

Original Article

NCCRP1 promotes ovarian cancer progression and is associated with activation of the PI3K/AKT/mTOR signaling pathway and altered mitochondrial respiration

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Abstract: Non-specific cytotoxic cell receptor protein 1 (NCCRP1) is a membrane protein involved in cell proliferation, but its role in ovarian cancer (OC) progression remains unclear. In this study, a prognostic model for OC was constructed using LASSO-penalized Cox regression analysis. NCCRP1 expression in ovarian tissues was detected by immunohistochemical staining, and its associations with clinicopathological features and prognosis were analyzed. The effects and underlying mechanism of NCCRP1 on the malignant biological behaviors of OC cells were further investigated in vitro and in vivo. The prognostic model consisted of 28 genes with significant prognostic value. Among these genes, NCCRP1 mRNA showed differential expression in ovarian tissues and potential prognostic significance. NCCRP1 protein was overexpressed in OC tissues compared with adjacent ovarian tissues, and high NCCRP1 expression was associated with ascites, tumor cells in ascites, lymph node metastasis, distant metastasis and advanced FIGO stage. Patients with high NCCRP1 expression tended to have poorer overall survival. Knockdown of NCCRP1 inhibited the proliferation, migration and invasion of OC cells and promoted apoptosis, whereas NCCRP1 overexpression produced the opposite effects. In addition, NCCRP1 knockdown decreased oxygen consumption rate, whereas NCCRP1 overexpression produced the opposite effect. NCCRP1 expression was also positively associated with the phosphorylation levels of PI3K, AKT and mTOR. These findings suggest that NCCRP1 promotes malignant biological behaviors in OC, is associated with altered mitochondrial respiratory activity and may exert its tumor-promoting effects, at least in part, through activation of the PI3K/AKT/mTOR signaling pathway. Therefore, NCCRP1 may serve as a novel prognostic marker and potential therapeutic target for OC patients.

Keywords: NCCRP1, ovarian cancer, mitochondrial respiration, PI3K/AKT/mTOR

Introduction

Ovarian cancer (OC) remains a clinically challenging gynecological malignancy, and improved methods for tumor characterization and assessment of treatment response are still needed [1, 2]. Although targeted therapies for OC are continuously being developed, a significant proportion of patients still struggle to benefit from existing targeted therapies because of low response rates and drug resistance [3]. Therefore, the identification of novel biomarkers for early diagnosis and prognostic assess-

ment, as well as potential therapeutic targets, remains an important clinical need in OC.

Non-specific cytotoxic cell receptor protein 1 (NCCRP1) is an important cell surface receptor on non-specific cytotoxic cells (NCCs) in teleost fish [4]. NCCRP1 plays an important role in target-cell recognition and the activation of NCCs, and its expression is associated with NCC proliferation and migration following *Nocardia seriolae* infection [5]. Recent studies have further characterized NCCRP1 as a conserved F-box-associated protein involved in teleost immune

responses [6]. In human cancers, altered NCCRP1 expression and epigenetic regulation have been associated with tumor-cell proliferation and patient prognosis, particularly in pancreatic cancer and triple-negative breast cancer (TNBC) [7-9]. However, the role and mechanism of NCCRP1 in OC have not been reported.

In this study, the prognostic gene NCCRP1 was singled out by LASSO-penalized Cox regression analysis, and the link between NCCRP1 expression and OC clinical characteristics, as well as the possible role of NCCRP1 in OC patients' prognosis were investigated. The function and underlying mechanism of NCCRP1 in OC were further evaluated *in vitro* and *in vivo*. Our study identified a potential tumor-promoting role of NCCRP1 in OC, suggesting that NCCRP1 may serve as a promising prognostic biomarker and potential therapeutic target.

Materials and methods

Data download and processing

Gene expression data and clinicopathological information for patients with OC were obtained from The Cancer Genome Atlas (TCGA) through cBioPortal for Cancer Genomics (www.cbioportal.org).

Prognostic risk assessment model construction for OC

A total of 2,000 genes meeting the criteria ($\log_{2}FC > 2$, $P < 0.05$) were identified using R and subsequently subjected to LASSO-penalized Cox regression analysis [10, 11]. To assess overall survival (OS) in patients with OC, Cox proportional hazards regression analysis was performed. Twenty-eight genes were selected based on the optimal λ value. A prognostic risk score was then calculated based on the regression coefficient and expression level of each gene.

Tissue samples

The study analyzed 280 tissue samples obtained from 218 patients with ovarian cancer treated at Bayannur Hospital between 2015 and 2018, including 218 ovarian cancer tissues and 62 corresponding adjacent normal tissues. The 62 adjacent normal tissues were matched with ovarian cancer tissues obtained

from the same patients. Clinical information was collected from medical records, and no patients received chemotherapy or radiotherapy before tissue collection. This study was approved by the Medical Ethics Review Committee of Bayannur Hospital (approval no. BSYH2023037) and was conducted in accordance with the approved research protocol.

Immunohistochemistry (IHC)

Tissue sections underwent standard processing: baking (60°C, 15 min), xylene/ethanol dehydration, peroxide blocking, and antigen retrieval (citrate buffer, pH 6.0). Subsequently, the sections were incubated with the NCCRP1 polyclonal antibody (1:500 dilution, PA5-62693, ThermoFisher, USA), secondary antibody, and diaminobenzidine (DA1010, Solarbio, China). The expression of NCCRP1 in each cell was scored as 0 (negative), 1 (yellow), 2 (brown-yellow), or 3 (dark brown). The staining score = (3× percentage of dark brown cells + 2× percentage of brown-yellow cells + 1× percentage of yellow cells) ×100. The IHC staining score was divided into high NCCRP1 group and low or no NCCRP1 group, according to the cutoff value = 120.

Cell lines

Human ovarian cancer A2780 and SKOV3 cell lines and human cervical cancer HeLa cells were obtained from FuHeng Biology. A2780 and HeLa cells were genetically modified using an NCCRP1-targeting short hairpin RNA (shRNA) construct or the corresponding control construct. To establish stable NCCRP1 overexpression, SKOV3 cells were transduced with a lentiviral vector carrying human NCCRP1 cDNA under the control of the cytomegalovirus (CMV) promoter or with the corresponding empty lentiviral vector. All constructs were obtained from GeneCopoeia. NCCRP1 knockdown or overexpression was confirmed by western blotting before subsequent experiments.

Western blot

Protein was extracted from cells according to the manufacturer's instructions. Protein samples were subjected to gel electrophoresis and transferred onto membranes. After blocking with 5% skimmed milk, the membranes were

incubated overnight at 4°C with rabbit primary antibodies against NCCRP1, PI3K, p-PI3K, AKT, p-AKT, mTOR and p-mTOR (all diluted 1:2,000), and β -actin (diluted 1:6,000) (Wuhan Sanying Biotechnology Co., Ltd., Wuhan, China). Subsequently, the membranes were incubated with an HRP-conjugated goat anti-rabbit IgG secondary antibody (1:1,000; Wuhan Sanying Biotechnology Co., Ltd., Wuhan, China). The membranes were imaged using a ChemiDoc XRS+ system (Bio-Rad, USA). Band intensities were quantified using ImageJ software. Non-phosphorylated target proteins were normalized to β -actin, whereas phosphorylated proteins were normalized to their corresponding total proteins.

CCK-8 assay

Cells from each group were seeded into 96-well plates at a density of 2×10^3 cells/well. At the indicated time points, CCK-8 reagent (#CK04, DOJINDO, Japan) was added to each well according to the manufacturer's instructions, followed by incubation for 2 h at 37°C. The absorbance at 450 nm was measured using a microplate reader.

Colony formation assay

Cells from each group were seeded into 6-well plates at a density of 500 cells/well and cultured at 37°C in a humidified atmosphere containing 5% CO₂ until most colonies contained at least 50 cells. The colonies were then fixed with methanol for 30 min and stained with 0.5% crystal violet for 1 h.

Apoptosis detection

Cells from each group were resuspended in binding buffer and incubated with Annexin V/ Alexa Fluor 488 in the dark for 5 min. Propidium iodide (PI) was then added, and apoptosis was analyzed using a FACS Aria II SORP flow cytometer (BD, USA).

Transwell assay

Cell migration and invasion were evaluated using Transwell chambers in 24-well plates. For the migration assay, uncoated Transwell inserts were used, whereas for the invasion assay, the inserts were coated with Matrigel. Fresh medium containing 20% fetal bovine serum (FBS)

was added to the lower chambers, and cells from each group were seeded into the upper chambers. After incubation for 24 h, cells that had migrated or invaded through the membrane were fixed with methanol for 30 min and stained with 0.5% crystal violet for 1 h. The stained cells were photographed and analyzed.

Wound healing assay

Cells from each group were seeded into six-well plates and cultured until a confluent monolayer was formed. A linear scratch was generated using a sterile pipette tip, and detached cells were removed by washing with PBS. Images of the same wound areas were captured at 0 and 24 h using an inverted microscope. Wound closure was quantified using ImageJ software.

Chick chorioallantoic membrane (CAM) assay

After opening the eggshell, the shell membrane overlying the air chamber was carefully separated from the CAM using a sterile needle and removed with forceps. The window was then sealed with sterile tape. After 24 h, a region of the CAM containing relatively few blood vessels was selected, and a gelatin sponge containing the cellular supernatant from each group was placed on the CAM. The window was resealed with sterile tape, and vascular growth was observed and photographed 72 h later.

Subcutaneous xenograft model

A total of 12 female BALB/c nude mice (6 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The animal experiments were approved by the Medical Ethics Review Committee of Bayannur Hospital (approval no. BSYY-A2023019) and were conducted in accordance with the approved animal experimental protocol. Cells from each group (1×10^7 cells/mouse) were injected subcutaneously into the axillary region. Tumor dimensions were measured every 3 days, and tumor volume was calculated as $0.5 \times \text{largest diameter} \times (\text{smallest diameter})^2$. At the experimental endpoint, mice were euthanized by cervical dislocation performed by trained personnel in accordance with the approved animal experimental protocol. Death was confirmed by the absence of spontaneous breathing and

heartbeat before tumor tissues were harvested.

Measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR)

Cells were seeded in Seahorse XF microplates and incubated in assay medium containing 10 mmol/L glucose, 1 mmol/L sodium pyruvate and 2 mmol/L glutamine. OCR and ECAR were measured using a Seahorse XFe/XF96 analyzer with the XF Real-Time ATP Rate Assay. Basal OCR and ECAR were recorded for three measurement cycles. Oligomycin (1.5 μ M) was then injected, followed by three measurement cycles, and rotenone/antimycin A (0.5 μ M) was subsequently injected, followed by three additional measurement cycles. OCR and ECAR changes were recorded and analyzed using Seahorse software.

Statistical analysis

Statistical analyses were performed using SPSS 26.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism software. Continuous variables are presented as the mean $\pm$ standard deviation (SD). Count data are presented as number and percentage [n (%)]. Differences in continuous variables between two independent groups were analyzed using Student's t-test. Basal ECAR values between two independent groups were compared using a two-tailed unpaired Welch's t-test. NCCRP1 immunohistochemical staining scores were compared between 62 matched pairs of ovarian cancer and adjacent normal tissues using a paired two-tailed Student's t-test. Associations between NCCRP1 expression and clinicopathological characteristics were analyzed using Pearson's chi-square test. Comparisons among groups with repeated measurements over time, including cell proliferation curves and tumor growth curves, were analyzed using repeated measures ANOVA. OCR and ECAR curves were analyzed using two-way repeated-measures ANOVA with group and time as factors. Overall survival was evaluated using the Kaplan-Meier method, and differences between survival curves were compared using the log-rank test. Cox proportional hazards regression analysis was used to identify prognostic factors. A two-sided *P* value < 0.05 was considered statistically significant.

Results

Construction of prognostic risk assessment model for OC

To identify potential biomarkers for the diagnosis and prognosis of OC, we constructed a prognostic risk assessment model using LASSO-penalized Cox regression analysis. Based on the optimal value of log(λ) (-2.5), 28 genes associated with overall survival (OS) were identified (**Figure 1A**). The AUCs for 1-, 3-, and 5-year OS were 0.74, 0.75, and 0.81, respectively (**Figure 1B**). The Kaplan-Meier (KM) curves of the prognostic risk assessment model indicated that the survival rate of the high-risk group was lower than that of the low-risk group (**Figure 1C**). Multivariate Cox regression models were used to remove 4 genes (*P* $\geq$ 0.05) (**Figure 1D**). Kaplan-Meier analysis of the remaining 24 genes resulted in the exclusion of eight genes that were not significantly associated with OS (*P* $\geq$ 0.05). Through comparisons of the expression levels of 16 genes from The Cancer Genome Atlas (TCGA)-serous ovarian cancer database (*n* = 450), the study identified nine genes with higher expression in tumor tissues (**Figure 1E**). The NCCRP1 gene, with its differential expression and significant prognostic value (**Figure 1F**) was selected as the gene for further analysis.

NCCRP1 protein was overexpressed in OC tissues compared with adjacent tissues

NCCRP1 protein expression was detected by immunohistochemistry in 218 ovarian cancer tissues and 62 adjacent normal tissues. As shown in **Figure 2A** and **2B**, NCCRP1 protein was mainly localized in the cytoplasm. Among the 218 ovarian cancer tissues, 96 showed high NCCRP1 expression, whereas 10 of the 62 adjacent normal tissues showed high NCCRP1 expression (**Table 1**). In the subset of 62 matched tissue pairs, NCCRP1 immunohistochemical staining scores were significantly higher in ovarian cancer tissues than in the corresponding adjacent normal tissues (paired two-tailed Student's t-test, *P* < 0.001).

NCCRP1 expression was associated with the clinicopathologic features of OC patients

To elucidate the relationship between NCCRP1 expression and clinicopathological characte-

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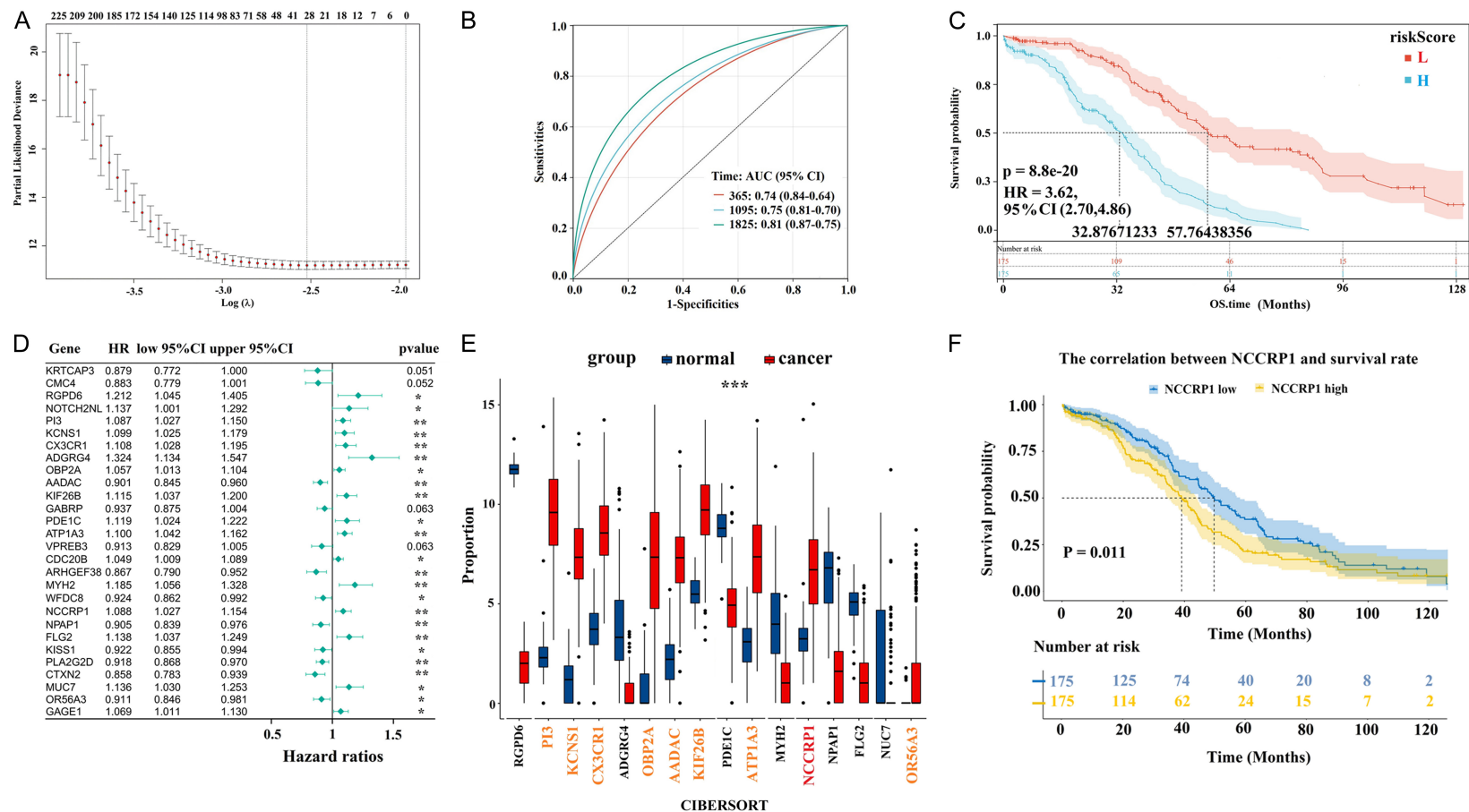


Figure 1. Construction of prognostic risk assessment model for OC. A. LASSO-penalized Cox regression analysis was performed to construct ovarian cancer prognostic-related gene signatures. B. Time-dependent receiver operating characteristic (ROC) curves for the risk score in ovarian cancer. C. The Kaplan-Meier (KM) curves for the high- and low-risk score groups of OC patients. D. The forest plot represents the hazard ratio and 95% confidence interval for the 28 prognostic-related genes. *P < 0.05; **P < 0.01. E. Box plots showed the mRNA expression levels of 16 prognostic genes in OC tissues and adjacent normal tissues. F. KM curves analysis was applied to evaluate the association of NCCRP1 expression with the overall survival (OS) of OC patients.

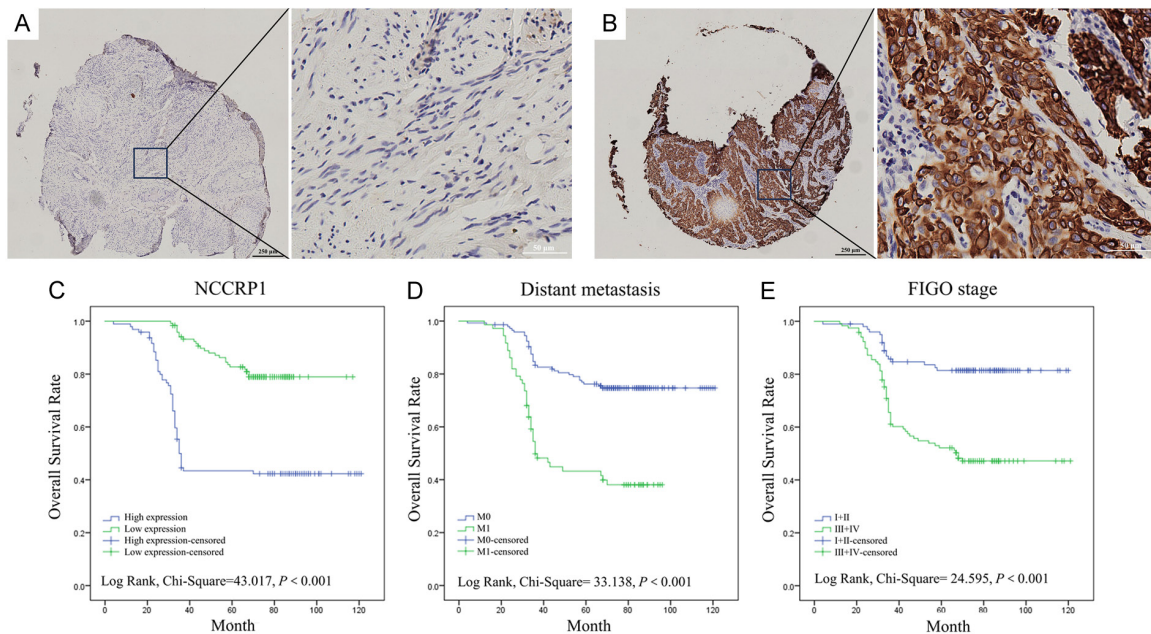


Figure 2. NCCRP1 protein in ovarian tissues and survival curves. A. Representative immunohistochemical images of NCCRP1 expression in normal ovarian tissues at low ($\times 40$; scale bar = 250 μm) and high ($\times 400$; scale bar = 50 μm) magnifications. B. Representative immunohistochemical images of NCCRP1 expression in OC tissues at low ($\times 40$; scale bar = 250 μm) and high ($\times 400$; scale bar = 50 μm) magnifications. C. Overall survival curves by NCCRP1 expression, low or negative (green) and high (blue). D. Overall survival curves by distant metastasis, M0 (blue) and M1 (green). E. Overall survival curves by FIGO stage, I + II (blue) and III + IV (green).

Table 1. NCCRP1 protein expression in ovarian tissues

Characteristics	n	Low or no, n (%)	High, n (%)
Carcinoma	218	122 (55.96)	96 (44.04)
Adjacent normal tissues	62	52 (83.87)	10 (16.13)

Data are presented as n (%).

istics, the medical data of 218 OC patients were analyzed. The results showed that NCCRP1 expression was significantly associated with ascites ($\chi^2 = 13.431$, $P < 0.001$), tumor cells in ascites ($\chi^2 = 7.577$, $P = 0.006$), lymph node metastasis ($\chi^2 = 30.990$, $P < 0.001$), distant metastasis ($\chi^2 = 13.451$, $P < 0.001$) and International Federation of Gynecology and Obstetrics (FIGO) stage ($\chi^2 = 45.756$, $P < 0.001$) (Table 2). However, NCCRP1 expression was not significantly associated with age, differentiation or menopausal status ($P > 0.05$).

NCCRP1 expression is associated with the prognosis of patients with OC

To evaluate the prognostic value of NCCRP1 in OC, univariate and multivariate regression analyses were performed. In univariate analysis, NCCRP1 expression (HR, 1.115; 95% CI, 1.045-1.190; $P < 0.001$), menopausal status (HR,

1.713; 95% CI, 1.022-2.213; $P = 0.045$), differentiation (HR, 2.237; 95% CI, 1.167-3.243; $P = 0.008$), tumor cells in ascites (HR, 1.822; 95% CI, 1.114-2.848; $P = 0.031$), distant metastasis (HR, 3.013; 95% CI, 1.714-4.805; $P < 0.001$) and FIGO stage (HR, 2.435; 95% CI, 1.502-3.623; $P = 0.001$) were associated with the prognosis of OC patients (Table 3). In multivariate regression analysis, NCCRP1 expression (HR, 1.112; 95% CI, 1.039-1.187; $P = 0.002$), distant metastasis (HR, 2.116; 95% CI, 1.932-3.517; $P = 0.006$), and FIGO stage (HR, 1.763; 95% CI, 1.532-2.845; $P = 0.002$) were confirmed as independent prognostic factors (Table 3). Kaplan-Meier survival analysis further showed that high NCCRP1 expression, distant metastasis, and advanced FIGO stage were associated with poorer OS (Figure 2C-E).

Knockdown of NCCRP1 inhibited the malignant biological properties of OC cells

NCCRP1 expression was knocked down to investigate its effects on the malignant biological properties of OC cells. Western blotting and

Table 2. NCCRP1 protein expression and OC patient clinicopathological characteristics

Characteristics	n	NCCRP1		χ^2	P value
		Low or no, n (%)	High, n (%)		
Total	218	122	96		
Age				0.189	0.664
< 50	60	35 (58.33)	25 (41.67)		
≥ 50	158	87 (55.06)	71 (44.94)		
Differentiation				0.988	0.610
Well	15	9 (60.00)	6 (40.00)		
Moderate	61	37 (60.66)	24 (39.34)		
Poor	142	76 (53.52)	66 (46.48)		
Menopausal status				0.036	0.850
No	81	46 (56.79)	35 (43.21)		
Yes	137	76 (55.47)	61 (44.53)		
Ascites				13.431	< 0.001*
No	132	87 (65.91)	45 (34.09)		
Yes	86	35 (40.70)	51 (59.30)		
Tumor cells in ascites				7.577	0.006*
No	167	102 (61.08)	65 (38.92)		
Yes	51	20 (39.22)	31 (60.78)		
Lymph node metastasis				30.990	< 0.001*
No	177	115 (64.97)	62 (35.03)		
Yes	41	7 (17.07)	34 (82.93)		
Distant metastasis				13.451	< 0.001*
No	110	75 (68.18)	35 (31.82)		
Yes	108	47 (43.52)	61 (56.48)		
FIGO stage				45.756	< 0.001*
I	93	76 (81.72)	17 (18.28)		
II	26	12 (46.15)	14 (53.85)		
III	89	32 (35.96)	57 (64.04)		
IV	10	2 (20.00)	8 (80.00)		

Data are presented as n (%). Associations between NCCRP1 expression and clinicopathological characteristics were analyzed using Pearson's chi-square test. *P < 0.05.

densitometric analysis confirmed that NCCRP1 protein expression was significantly reduced in shNCCRP1 cells compared with shNC cells (**Figure 3A**). The CCK-8 assay and colony formation assay confirmed that NCCRP1 knockdown inhibited the proliferation of OC cells (**Figure 3B, 3C**). The flow cytometry analysis showed that NCCRP1 inhibition promoted the apoptosis of OC cells (**Figure 3D**). Transwell assays showed that NCCRP1 knockdown inhibited cell migration and invasion (**Figure 3E, 3F**). The CAM assay demonstrated that NCCRP1 silencing decreased the angiogenesis induced by OC cells (**Figure 3G**). The in vivo experiment showed that NCCRP1 knockdown significantly suppressed tumor growth (**Figure 3H-J**).

Overexpression of NCCRP1 promoted the malignant biological properties of OC cells

Stable NCCRP1-overexpressing SKOV3 cells were established by lentiviral transduction. Western blotting and densitometric analysis confirmed that NCCRP1 protein expression was significantly increased in ovNCCRP1 cells compared with ovNC cells (**Figure 4A**). The CCK-8 assay and colony formation assay confirmed that NCCRP1 overexpression promoted the proliferation of OC cells (**Figure 4B, 4C**). The flow cytometry analysis showed that NCCRP1 upregulation inhibited the apoptosis of OC cells (**Figure 4D**). Transwell assays showed that NCCRP1 overexpression promoted cell migra-

Table 3. Univariate and multivariate analyses of the prognostic factors for overall survival in OC

	Univariate analysis			Multivariate analysis		
	HR	P value	95% CI	HR	P value	95% CI
NCCRP1 expression						
High vs. Low or no	1.115	< 0.001*	1.045-1.190	1.112	0.002*	1.039-1.187
Age (year)						
< 60 vs. ≥ 60	1.457	0.064	0.890-2.357			
Menopausal status						
Yes vs. No	1.713	0.045*	1.022-2.213			
Differentiation						
Well vs. Moderate vs. Poor	2.237	0.008*	1.167-3.243			
Ascites						
Yes vs. No	1.485	0.162	0.768-2.307			
Tumor cells in ascites						
Yes vs. No	1.822	0.031*	1.114-2.848			
Lymph node metastasis						
Yes vs. No	1.421	0.205	0.745-2.436			
Distant metastasis						
M1 vs. M0	3.013	< 0.001*	1.714-4.805	2.116	0.006*	1.932-3.517
FIGO stage						
III + IV vs. I + II	2.435	0.001*	1.502-3.623	1.763	0.002*	1.532-2.845

*P < 0.05. HR: Hazard ratio; CI: Confidence interval.

tion and invasion (**Figure 4E, 4F**). The CAM assay demonstrated that NCCRP1 upregulation facilitated the angiogenesis induced by OC cells (**Figure 4G**).

Effects of NCCRP1 expression on OCR and ECAR and activation of the PI3K/AKT/mTOR pathway

To investigate whether NCCRP1 expression was associated with mitochondrial respiratory activity in OC cells, OCR was measured in cells with NCCRP1 knockdown or overexpression. NCCRP1 downregulation decreased OCR, whereas NCCRP1 overexpression increased OCR (**Figure 5A, 5B**). Two-way repeated-measures ANOVA showed significant overall differences in OCR between the shNC and shNCCRP1 groups ($P = 0.0121$) and between the ovNC and ovNCCRP1 groups ($P = 0.0318$). Complete ECAR profiles were also evaluated during the basal phase and following oligomycin and rotenone/antimycin A injection. No significant overall differences in ECAR were observed between the shNC and shNCCRP1 groups or between the ovNC and ovNCCRP1 groups (**Supplementary Figure 1A, 1B**). Quantification of basal ECAR also showed no significant differences between

the corresponding groups (**Supplementary Figure 1C, 1D**). Quantification of basal respiration further showed that NCCRP1 knockdown decreased basal respiration, whereas NCCRP1 overexpression increased basal respiration (**Supplementary Figure 2A, 2B**). These findings suggest that NCCRP1 expression is associated with changes in cellular respiratory activity. Subsequently, NCCRP1 expression was found to be positively associated with the phosphorylation levels of PI3K, AKT and mTOR (**Figure 5C**). Densitometric analyses further confirmed these changes (**Supplementary Figure 3A, 3B**). Finally, treatment with the PI3K inhibitor LY294002 attenuated the NCCRP1 overexpression-induced increases in cell proliferation and migration (**Figure 5D, 5E**).

NCCRP1 knockdown did not significantly affect the biological behaviors of HeLa cells

To further evaluate the effects of NCCRP1 knockdown in a non-ovarian cancer cell line, NCCRP1 was knocked down in HeLa cells. Western blotting confirmed reduced NCCRP1 protein expression in shNCCRP1 cells compared with shNC cells (**Figure 6A**). However, NCCRP1 knockdown did not significantly affect

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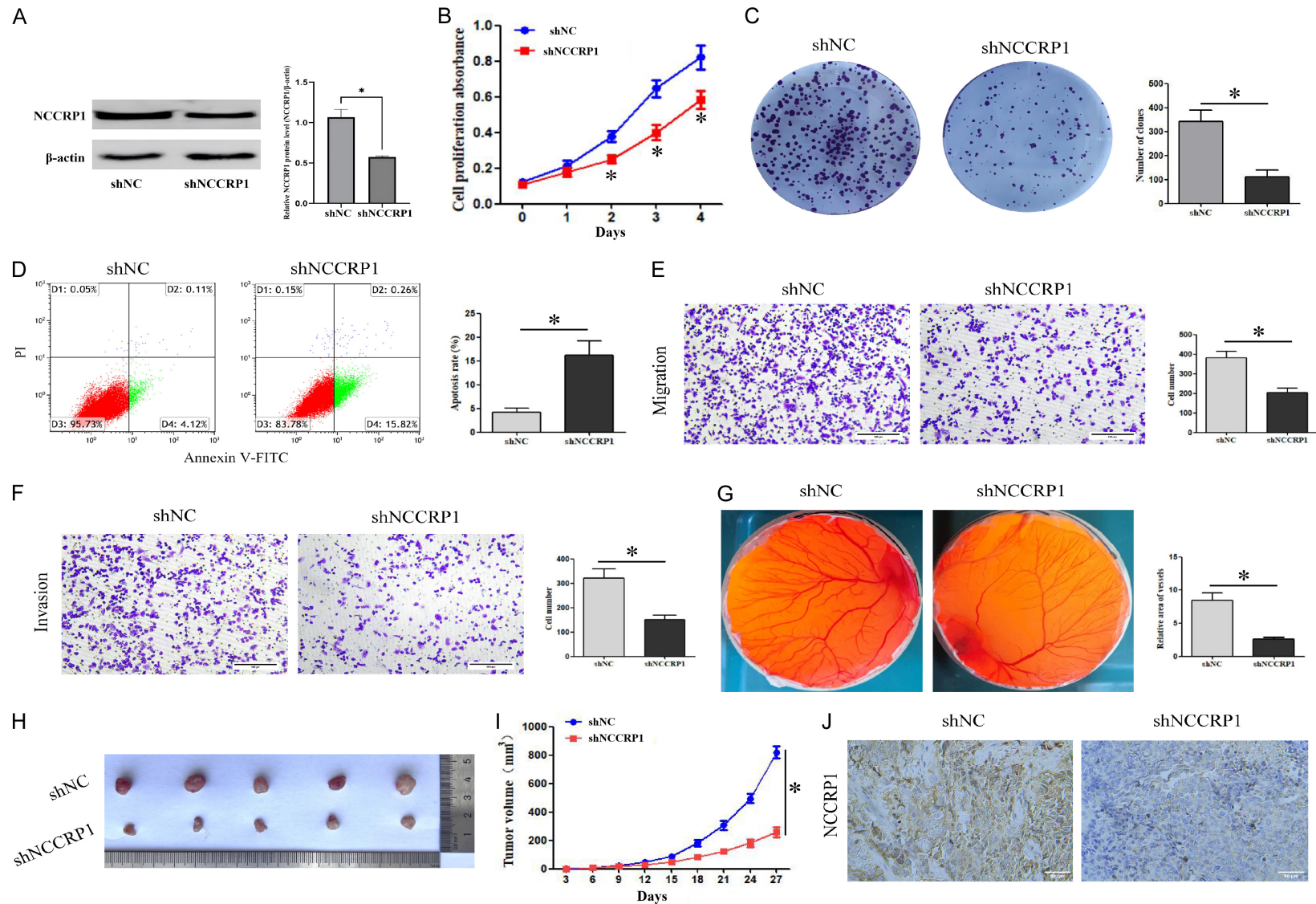


Figure 3. Influence of NCCRP1 knockdown on OC cells. (A) NCCRP1 knockdown was verified by western blotting. Relative NCCRP1 protein expression was quantified by densitometric analysis and normalized to β -actin. Data are presented as the mean $\pm$ SD from three independent experiments. Statistical significance was assessed using Student's t-test. *P < 0.05 versus shNC. (B) Cell proliferation after NCCRP1 knockdown was assessed using the CCK-8 assay. (C) Colony-forming ability was evaluated using a colony formation assay. (D) Cell apoptosis after NCCRP1 knockdown was analyzed by flow cytometry. (E) Cell migration after NCCRP1

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knockdown was evaluated using a Transwell assay. (F) Cell invasion after NCCRP1 knockdown was evaluated using a Transwell assay. Images in (E) and (F) were captured at an original magnification of $\times 200$; scale bar = 100 μm . (G) Angiogenesis induced by OC cells after NCCRP1 knockdown was evaluated using the chick chorioallantoic membrane assay. (H) Representative images of excised xenograft tumors. (I) Tumor growth curves in the xenograft mouse model. (J) NCCRP1 expression in subcutaneous tumors was detected by immunohistochemistry. Images in (J) were captured at an original magnification of $\times 400$; scale bar = 50 μm . * $P < 0.05$ versus shNC.

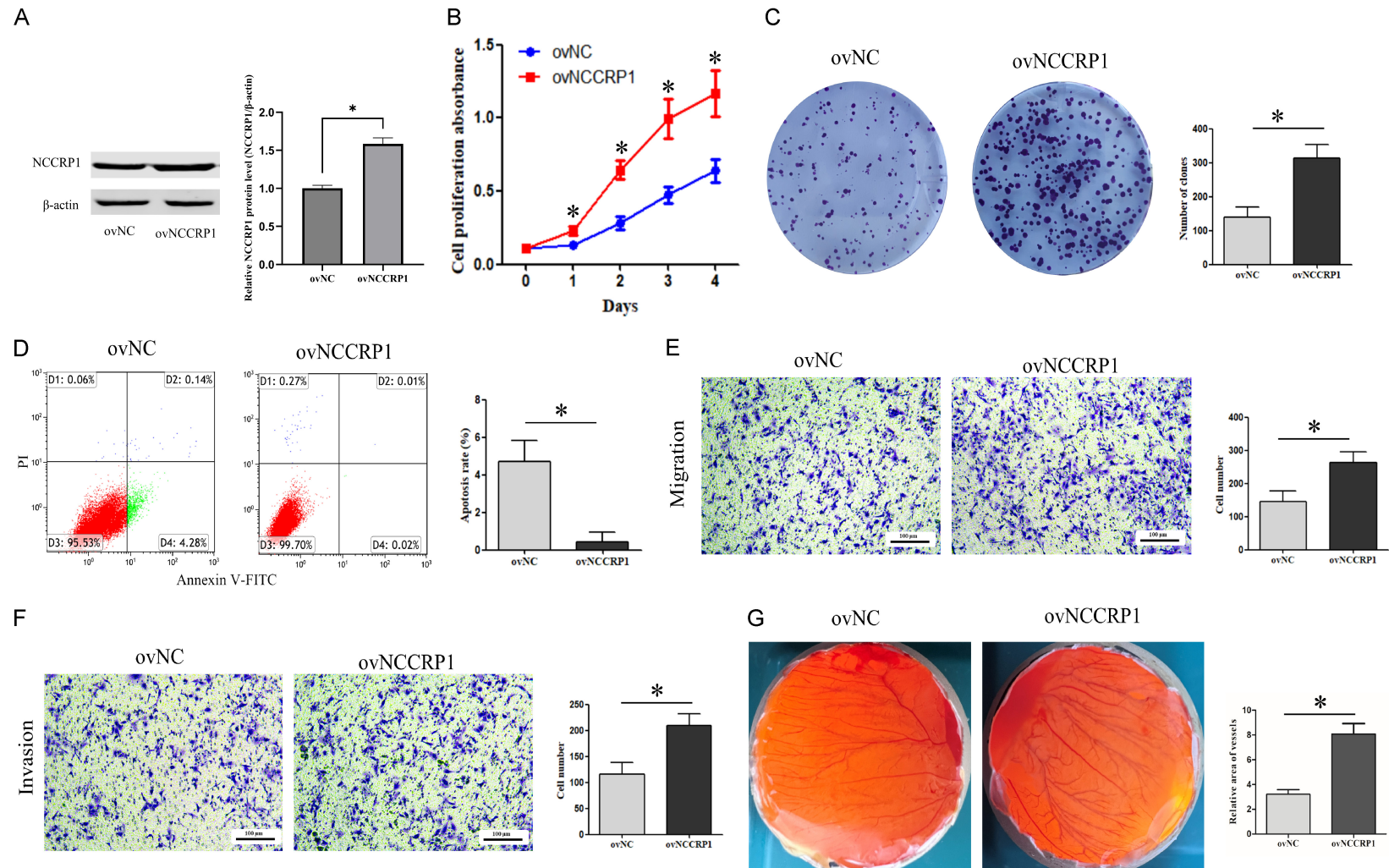


Figure 4. Influence of NCCRP1 upregulation on OC cells. (A) Stable NCCRP1 overexpression in SKOV3 cells following lentiviral transduction was verified by western blotting. Relative NCCRP1 protein expression was quantified by densitometric analysis and normalized to β -actin. Data are presented as the mean $\pm$ SD from three

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independent experiments. Statistical significance was assessed using Student's t-test. * $P < 0.05$ versus ovNC. (B) Cell proliferation was assessed using the CCK-8 assay. (C) Colony-forming ability was evaluated using a colony formation assay. (D) Cell apoptosis was analyzed by flow cytometry. (E) Cell migration was evaluated using a Transwell assay. Images in (E) and (F) were captured at an original magnification of $\times 200$; scale bar = 100 μm . (G) Angiogenic activity induced by ovarian cancer cells was assessed using the chick chorioallantoic membrane assay. Data are presented as the mean $\pm$ SD. Cell proliferation curves were analyzed using repeated-measures ANOVA. * $P < 0.05$ versus ovNC.

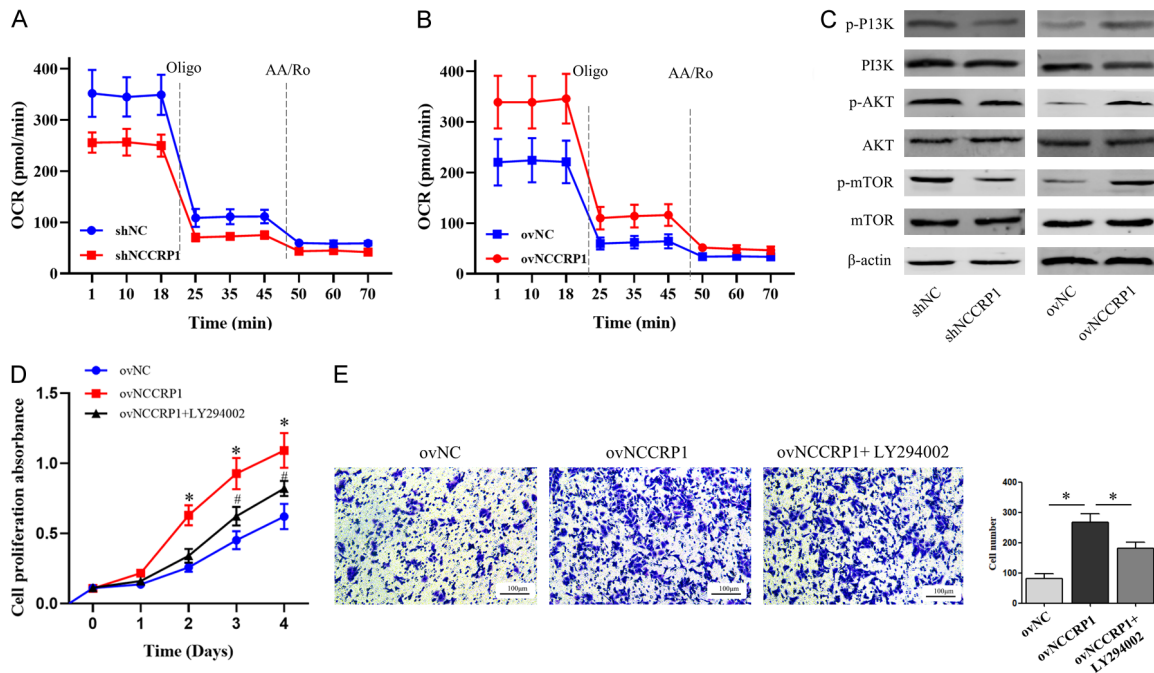


Figure 5. Mechanism of NCCRP1 in OC progression. (A) OCR was measured in OC cells after NCCRP1 knockdown. (B) OCR was measured in OC cells after NCCRP1 overexpression. Two-way repeated-measures ANOVA showed significant differences between shNC and shNCCRP1 cells ($P = 0.0121$) and between ovNC and ovNCCRP1 cells ($P = 0.0318$). Complete ECAR profiles and quantification of basal ECAR are shown in [Supplementary Figure 1A-D](#). Quantification of basal respiration is shown in [Supplementary Figure 2A, 2B](#). (C) The phosphorylation levels of PI3K, AKT and mTOR were assessed by western blotting. Densitometric quantification of the Western blots is shown in [Supplementary Figure 3A, 3B](#). (D) Cell proliferation after LY294002 treatment was detected by CCK-8 assay. * $P < 0.05$ versus ovNC; # $P < 0.05$ versus ovNCCRP1. (E) Cell migration after LY294002 treatment was detected by Transwell assay. Images in (E) were captured at an original magnification of $\times 200$; scale bar = 100 μm . Oligo, oligomycin; AA/Ro, antimycin A plus rotenone.

HeLa cell proliferation as assessed by the CCK-8 assay (**Figure 6B**). Consistently, no significant differences in colony-forming ability or cell migration were observed between the shNC and shNCCRP1 groups, as determined by colony formation and wound healing assays, respectively (**Figure 6C, 6D**).

Discussion

In this study, we first constructed a prognostic risk assessment model using LASSO-penalized Cox regression analysis. The model consisted of 28 genes and showed significant prognostic value for patient survival. Furthermore,

we found that NCCRP1 mRNA is highly expressed in OC tissues and might be an independent prognostic factor for OC patients. Zhou et al. [9] found elevated NCCRP1 expression in TNBC tissues, whereas Nakamura et al. [7] reported frequent loss of NCCRP1 expression associated with promoter hypermethylation in pancreatic cancer tissues. IHC analysis further confirmed that NCCRP1 protein expression was higher in OC tissues than in adjacent normal tissues. These findings support the potential value of NCCRP1 as a biomarker for OC.

To further investigate the clinical value of NCCRP1 in OC patients, this study analyzed the

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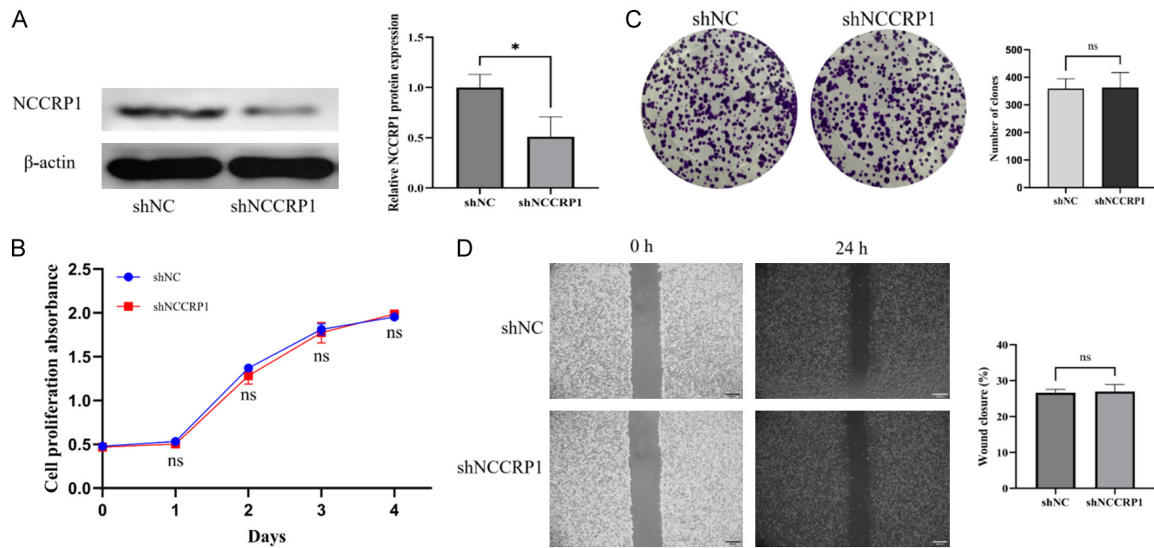


Figure 6. Effects of NCCRP1 knockdown in HeLa cells. A. NCCRP1 knockdown in HeLa cells was verified by western blotting, and relative NCCRP1 protein expression was quantified by densitometric analysis and normalized to β -actin. B. Cell proliferation after NCCRP1 knockdown was assessed using the CCK-8 assay. C. Colony-forming ability was evaluated using a colony formation assay. D. Cell migration was evaluated using a wound healing assay. Representative images were captured at 0 and 24 h at an original magnification of $\times 40$; scale bar = 250 μ m. Data are presented as the mean $\pm$ SD. Cell proliferation curves were analyzed using repeated-measures ANOVA. * $P < 0.05$; ns, not significant.

relationship between NCCRP1 expression and the clinicopathologic features of 218 OC patients. High NCCRP1 expression was significantly associated with ascites, tumor cells in ascites, lymph node metastasis, distant metastasis and advanced FIGO stage. Previous literature has not reached a consensus on the prognostic value of NCCRP1. In pancreatic cancer, NCCRP1 promoter hypermethylation was associated with poor prognosis [7]. In TNBC, NCCRP1 was included in a recent prognostic model [8], and high NCCRP1 expression was related to poor prognosis [9]. In addition, Wu et al. [12] constructed an N6-methyladenosine (m6A)-related risk signature including 12 genes for survival prediction in melanoma, and NCCRP1 was one of them. NCCRP1 was among the differentially expressed genes that were associated with the prognosis of patients with pancreatic cancer [13]. Our results based on univariate and multivariate regression analyses confirmed that NCCRP1 is an independent risk factor for OC patients, and OC patients with high NCCRP1 expression tended to have poor OS. These results suggest that NCCRP1 may promote OC progression and predict poor prognosis.

It has been reported that NCCRP1 is involved in cell proliferation regulation. Silencing NCCRP1

inhibited the proliferation of MDA-MB-231 cells, whereas NCCRP1 overexpression produced the opposite effect [9]. In this study, NCCRP1 knockdown significantly decreased the proliferation, migration and invasion of OC cells and increased cell apoptosis. Conversely, NCCRP1 overexpression promoted cell proliferation, migration and invasion while reducing apoptosis. Interestingly, NCCRP1 not only directly regulated the functions of OC cells but also affected the tumor microenvironment by promoting vascularization. Together, these findings support a tumor-promoting role for NCCRP1 in OC.

Alterations in cellular energy metabolism have been associated with tumor progression and can contribute to tumor cell proliferation, survival, invasion and metastasis [14-20]. In the present study, NCCRP1 knockdown decreased OCR and basal respiration, whereas NCCRP1 overexpression produced the opposite effects. In contrast, no significant overall differences in ECAR were observed in either the NCCRP1 knockdown or overexpression experiments. These findings suggest that NCCRP1 expression is associated with altered mitochondrial respiratory activity in OC cells. However, these findings should not be interpreted as direct evidence that NCCRP1 induces comprehensive

metabolic reprogramming or a metabolic shift between oxidative phosphorylation and glycolysis. Further studies using more comprehensive metabolic flux analyses are warranted to clarify the role of NCCRP1 in OC metabolism.

The PI3K/AKT/mTOR signaling pathway plays an important role in tumor progression and metabolic regulation [21]. Previous studies have also linked activation of this pathway to altered metabolic phenotypes in different cancers, including high-grade serous ovarian cancer [22, 23]. In the present study, NCCRP1 expression was positively associated with the phosphorylation levels of PI3K, AKT and mTOR. Furthermore, treatment with the PI3K inhibitor LY294002 attenuated the NCCRP1 overexpression-induced increases in cell proliferation and migration. These findings suggest that NCCRP1 may promote OC progression, at least in part, through activation of the PI3K/AKT/mTOR signaling pathway. Together with the observed changes in OCR, our results also suggest an association between NCCRP1 expression and mitochondrial respiratory activity. However, whether PI3K/AKT/mTOR pathway activation directly mediates the NCCRP1-associated changes in mitochondrial respiration remains to be determined.

Conclusions

NCCRP1 is overexpressed in OC tissues, and high NCCRP1 expression is associated with poor prognosis in OC patients. NCCRP1 promotes malignant biological properties and is associated with activation of the PI3K/AKT/mTOR signaling pathway and changes in mitochondrial respiratory activity. Thus, NCCRP1 may serve as a novel prognostic marker and potential therapeutic target for OC patients.

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Disclosure of conflict of interest

None.

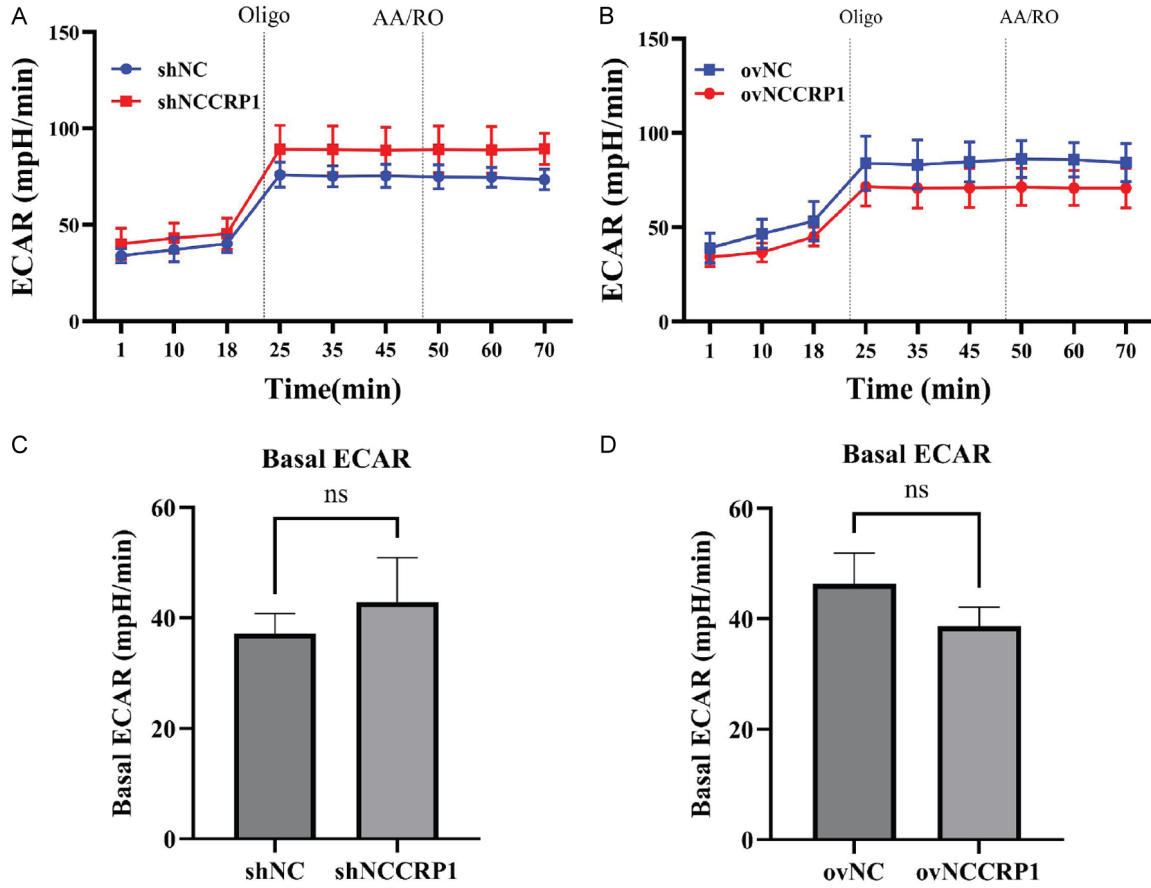
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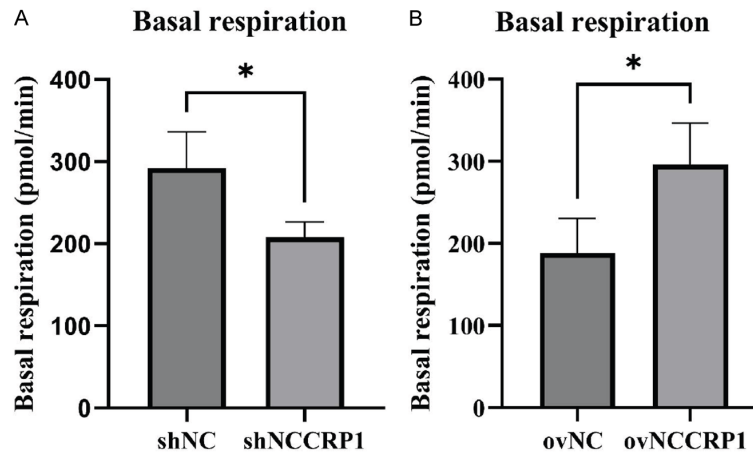
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NCCRP1 promotes ovarian cancer

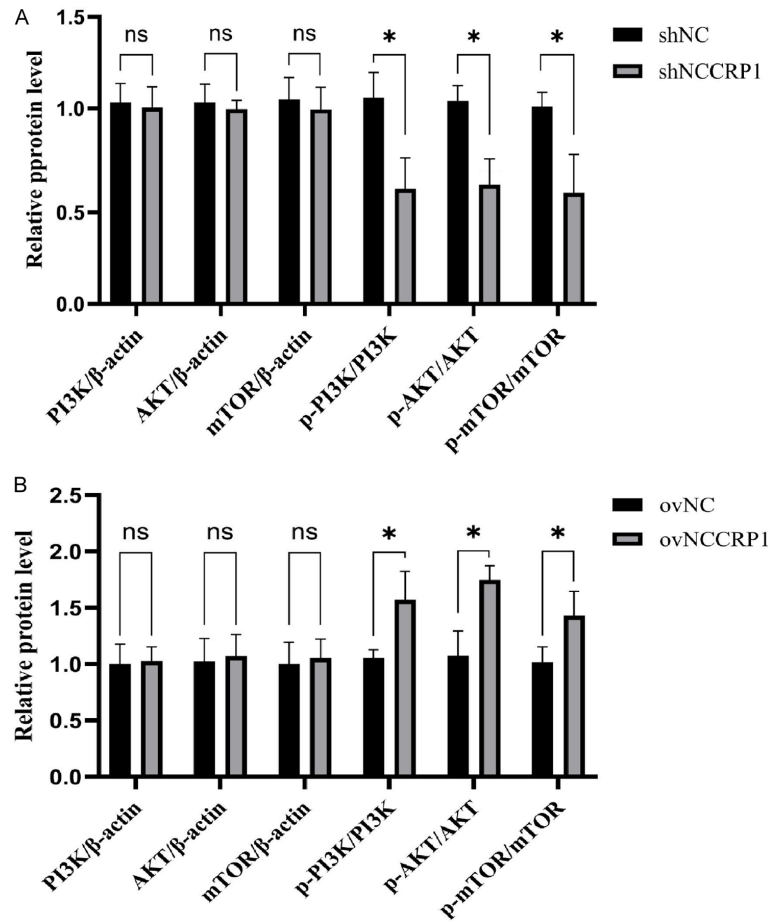


Supplementary Figure 1. ECAR profiles and quantification of basal ECAR in ovarian cancer cells. (A, B) ECAR profiles of shNC and shNCCRP1 cells (A), and ovNC and ovNCCRP1 cells (B), during the basal phase and following sequential injection of oligomycin (Oligo) and antimycin A plus rotenone (AA/RO). (C, D) Quantification of basal ECAR in shNC and shNCCRP1 cells (C), and ovNC and ovNCCRP1 cells (D). Basal ECAR was calculated as the mean of the three measurements obtained before oligomycin injection. Data are presented as the mean $\pm$ SD ($n = 3$). Statistical significance was assessed using a two-tailed unpaired Welch's t-test. ns, not significant.



Supplementary Figure 2. Quantification of basal respiration in ovarian cancer cells. A. Basal respiration in shNC and shNCCRP1 cells, corresponding to Figure 5A. B. Basal respiration in ovNC and ovNCCRP1 cells, corresponding to Figure 5B. Data are presented as the mean $\pm$ SD ($n = 3$). Statistical significance was assessed using Student's t-test. * $P < 0.05$.

NCCRP1 promotes ovarian cancer



Supplementary Figure 3. Densitometric quantification of PI3K/AKT/mTOR pathway proteins. A. Quantification of total and phosphorylated PI3K, AKT and mTOR in shNC and shNCCRP1 cells. B. Quantification of total and phosphorylated PI3K, AKT and mTOR in ovNC and ovNCCRP1 cells. Total proteins were normalized to β -actin, and phosphorylated proteins were normalized to their corresponding total proteins. Data are presented as the mean $\pm$ SD (n = 3). Statistical significance was assessed using Student's t-test. *P < 0.05; ns, not significant.