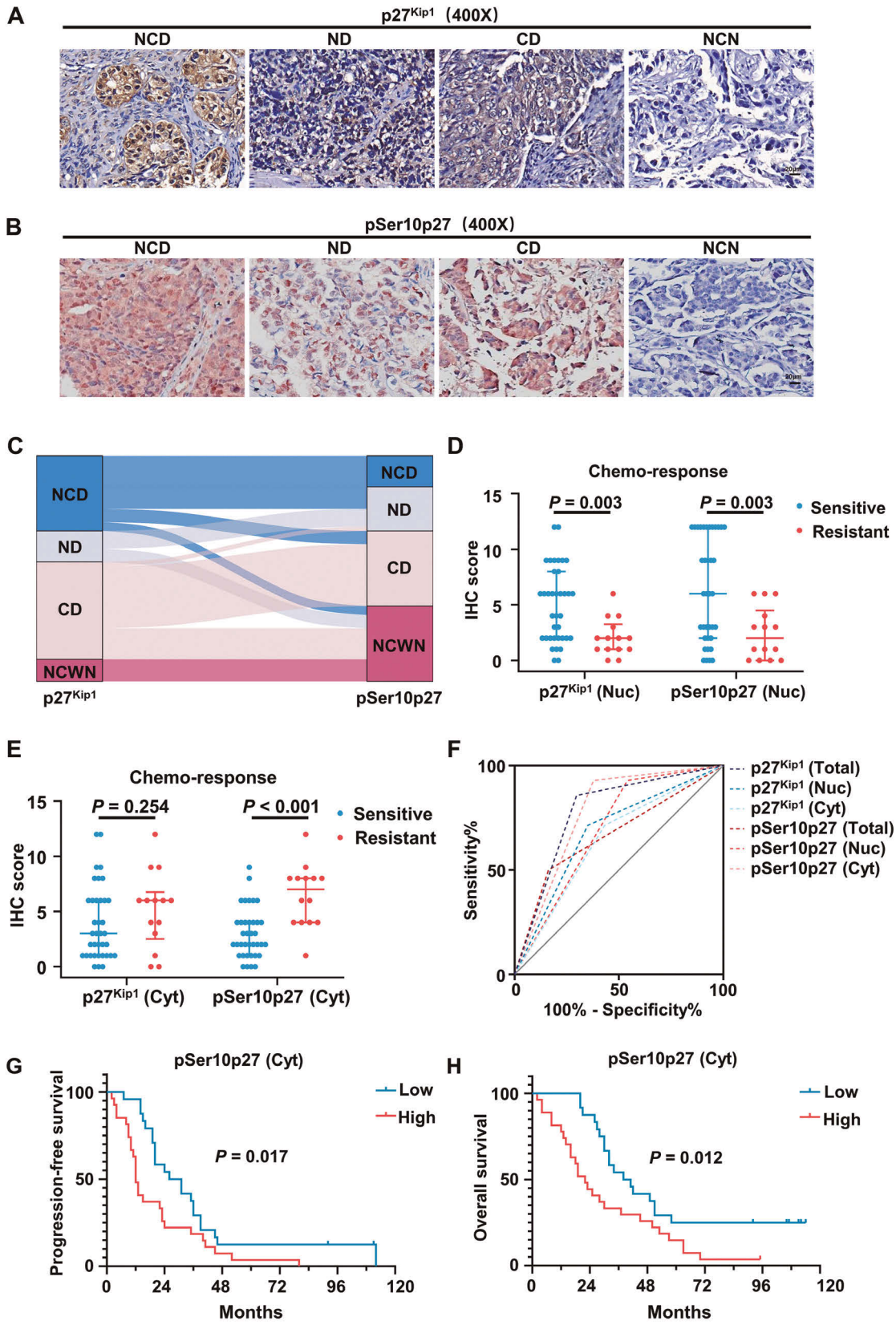


Fig. 5. Different subcellular localization of $p27^{Kip1}$ and pSer10p27 predicted chemotherapy response and prognosis of ovarian cancer. **A, B.** Different expression patterns of $p27^{Kip1}$ and pSer10p27 staining, such as nuclear and cytoplasmic dominant (NCD), nuclear dominant (ND), cytoplasmic dominant (CD), and nuclear and cytoplasmic weak-to-none (NCWN) in ovarian cancer tissues. **C.** Sankey diagram of the relationship between different expression patterns of $p27^{Kip1}$ and pSer10p27. **D, E.** Boxplots of semi-quantification of nuclear expression (Nuc) and cytoplasmic expression (Cyt) of $p27^{Kip1}$ and pSer10p27 in sensitive and resistant groups (Mann-Whitney test). **F.** ROC curves of different subcellular localizations of $p27^{Kip1}$ and pSer10p27 predicting chemotherapy response. **G, H.** Kaplan-Meier plots for progression-free survival and overall survival in ovarian cancer patients with cytoplasmic pSer10p27 levels.

worse chemotherapy response; patients with low cytoplasmic pSer10p27 expression (OR=0.047; 95% CI [0.006 - 0.398], *P*=0.005) had better chemotherapy response (Table 4). The ROC curves showed that the area under the curve (AUC) for total $p27^{Kip1}$ and cytoplasmic pSer10p27 were 0.780 and 0.775, respectively (Fig. 5F). Univariate Cox regression analysis and Kaplan-Meier survival analysis showed that low cytoplasmic pSer10p27 expression was associated with better OS (HR=0.473; 95% CI [0.258 - 0.865], *P*=0.015) and PFS (HR=0.509; 95% CI [0.286 - 0.904], *P*=0.021) (Fig. 5G,H, Table 2). Multivariate cox regression analyses showed that low total $p27^{Kip1}$ remained an independent risk factor for OS (HR=2.097; 95% CI [1.121 - 3.922], *P*=0.021) and PFS (HR=2.483; 95% CI [1.364 - 4.518], *P*=0.003), while high cytoplasmic pSer10p27 had independent protective effects in terms of OS (HR=0.472; 95% CI [0.248 - 0.898], *P*=0.022) and PFS (HR=0.488; 95% CI [0.261 - 0.910], *P*=0.024) (Table 5). These results suggested that total $p27^{Kip1}$ and cytoplasmic pSer10p27 had promising predictive values for chemotherapy response and prognosis.

Low expression of p27Kip1 and pSer10p27 is associated with cisplatin resistance in platinum-resistant ovarian cancer cell lines

To further investigate the relationship between $p27^{Kip1}$/pSer10p27 and platinum resistance in ovarian cancer, we constructed the cisplatin-resistant ovarian cancer cell lines SKOV3-cDDP and A2780-cDDP. The SKOV3-cDDP (IC50=44.40 μM) and A2780-cDDP (IC50=16.93 μM) cell lines were more resistant to cisplatin than the parental SKOV3 (IC50=12.22 μM) and A2780 (IC50=3.04 μM) validated by MTT (Fig. 6A,B). The protein levels of $p27^{Kip1}$ and pSer10p27 were significantly reduced in SKOV3-cDDP and A2780-cDDP (Fig. 6C). Similarly, *CDKN1B* mRNA levels were also reduced in the cisplatin-resistant cell lines (Fig. 6D). These results suggested that increased $p27^{Kip1}$ and pSer10p27 levels were associated with increased cisplatin sensitivity in ovarian cancer. KEGG and GO enrichment analyses of DEGs between the $CDKN1B^{high}$ and $CDKN1B^{low}$ group from the TCGA-OV database were mainly associated with cell cycle, cellular senescence, DNA replication, and the PI3K-Akt signaling pathway (Fig. 6E, Fig. 7A). Considering that some studies suggest that $p27^{Kip1}$ is phosphorylated at Ser10 by Akt (Fujita et al., 2002; Liang et al., 2002), we further investigated whether the level of pSer10p27 was subjective to Akt activation. The level of pS473Akt1 in cisplatin-resistant cell lines was generally higher than in the parental cell lines (Fig. 6F,G). Inhibition of pS473Akt1 by MK2206 was accompanied by an evident increase of $p27^{Kip1}$/pSer10p27 in SKOV3-cDDP but not in A2780-cDDP (Fig. 6F,G). These results suggested that inhibition of pS473Akt1 by MK2206 could to some extent restore $p27^{Kip1}$ and pSer10p27 in certain cell lines.

Discussion

In this study, we first investigated the overall prognostic values of $p27^{Kip1}$ and pSer10p27 in ovarian cancer by meta-analysis and bioinformatic data analysis, then evaluated the expression pattern of $p27^{Kip1}$/pSer10p27 and subsequently assessed the correlation of $p27^{Kip1}$/pSer10p27 with different subcellular localizations to OS, PFS, and chemotherapy response of ovarian cancer patients through IHC staining using tissue microarrays. Finally, the association of $p27^{Kip1}$/pSer10p27 with cisplatin resistance was further validated in two cisplatin-resistant ovarian cancer cell lines. These results suggest that not only the total levels of $p27^{Kip1}$/pSer10p27 but also nuclear $p27^{Kip1}$ and cytoplasmic pSer10p27 may influence ovarian cancer progression and chemotherapy response.

$p27^{Kip1}$ was originally identified as a 27 kD non-tyrosine protein, which binds to various cyclin-CDK complexes in non-proliferating cells and leads to CDK inhibition and G1 arrest (Polyak et al., 1994; Toyoshima and Hunter, 1994). The canonical role of $p27^{Kip1}$ as a major cell cycle regulator had become even more conclusive when the evidence mounted and the protein-coding gene of $p27^{Kip1}$, *CDKN1B*, was, therefore, regarded as a representative putative tumor suppressor. Conformably, the tissue sample-based $p27^{Kip1}$ level in various types of cancer is concordantly correlated with patient prognosis (Porter et al., 1997; Chiarle et al., 2000). Earlier findings suggested that, in addition to the anti-tumor effect, $p27^{Kip1}$ was also involved in the regulation of embryonic stem cell differentiation (Li et al., 2012), cytokinesis (Serres et al., 2012), and actomyosin contractions (Godin et al., 2012). More recently, novel functions of $p27^{Kip1}$ were characterized,

Table 5. Multivariate Hazard Cox regression survival analysis.

	PFS			OS		
	HR	95% CI	*P*	HR	95% CI	*P*
Age (<50 vs. ≥50y)	1.350	0.699-2.609	0.372	0.916	0.455-1.845	0.806
Histology (high-grade serous vs. others)	0.339	0.151-0.760	0.009	0.280	0.124-0.634	0.002
FIGO stage (I-II vs. III-IV)	0.645	0.306-1.359	0.249	0.844	0.372-1.913	0.685
$p27^{Kip1}$ (Total) expression (low vs. high)	2.483	1.364-4.518	0.003	2.097	1.121-3.922	0.021
pSer10p27 (Cyt) expression (low vs. high)	0.488	0.261-0.910	0.024	0.472	0.248-0.898	0.022

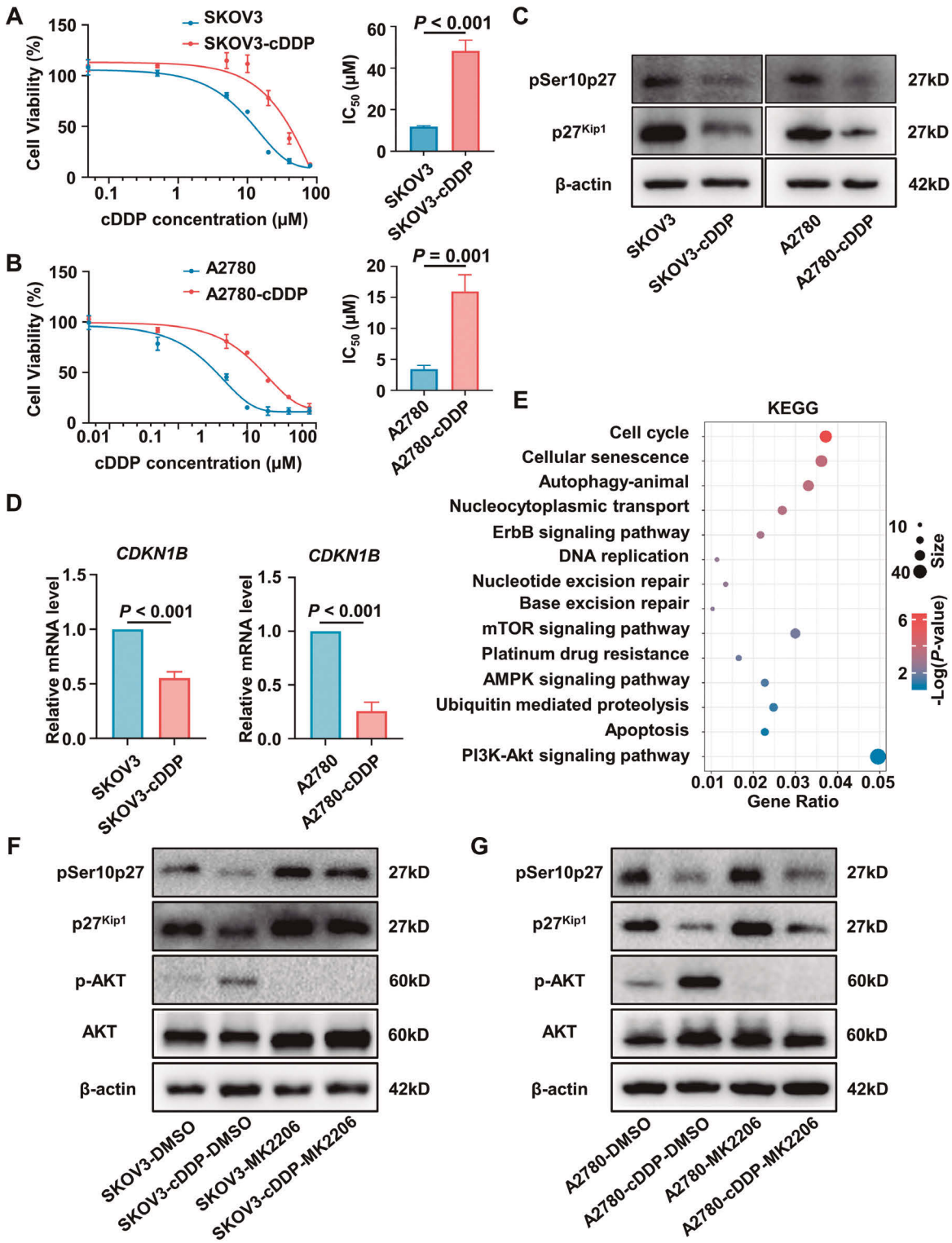


Fig. 6. p27Kip1 and pSer10p27 associated with cisplatin resistance in ovarian cancer cells. **A, B.** Cell viability analysis of parental and cisplatin-resistant cells under treatment with cisplatin at various concentrations for 72h. IC50 of the indicated cells under treatment with cisplatin. Bars represent mean ± SD (n=3). **C.** Western blot for phosphorylated Ser10 and total p27Kip1 in the two paired parental and cisplatin-resistant cell lines. β-actin served as a loading control (n=3). **D.** The mRNA levels of *CDKN1B* in parental and cisplatin-resistant cells. Bars represent mean ± SD (n=3). **E.** KEGG enrichment analysis of *CDKN1B*-related upregulated genes. Fourteen representative pathways are displayed. **F, G.** Western blot for phosphorylated and total p27Kip1 and phosphorylated and total Akt in the two paired parental and cisplatin-resistant cell lines after being treated with DMSO and 5 μM MK2206 for 24h. β-actin served as a loading control (n=3).

including regulation of the DNA damage response in P53-deficient cells (Cannell et al., 2015), transcriptional regulation, and control of autophagic vesicle trafficking (Yoon et al., 2019).

Since driver gene and tumor-suppressor mutation is one of the major theories of tumorigenesis, the mutational profile of *CDKN1B* is naturally studied. Multiple sets of data based on tissue specimens and cell lines all indicated that *CDKN1B* was rarely mutated in almost all tumor types (Kawamata et al., 1995; Shin et al., 2000), which suggested the importance of post-transcriptional modification on *CDKN1B*. The correlation between decreased p27^{Kip1} protein levels and poor prognosis of ovarian cancer patients was validated by our results, together with several previous studies (Shigemasa et al., 2001; Schmider-Ross et al., 2006; Lu et al., 2011; Felix et al., 2015). In previous studies, cytoplasmic p27^{Kip1} was observed but only nuclear p27^{Kip1} was evaluated in ovarian cancer cohorts possibly because only nuclear p27^{Kip1} had access to CDKs. However, several recent studies suggested that, in addition to nuclear p27^{Kip1}, cytoplasmic p27^{Kip1} also had a prognostic effect in certain types of cancer, such as nasopharyngeal carcinoma and osteosarcoma (Chen et al., 2020; Teng et al., 2020). According to our results, following previous studies, low nuclear p27^{Kip1} was associated with poor prognosis and a higher risk of chemotherapy resistance. Although lacking statistical significance, patients with low cytoplasmic p27^{Kip1} were apt to be chemotherapy-sensitive suggesting that a high nuclear and low cytoplasmic pattern might be most conducive to p27^{Kip1} exerting its anti-cancer effect.

Accumulating evidence reveals that cytoplasmic has distinct biological functions to nuclear p27^{Kip1} (Serres et al., 2011; Li et al., 2016; Calvayrac et al., 2019). The translocation of p27^{Kip1} from nucleus to cytoplasm could either result in degradation (Morishita et al., 2008), cytoplasmic sequestration (Kim et al., 2009), or even tumor promotion (Shin et al., 2002). The fate and function of cytoplasmic p27^{Kip1} are tightly linked to p27^{Kip1} phosphorylation. To date, around 10 sites of p27^{Kip1} phosphorylation have been identified. Ser10 phosphorylation of p27^{Kip1} represented a nuclear export signal, while Thr157 and Thr198 phosphorylation of p27^{Kip1} blocked its nuclear entry and held p27^{Kip1} for cytoplasmic retention (Lian et al., 2019). The dynamic process of nuclear-cytoplasmic p27^{Kip1} translocation driven by different phosphorylation statuses might present as various p27^{Kip1} subcellular expression patterns when evaluated cross-sectionally. Since pSer10p27 triggered p27^{Kip1} export and blocked it from CDK, we further analyzed the relationship between different subcellular locations of pSer10p27 and clinical characteristics (e.g., age, histological type, FIGO stage, tumor type, response to chemotherapy, and prognosis) in ovarian cancer patients. We found that patients with a high cytoplasmic pSer10p27 state had a higher risk of chemotherapy resistance and poor OS/PFS, which might suggest that cytoplasmic export of p27^{Kip1} diminished the anti-cancer effect of p27^{Kip1}.

Recently, a number of studies reported the involvement of p27^{Kip1} in cisplatin resistance (Huang et al., 2022; Li et al., 2022; Su et al., 2023). Cisplatin exerts an anti-cancer effect by forming cisplatin-DNA adducts. Cells damaged by cisplatin usually go through G1 arrest and then apoptosis. A recent study showed that cells relied on p27^{Kip1} for G1 arrest when P53 was deficient (La et al., 2023). As a P53 mutation occurred in nearly 90% of high-grade serous ovarian cancer patients, the level of p27^{Kip1} could be of notable clinical significance. Here, we found that p27^{Kip1} and pSer10p27 were significantly decreased in the cisplatin-resistant cell lines SKOV3-cDDP and A2780-cDDP. After inhibition of pS473Akt1 by MK2206, the levels of p27^{Kip1} and pSer10p27 were restored in SKOV3-cDDP but not in A2780-cDDP. This was possibly due to a natural

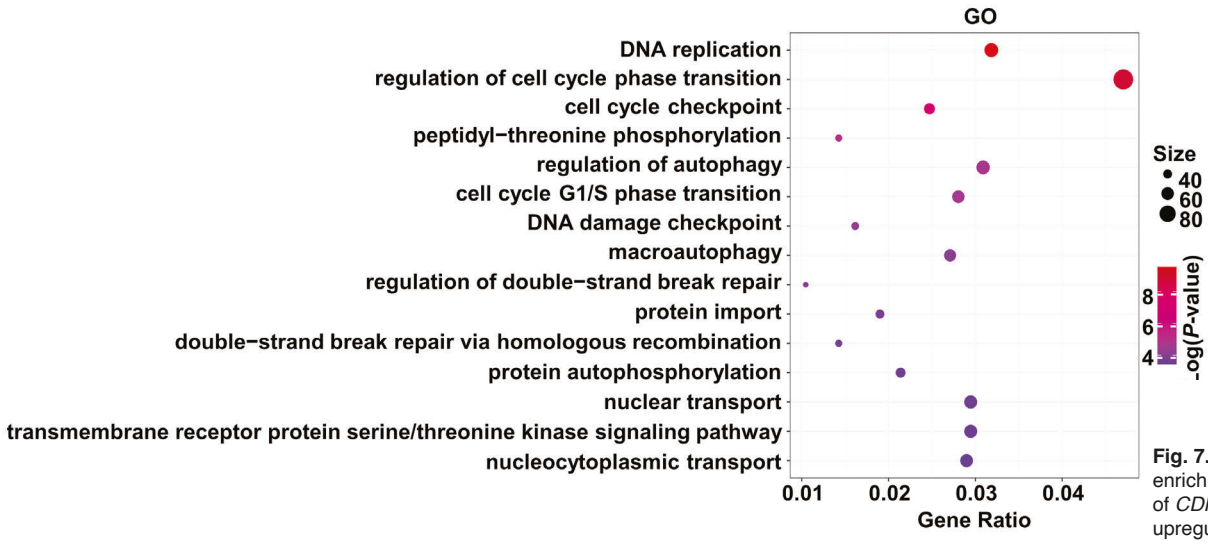


Fig. 7. GO enrichment analysis of *CDKN1B*-related upregulated genes.

deficiency of P53 in SKOV3 cells.

In summary, our study provides evidence that the different subcellular localizations of $p27^{Kip1}$ and pSer10p27 correlate with chemotherapy response and prognosis and can be used as potential biomarkers to assess chemotherapy response and prognosis in ovarian cancer.

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Ethics approval. This study was approved by the Independente Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (20210567).

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Conflict of Interest. The authors do not have any personal financial interests related to the subject matters discussed in this manuscript.

Competing Interests. The authors declare that they have no competing interests.

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