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The up-regulation of PAK2 indicates unfavorable prognosis in patients with serous epithelial ovarian cancer and contributes to paclitaxel resistance in ovarian cancer cells

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Abstract

Background The main challenge in treating ovarian cancer is chemotherapy resistance. Previous studies have shown that PAK2 is highly expressed in various cancers. This research investigates whether increased PAK2 expression contributes to chemo-resistance and poor prognosis in ovarian cancer.

Methods Initially, bioinformatics analysis was used to assess the importance of PAK2 mRNA up-regulation in ovarian cancer. This was then validated using tissue microarray to confirm PAK2 protein expression and localization in clinical samples. Univariate and multivariate logistic regression analyses were carried out to identify potential risk factors for chemo-resistance in serous epithelial ovarian cancer (EOC), while multivariate Cox regression and Kaplan-Meier analysis were conducted to ascertain prognostic factors for overall survival (OS) and disease-free survival (DFS) in patients with serous EOC. In vitro experiments were conducted to verify if inhibiting PAK2 expression could increase A2780/Taxol cells' sensitivity to paclitaxel, as shown by evaluating cell proliferation, apoptosis, transwell, and clone formation. Additionally, the interaction between PAK2, lnc-SNHG1, and miR-216b-5p was verified using RIP and luciferase reporter assays. Rescue experiments were undertaken to examine the influence of the lnc-SNHG1/miR-216b-5p/PAK2 axis on the development of paclitaxel resistance in A2780/Taxol cells.

Results The bioinformatics analysis indicated a notable increase in PAK2 expression in ovarian malignant tumors compared to adjacent tissues, particularly in patients with stage III-IV disease compared to those with stage I-II disease ($P=0.0056$). Elevated levels of PAK2 were linked to reduced OS in ovarian cancer patients, although no significant association was observed with DFS. Immunohistochemistry findings further supported these results, showing positive PAK2 protein expression in chemo-resistant serous EOC tissues, predominantly localized in the cytoplasm, which correlated with poorer OS and DFS outcomes. In vitro experiments demonstrated that the downregulation of PAK2 in A2780/Taxol cells led to a reduction in colony formation, an increase in apoptosis, and a diminished capacity for cell invasion. Subsequent analysis confirmed that lnc-SNHG1 functions as a competitive endogenous RNA (ceRNA)

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by interacting with miR-216b-5p and regulating PAK2 expression. Rescue experiments demonstrated that lnc-SNHG1 induces resistance to paclitaxel in A2780/Taxol cells by modulating the miR-216b-5p/PAK2 axis.

Conclusions PAK2 shows promise as a predictor of chemotherapy resistance and poor outcomes in ovarian cancer, indicating its potential use as a treatment target to overcome this resistance.

Keywords Serous epithelial ovarian cancer, Chemo-resistance, PAK2, Prognosis, ceRNA

Introduction

Ovarian cancer is ranked as the seventh most common type of cancer and is the primary cause of mortality among gynecological cancers in women globally [1]. Projections derived from cancer data suggest that there will be an approximate 19,710 new instances of ovarian cancer and 13,270 fatalities attributed to ovarian cancer in the United States by 2023 [2]. Around 70% of ovarian cancer cases are diagnosed in advanced stages, underscoring the significance of utilizing platinum and paclitaxel chemotherapy following cytoreductive surgery as the principal therapeutic strategy. A notable obstacle in enhancing survival rates for ovarian cancer is the prevalence of chemotherapy resistance, affecting at least 70% of patients. This resistance has presented a substantial obstacle, leading to a 5-year survival rate of merely 47% in developed countries [3]. Therefore, addressing this pressing issue is of paramount importance in the clinical care of individuals with ovarian cancer.

P21-activated kinases (PAKs) are a conserved group of serine/threonine kinases that can be activated by upstream signals, such as Rac and Cdc42 from the Rho family of small G proteins. The activation of PAKs by these signals plays a significant role in regulating multiple signaling pathways and cellular functions. Aberrant expression of PAKs has been implicated in tumorigenesis, impacting processes such as cytoskeletal remodeling, cell motility, proliferation, differentiation, apoptosis, mitosis and angiogenesis [4–6]. PAK kinases have been implicated in the pathogenesis of tumors, thus prompting exploration into targeted therapies aimed at inhibiting PAK activity.

The identification of six isoforms of PAK kinases (PAK1-6) in mammals has allowed for their categorization into two distinct groups: Group I (PAK1-3) and Group II (PAK4-6) [7]. PAK1 has been extensively researched in various cancers, such as breast cancer, colorectal cancer, and ovarian cancer [8–11]. In contrast, PAK2 exhibits a dual function, regulating both cell survival and cell death pathways based on the cellular environment. Activation of the full-length PAK2 gene facilitates cell survival by phosphorylating Bad, thereby disrupting its interaction with Bcl-2 or Bcl-x(L) and promoting its association with 14-3-3tau, ultimately enhancing cell survival [12]. Nevertheless, the proteolytic activation of PAK-2p34 results in apoptosis [13].

The preceding research demonstrated a notable correlation between heightened PAK1 staining and adverse overall survival results in ovarian cancer patients with disease recurrence post-chemotherapy treatment [8]. Conversely, there is limited investigation on PAK2 in the context of ovarian cancer. Siu MK et al. observed reduced migration and invasion of ovarian cancer cells upon knockdown of both PAK1 and PAK2 [9]. A report suggested that PAK2 plays a role in the movement of ovarian cancer cells. Inhibiting PAK2 activity reduces the cells' ability to migrate on collagen. This suggests that PAK2 may contribute to collagen's effect on cell migration and should be studied further as a potential pro-metastatic gene in ovarian cancer [14]. In 2023, Xiao Zhang et al. utilized single-cell RNA sequencing (ScRNA-seq) to determine that apoptosis-related genes, particularly PAK2, exhibited elevated expression levels in high-grade serous ovarian cancer (HGSOC) cells when compared to healthy ovarian cells [15]. Prior studies have yet to examine the expression and localization of PAK2 in ovarian cancer tissues, as well as its correlation with clinical characteristics and prognosis in patients with ovarian cancer. Additionally, the mechanism by which PAK2 up-regulation contributes to chemo-resistance in ovarian cancer is still not fully understood.

In our prior study, we established that PAK2 is a direct target of miR-134, indicating that the downregulation of miR-134 and subsequent upregulation of PAK2 could potentially play a role in the emergence of paclitaxel resistance in ovarian cancer cells [16]. The current investigation seeks to examine the expression levels of PAK2 in chemo-resistant and chemo-sensitive serous EOC patients through the utilization of tissue microarray and bioinformatics analysis. Furthermore, this research endeavor seeks to investigate the variation in PAK2 expression levels among serous EOC patients at various International Federation of Gynecology and Obstetrics (FIGO) stages, and to determine if increased PAK2 expression is associated with reduced OS or DFS in these patients. In vitro experiments will also be carried out to evaluate the effects of suppressed PAK2 expression on the sensitivity of A2780/Taxol cells to paclitaxel. Our previous research has demonstrated that the long non-coding RNA lnc-SNHG1 acts as a ceRNA for miR-216b-5p in ovarian cancer cells, playing a crucial role in modulating the sensitivity of ovarian cancer cells to paclitaxel.

It has been postulated that PAK2 is a specific target of miR-216b-5p. Therefore, the aim of this study is to investigate the potential function of lnc-SNHG1 as a ceRNA in sequestering miR-216b-5p and regulating the expression of PAK2, ultimately impacting the resistance of ovarian cancer cells to paclitaxel.

Materials and methods

Cell culture and transfection

The current study employed the A2780/Taxol cell line, which was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were treated with 800 ng/mL paclitaxel, which was discontinued one week prior to conducting the experiments. GeneCreate Biotech in Wuhan, China, synthesized siRNAs targeting PAK2 and lnc-SNHG1, named PAK2-siRNA-1,PAK2-siRNA-2,PAK2-siRNA-3,lnc-SNHG1-siRNA-1,lnc-SNHG1-siRNA-2, and lnc-SNHG1-siRNA-3. These siRNAs were transfected into A2780/Taxol cells at 100 nmol/L concentration using Lipofectamine 3000. miR-216-5p-mimic and miRNA mimic NC were also transfected into A2780/Taxol cells at 100 nmol/L concentration using Lipofectamine 3000. The PAK2-pcDNA3.1 plasmid, which overexpresses PAK2, was synthesized by GeneCreate Biotech. The cells were cultured under controlled conditions at 37℃, 5% CO_2, and saturated humidity. After transfection, the cells were incubated for 48 h before commencing subsequent experiments.

Cell proliferation assay

After transfection, the cells were seeded into 96-well plates at a density of 5×10^3 cells/well and incubated at 37℃ with 5% CO_2 for varying time intervals of 0, 24, 48, and 72 h. Cell proliferation was evaluated utilizing the CCK-8 assay according to the manufacturer's instructions (Dojindo, Kumamoto, Japan). Absorbance readings were taken at a wavelength of 450 nm using a spectrophotometer (XFLUOR4 Version: V 4.51).

Cell apoptosis

A2780/Taxol cells were cultured in a 6-well plate at a density of 1×10^5 cells/mL and incubated for 12 h. Following transfection with PAK2-siRNA-3 and siRNA NC for 48 h, the cells were treated with a paclitaxel-containing medium at a concentration of 5 μmol/L. After a 24-hour incubation period, the cells were harvested and subjected to apoptosis analysis. This involved rinsing the cells twice with PBS and staining them with the Annexin V-FITC cell apoptosis detection kit (BD, New Jersey, USA), followed by flow cytometry analysis to quantify apoptosis levels.

Transwell assay

The experiment commenced by inserting the transwell insert into a 24-well plate and introducing 60μL of Matrigel gel, which had been diluted with serum-free DMEM at a 1:8 ratio. Subsequently, the assembly was incubated in a cell culture incubator for 4–5 h to facilitate solidification of the gel in the upper chamber. Following the removal of excess liquid, 100μL of serum-free DMEM medium was introduced, and the assembly was re-incubated for a 20-minute hydration period. After a 48-hour period of cell transfection, the cells were harvested, resuspended in serum-free DMEM, and quantified using a hemocytometer. Subsequently, 1×10^4 cells were seeded in the upper chamber of a transwell insert. In the lower chamber, DMEM medium supplemented with 10% FBS was added, and the cells were maintained in a 37℃, 5% CO_2 environment. Following a 24-hour incubation period, the medium was refreshed, and the cells were fixed with 4% paraformaldehyde for 15 min, stained with crystal violet for 20 min, washed with PBS, and visualized under a microscope for imaging purposes.

Test for the ability of clone formation

The cells were transfected and subsequently seeded into 6-well plates at a density of 1000 cells/well. The plates were then incubated in a 5% CO_2 environment at 37℃ overnight. The following day, the cells were treated with 5 μmol/L paclitaxel for 24 h. After treatment, the cells were maintained in fresh complete medium for two weeks. Subsequently, the cells were stained with 0.1% crystal violet for 30 min, washed with PBS three times, and subjected to statistical analysis to evaluate colony formation. The disparities in colony formation were subsequently analyzed and contrasted.

Western blot

The cellular protein was extracted using RIPA buffer (ThermoFisher Scientific) and quantified with a BCA Protein Array Kit (Biosharp, Guangzhou, China). Subsequently, samples containing 150 μg of protein from each group were combined with 6 × SDS loading buffer and heated for 10 min. The protein samples were then separated by electrophoresis on 12% SDS-PAGE gels, and the resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes via electro-transfer. After blocking with 5% skimmed milk, the membranes were subjected to incubation with anti-PAK2 (Abcam, Cambridge, UK, 1:500) and anti-GAPDH antibodies (Abcam, Cambridge, UK, 1:1000), followed by incubation with HRP-conjugated anti-rabbit secondary antibodies (Abcam, Cambridge, UK, 1:2000). The protein bands were detected using an ECL system and quantified using Chemi-Doc XRS imaging software (Bio-Rad).

Gene expression

The total RNA from all cell groups were isolated utilizing Trizol reagent (Invitrogen, CA, USA) according to the provided protocol. Subsequently, cDNA was synthesized employing the PrimeScript RT reagent Kit (Takara, Dalian, China). The expression levels of PAK2, lncSNHG1, and miR-216b-5p were assessed via quantitative reverse transcription PCR (RT-qPCR) utilizing the Bio-Rad S1000 with Bestar SYBR Green RT-PCR Master Mix (Toyobo), with the human Actin gene serving as a reference control. The relative gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), with each sample being analyzed in triplicate.

RIP

The Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit, manufactured by Millipore in Massachusetts, USA, was utilized for conducting the RIP assay in A2780/Taxol cells. During this process, cell lysates were combined with magnetic beads that were coated with either IgG or a human anti-Ago2 antibody in RIP buffer. Subsequently, the extracted RNA was purified in preparation for RT-qPCR.

Luciferase activity assay

A2780/Taxol cells were plated in 96-well culture plates at a concentration of 2×10^4 cells per well and then transfected with luciferase reporter constructs (400 ng), miR-216b-5p mimics (500 nmol/L), and internal control vectors pRL-TK, pRL-SV40, or pRL-CMV (Promega, WI, USA) at a 20:1 ratio (reporter construct: control vector) using Lipofectamine 3000 (Invitrogen) following the manufacturer's instructions. After a 5-hour incubation period, the transfection medium was replaced with a solution containing 6 μmol/L curcumin (Sigma-Aldrich, MO, USA) dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, MO, USA). The luciferase activity was evaluated 48 h post-transfection using the Dual-Luciferase Reporter Assay System (Promega, WI, USA).

Bio-informatics analysis

The Cancer Genome Atlas (TCGA) is a cancer genomics initiative that utilizes genome analysis technology through extensive sequencing methods [17]. In this study, datasets of RNAseq for ovarian cancer patients were specifically chosen and examined from the TCGA database. The Gene Expression Omnibus (GEO) is an international gene expression repository that was founded in 2000 and is currently managed by the National Center for Biotechnology Information. The study utilized data from the TCGA and GEO databases to investigate the correlation between PAK2 gene expression and the clinical outcomes of ovarian cancer.

Tissue microarray and immunohistochemical staining

The ovarian cancer tissue microarray utilized in this study was obtained from Shanghai Outdo Biotech and included 2 cases of benign ovarian tumors and 152 cases of ovarian cancer. A follow-up period of 5–9 years was conducted for all cancer patients. Among the ovarian cancer tissues, 92 cases of serous EOC were identified, with 31 cases classified as chemo-resistant and 61 cases as chemo-sensitive. Ethical approval for the study was granted by the ethics committees of the First Affiliated Hospital of Xi'an Jiao Tong University (approval number: 2020-G143) and Shanghai Outdo Biotechnology Company (approval number: YB M-05-02). Immunohistochemical staining of PAK2 was conducted utilizing a rabbit anti-human polyclonal antibody sourced from Abcam. The cytoplasmic expression of PAK2 was evaluated through the assessment of staining intensity, which was classified into four levels: absence of staining (scored as 0), light yellow staining (scored as 1), brown yellow staining (scored as 2), and dark brown staining (scored as 3). Furthermore, the proportion of positively stained cells was measured as follows: less than 5% (scored as 0), 5–25% (scored as 1), 26–50% (scored as 2), 51–75% (scored as 3), and more than 75% (scored as 4). The final score was calculated by multiplying the staining intensity score with the positive cell rate score. Scores ranging from 0 to 2 were categorized as negative (-), while scores between 3 and 4 were labeled as weakly positive (+), 5 to 8 as slightly positive (++), and 9 to 12 as strongly positive (+++). A score of 7 or higher indicated a high level of PAK2 expression. To minimize potential reading errors, two unbiased observers independently assessed and scored the samples.

Statistical analysis

The statistical analysis was conducted using the SPSS software package (version 19.0, IBM SPSS, IL, USA), with data presented as mean ± standard deviation. The relationship between PAK2 and clinicopathological characteristics of patients diagnosed with serous EOC was evaluated through the chi-square test. Additionally, a multivariate logistic regression analysis model was employed to predict the chemo-resistance of serous EOC patients based on the aforementioned variables. Furthermore, the research utilized a multivariate Cox proportional hazard model to investigate the prognostic factors impacting OS and DFS in the patients. Subsequently, Kaplan-Meier analysis was employed to assess OS and DFS outcomes. The cytology experiment data was analyzed using GraphPad Prism software, while transwell and apoptosis data were analyzed utilizing Student's t-test. CCK8 and colony formation data were analyzed using Two-way ANOVA, and the results derived from

the RT-qPCR test were subjected to statistical analysis utilizing One-way Analysis of Variance (ANOVA).

Results

Differential expression of PAK2 in patients with serous EOC

The RNASEQ dataset for ovarian cancer was acquired from the TCGA and GTEx databases, comprising 376 samples categorized as cancer and 88 samples categorized as adjacent to cancer. The RPKM data for PAK2 was transformed using the base 2 logarithm (log2) prior to the generation of a box plot utilizing the ggplot package within the R programming language. The samples were grouped into cancer and adjacent categories, revealing a significant up-regulation of PAK2 expression in ovarian cancer tissues compared to adjacent tissues (Fig. 1A). The table presented below utilizes the GEO database, consisting of 51 data sets, and conducts regression analysis using the R programming language. The resulting table displays findings associated with the GSE number GSE165808. The outcomes reveal a statistically significant relationship between age and prognosis in the single-factor regression analysis, indicating that individuals aged 60 and above exhibit a heightened mortality rate. Moreover, the results of both univariable and multivariable analyses indicate a significant correlation between the levels of PAK2 expression and prognosis. Specifically, elevated levels of PAK2 expression are linked to increased mortality rates, as shown in Table 1.

Furthermore, we employed the tissue microarray method to evaluate the expression of the PAK2 protein in patients with ovarian cancer. Considering that EOC comprises around 90% of all malignant ovarian tumors, and serous epithelial ovarian cancer makes up about 75% of EOC cases, our research focuses on examining the chemo-resistance properties of this specific subtype of ovarian cancer. In pursuit of this objective, a total of 92 serous EOC tissue samples were analyzed, with 31 cases classified as chemo-resistant and 61 cases as

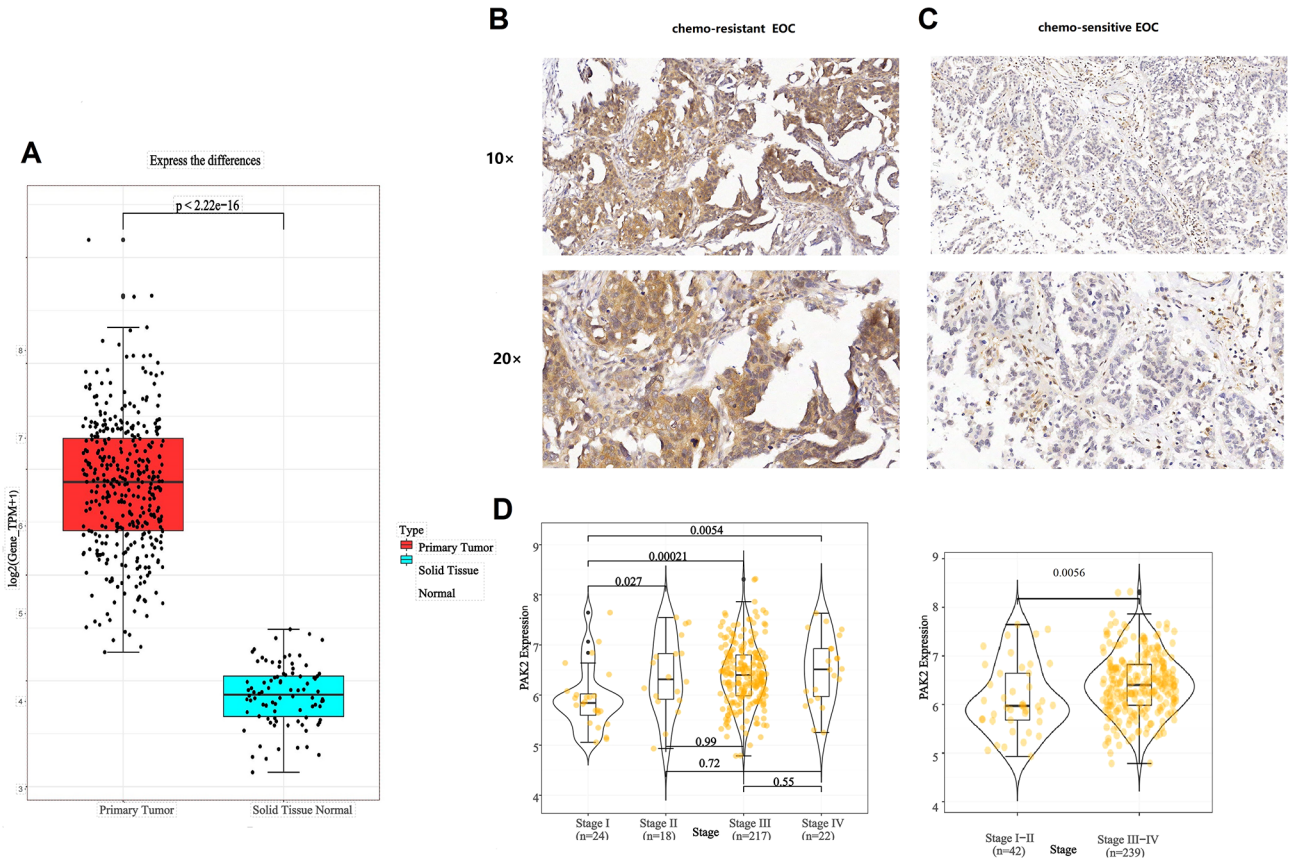

Fig. 1 Differential expression of PAK2 within ovarian cancer patients. (**A**) The RNASEQ dataset includes 376 samples classified as ovarian cancer and 88 samples classified as adjacent to cancer. The analysis demonstrates a notable increase in PAK2 expression in ovarian cancer tissues compared to adjacent tissues. (**B-C**) PAK2 expression was primarily observed in the cytoplasm, with a significantly higher expression ratio of 68% in chemo-resistant serous EOC tissues. In contrast, chemo-sensitive serous EOC tissues demonstrated lower PAK2 expression, with a comparatively lower expression ratio of 23%. (**D**) The data utilized in this study was sourced from the GEO database, specifically from the GSE9891 dataset. The differentiation among the four groups was assessed using the WILCOX test. The results indicate significant differences between stage I and II ($P=0.027$), highly significant differences between stage I and III ($P=0.00021$), and extremely significant differences between stage I and IV ($P=0.0054$). Furthermore, the disparity in PAK2 expression levels between stage I-II and stage III-IV ovarian cancer is deemed to be statistically significant with a p-value of 0.0056

Table 1 The relationship between PAK2 and prognosis of ovarian cancer patients using regression analysis

Characteristics	Univariable analyses			Multivariable analyses		
	HR (95% CI)	P-value		HR (95% CI)	P-value	
Diagnosed age (>60 vs. ≤60)	2.757 (1.19,6.387)	0.018	*	1.766 (0.6542, 4.767)	0.262	
Stage (I-II vs. III-IV)	0.8512 (0.327,2.215)	0.741		0.603 (0.2215, 1.642)	0.322	
Expression (low vs. high)	4.105 (1.687,9.99)	0.00186	**	3.413 (1.2135,9.597)	0.02	*

* P<0.05, ** P<0.01

Table 2 The expression of PAK2 in serous EOC patients

	Case	PAK2 cytoplasm expression (≦6)		PAK2 cytoplasm expression (≧7)		
	n	n	n%	n	n%	*P*
Age (years)						0.2875
Postmenopausal(≧50)	51	29	0.57	22	0.43	
Premenopausal (<50)	41	28	0.68	13	0.32	
Tumor stage (FIGO)						0.0343
I-II	20	17	0.85	3	0.15	
III-IV	72	41	0.57	31	0.43	
Histological grade (*n*=86)						0.7593
H	13	9	0.69	4	0.31	
L	73	45	0.62	28	0.38	
Nodal status(n)						0.1892
Negative	58	39	0.67	19	0.33	
Positive	34	18	0.53	16	0.47	
Chemo-resistance						<0.0001
Y	31	10	0.32	21	0.68	
N	61	47	0.77	14	0.23	
Metastasis						0.1892
Y	28	14	0.5	14	0.5	
N	64	43	0.67	21	0.33	

chemo-sensitive tumors. The upregulation of PAK2 was notably observed in chemo-resistant serous EOC tissues, predominantly localized in the cytoplasm, with a significant expression ratio of 68% (Table 2; Fig. 1B). In contrast, chemo-sensitive serous EOC tissues exhibited low expression of PAK2, with a comparatively lower expression ratio of 23% (Table 2; Fig. 1C). Moreover, the investigation analyzed the PAK2 expression levels in ovarian cancer patients based on the FIGO staging system, which categorizes individuals into four distinct stages: Stage I, Stage II, Stage III, and Stage IV. Violin plots were created utilizing the R programming language packages ggplot2 and ggsignif to illustrate the expression trends within each stage. The data utilized for this analysis was obtained from the GEO database, specifically the GSE9891 dataset. The differences among the four groups were assessed utilizing the WILCOX test, with the p-value being computed directly. The findings demonstrate significant variances between stage I and II (P=0.027), highly significant variances between stage I and III (P=0.00021), and extremely significant variances between stage I and IV (P=0.0054). Additionally, the noted difference in PAK2 expression levels between stage I-II and stage III-IV ovarian cancer is statistically significant (P=0.0056). These results were visually represented in Fig. 1D.

Risk factors related to chemo-resistance of patients with serous EOC

This study utilized a multivariate logistic regression analysis to investigate the correlation between different clinical characteristics and chemo-resistance in patients diagnosed with serous EOC. The clinical features taken into consideration in the analysis encompassed PAK2 expression, age, nodal status, metastasis, and FIGO stage. The forward stepwise selection method was employed to pinpoint significant variables. The results indicated that the varying levels of PAK2 expression (P<0.0001) and nodal status (P=0.011) were identified as factors linked to chemo-resistance in patients with serous EOC (Table 3).

Table 3 Analysis of multiple variables to determine factors linked to chemotherapy resistance in serous EOC

	B	S.E.	Sig.	Exp(B)	95.0% C.I. for EXP(B)	
					Lower	Upper
PAK2 expression	2.065	0.576	0.0001	7.888	2.553	24.37
Age	-0.612	0.577	0.278	0.542	0.175	1.679
Nodal status	1.83	0.716	0.011	6.232	1.531	25.373
Metastasis	0.197	0.699	0.797	1.217	0.309	4.789
FIGO stage	0.665	0.921	0.479	1.944	0.32	11.828

Table 4 Multivariate Cox regression analysis to evaluate the influence of various factors on disease-free survival

	B	SE	Sig.	Exp(B)	95.0% CI for Exp(B)	
					Lower	Upper
Chemo-resistance	3.832	0.636	0.0001	46.135	13.271	160.382
PAK2 expression	0.312	0.284	0.273	1.366	0.783	2.383
Age	-0.111	0.252	0.661	0.895	0.546	1.468
Nodal status	0.143	0.343	0.677	1.153	0.589	2.259
Metastasis	0.168	0.33	0.611	1.183	0.62	2.258
FIGO stage	1.073	0.367	0.003	2.925	1.424	6.007

Table 5 Multivariate Cox regression analysis to assess the impact on overall survival

	B	S.E.	Sig.	Exp(B)	95.0% C.I. for EXP(B)	
					Lower	Upper
Chemo-resistance	1.637	0.321	0.0001	5.142	2.743	9.64
PAK2 expression	0.705	0.3	0.019	2.023	1.124	3.642
Age	-0.361	0.279	0.196	0.697	0.403	1.205
Nodal status	0.64	0.367	0.081	1.897	0.925	3.893
Metastasis	0.473	0.35	0.177	1.604	0.808	3.185
FIGO stage	2.042	0.744	0.006	7.709	1.795	33.111

The outcome of the multivariate Cox regression model analysis for OS and DFS of serous EOC patients

Multivariate Cox regression analysis was utilized to assess prognostic factors for OS and DFS in serous EOC patients. The clinical variables examined included chemo-resistance, PAK2 expression, age, nodal status, metastasis, and FIGO stage. Results indicated that chemo-resistance ($P<0.0001$) and FIGO stage ($P=0.003$) were independent prognostic factors for DFS (Table 4), while chemo-resistance ($P<0.0001$), PAK2 expression ($P=0.019$), and FIGO stage ($P=0.006$) were associated with OS (Table 5).

The impact of elevated PAK2 expression on the OS and DFS of individuals diagnosed with ovarian cancer

The data on PAK2 expression was obtained from the previously mentioned GEO database, comprising 51 data points. Utilizing X-tile plots, a cutoff value of 52.92 was established based on the PAK2 expression levels of each sample. Subsequently, patients were stratified into two categories: high expression and low expression groups. Patients with expression levels surpassing the cutoff were assigned to the high expression group, whereas those below were assigned to the low expression group. The "survival" R package was employed to generate survival curves, which calculated the probability of survival by considering factors such as survival time, survival status, and different groupings. These curves were utilized to depict the survival probabilities of both the low expression and high expression cohorts.

It is clear that the OS probability of patients in the low expression group is significantly higher than that of patients in the high expression group. The Log Rank (Mantel-Cox) test yielded a p-value of 0.0008, indicating a highly significant difference. Therefore, the prognosis of the PAK2 high expression group is poor (Fig. 2A). The statistical analysis revealed that patients with low expression of PAK2 have a higher probability of survival without disease progression compared to patients with high expression. However, the Log Rank (Mantel-Cox) test showed a p-value of 0.061, suggesting that the observed difference is not statistically significant (Fig. 2B).

The examination of PAK2 expression in the tissue microarray indicated that individuals with elevated levels of PAK2 expression displayed notably diminished OS in comparison to those with lower levels of PAK2 expression ($P<0.0001$) (Fig. 2C). Furthermore, patients with high PAK2 expression demonstrated reduced DFS ($P<0.0001$) (Fig. 2D).

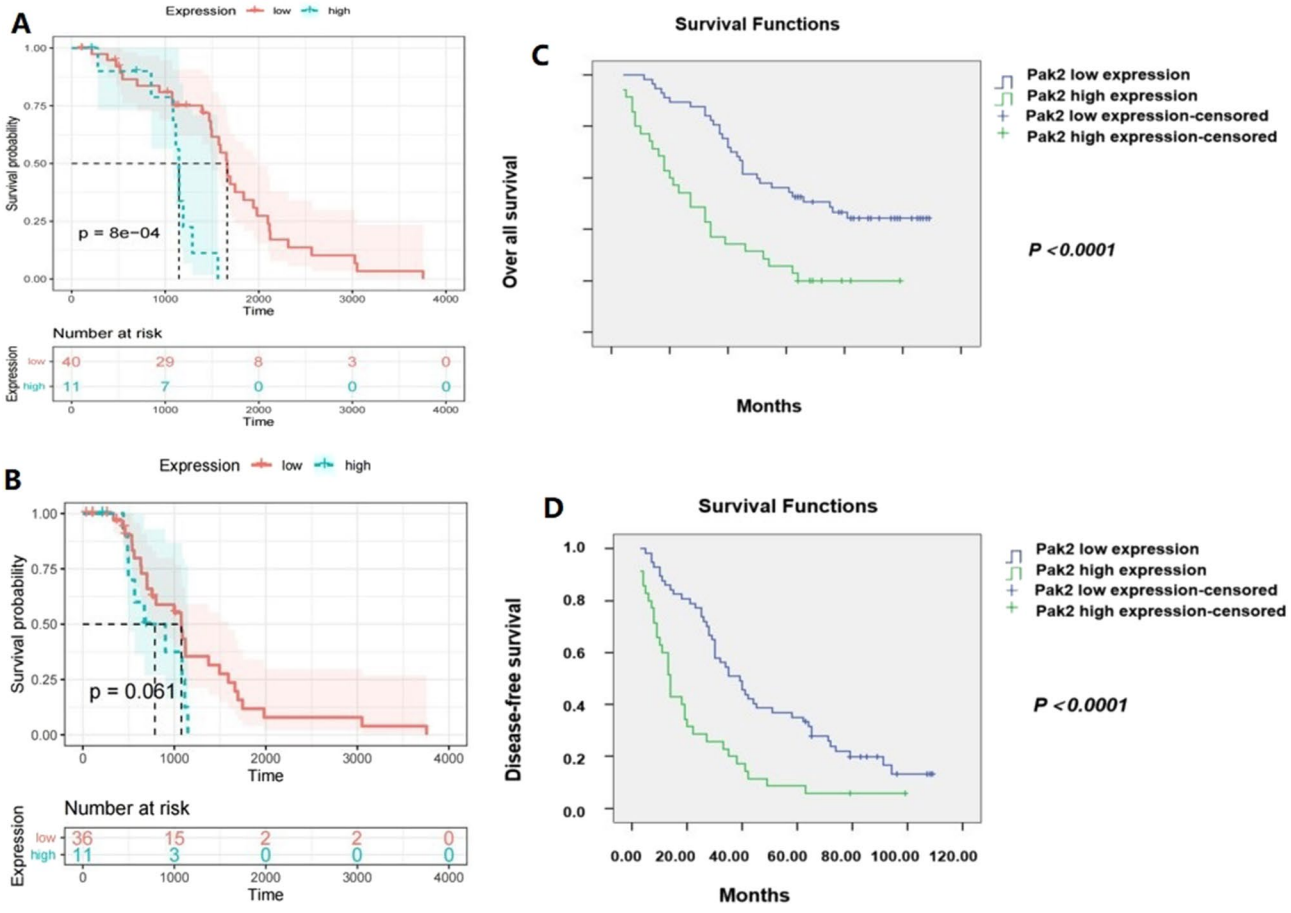

Fig. 2 Survival curves were examined in relation to the expression patterns of PAK2 among patients with ovarian cancer. (**A-B**) PAK2 expression data, comprising 51 points, was sourced from the GEO database. Patients were stratified into high expression and low expression cohorts based on whether their expression values exceeded or fell below a predefined threshold. (**A**) The survival probability of patients exhibiting low expression was determined to be significantly greater than that of patients with high expression ($P=0.0008$). (**B**) Patients with low expression of PAK2 had a higher likelihood of survival without disease progression compared to those with high expression, although the difference was not statistically significant ($P=0.061$). (**C**) In the tissue microarray analysis, it was observed that patients exhibiting elevated PAK2 expression levels demonstrated significantly reduced OS in comparison to those with lower expression levels ($P<0.0001$). (**D**) Patients exhibiting elevated PAK2 expression demonstrated a decrease in DFS ($P<0.0001$)

Targeting PAK2 with siRNA has the potential to enhance the sensitivity of A2780/Taxol cells to paclitaxel

Initially, an elevation in PAK2 expression at both the protein and RNA levels was noted in A2780/Taxol cells compared to their parental A2780 counterparts (Fig. 3A-B). To examine the influence of PAK2 on the sensitivity of ovarian cancer cells to paclitaxel, A2780/Taxol cells were transfected with three PAK2-specific siRNAs (PAK2-siRNA-1, PAK2-siRNA-2, PAK2-siRNA-3) alongside a negative control siRNA (NC-siRNA). The efficiency of transfection was evaluated through RT-qPCR and western blot analysis, indicating that PAK2-siRNA-3 exhibited the highest efficacy (Fig. 3A-B). Following transfection with PAK2-siRNA-3, A2780/Taxol cells exhibited a significant reduction in cell proliferation relative to both the NC-siRNA group and the untransfected A2780/Taxol cells (Fig. 3C). Functional assays were performed after transfection with PAK2-siRNA-3 (NC-siRNA) and treatment with paclitaxel for 24 h. The results of the colony formation assay substantiated that PAK2 deficiency resulted in a decrease in colony count (Fig. 3D). The findings from the cell apoptosis assay indicate that the silencing of PAK2 enhances apoptotic activity, as illustrated in Fig. 3E. Furthermore, the transwell assay revealed a reduction in cell invasion capability following PAK2 knockdown, as depicted in Fig. 3F. Collectively, these observations indicate that the downregulation of PAK2 may enhance the sensitivity of A2780/Taxol cells to paclitaxel.

Lnc-SNHG1 functioned as a ceRNA by binding miR-216b-5p, consequently modulating the expression of PAK2

In our previous study, we established a negative correlation between the up-regulation of lnc-SNHG1 and the down-regulation of miR-216b-5p in relation to chemoresistance in patients with serous EOC patients. Our

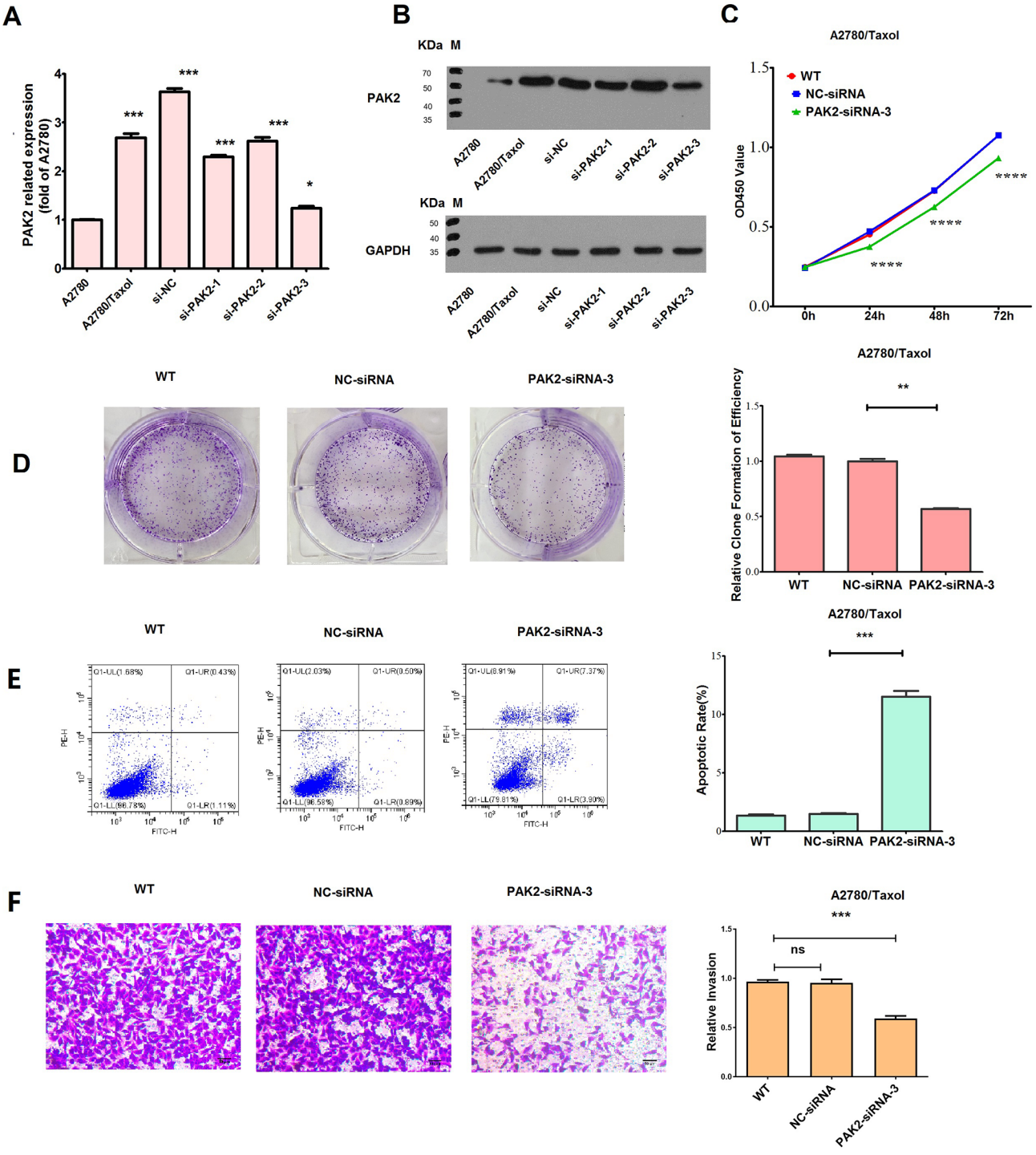

Fig. 3 The enhanced sensitivity of A2780/Taxol cells to paclitaxel is attributed to the inhibition of PAK2. (**A**) Analysis of mRNA expression revealed a significant up-regulation of PAK2 in A2780/Taxol cells compared to parental A2780 cells. Among the PAK2-siRNA variants tested, PAK2-siRNA-3 demonstrated the highest interference efficiency. (**B**) Western blot analysis revealed that PAK2 expression was elevated in A2780/Taxol cells relative to parental A2780 cells. Notably, the most significant down-regulation of PAK2 was observed in cells transfected with PAK2-siRNA-3. (**C**) PAK2-siRNA-3 transfection significantly reduced A2780/Taxol cell proliferation compared to NC-siRNA and untransfected cells. (**D**) The colony formation assay provided evidence that the absence of PAK2 resulted in a significant reduction in colony numbers. (**E**) The results of the cell apoptosis assay indicated that the inhibition of PAK2 significantly increased the rate of apoptosis. (**F**) The transwell assay revealed a significant reduction in cellular invasion capability following the knockdown of PAK2. GraphPad Prism software was utilized for data analysis, with Student's t-test employed for colony formation assessments, apoptosis and transwell. Two-way ANOVA utilized for CCK8 and at three distinct time points. Statistical significance was denoted as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$

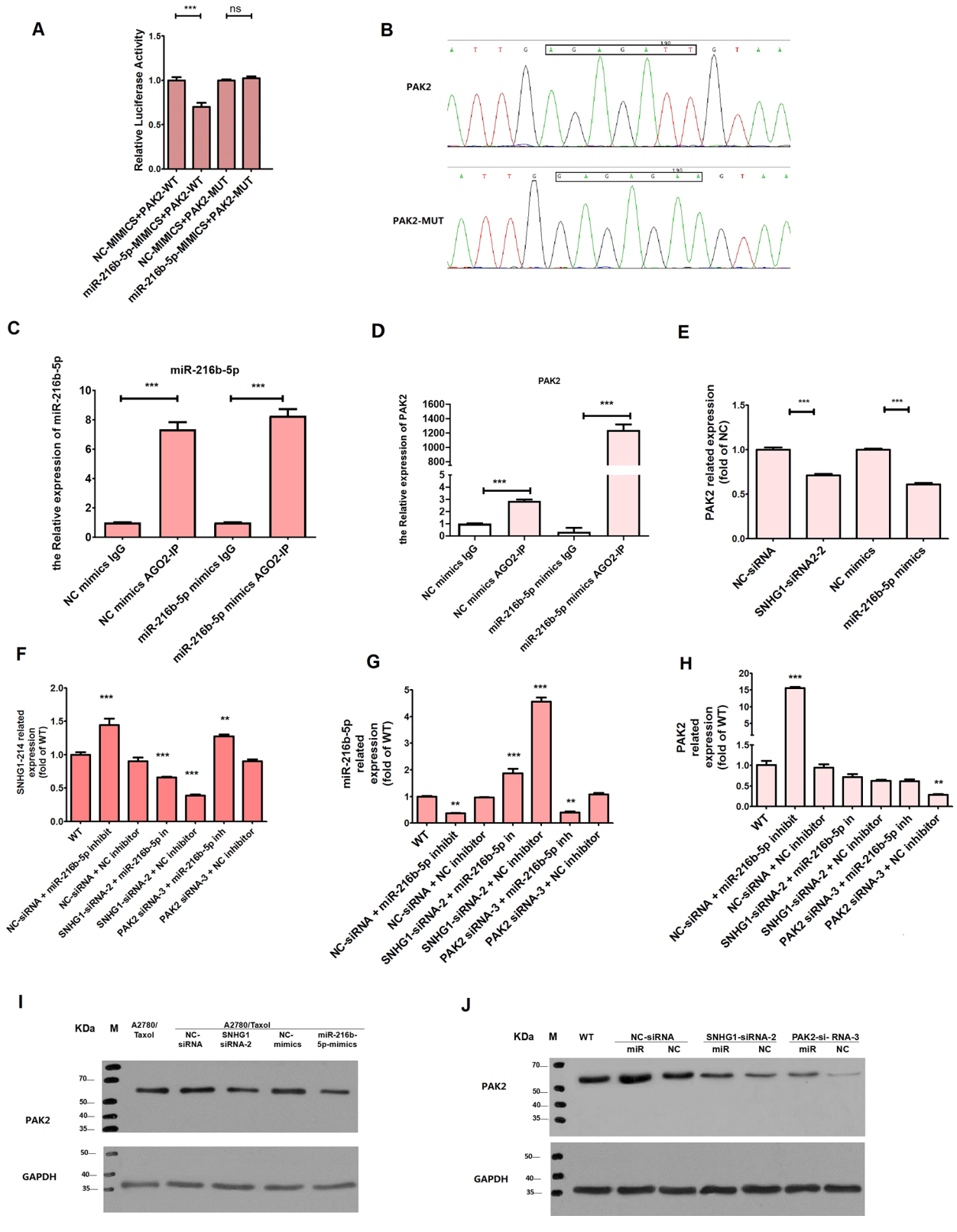

Fig. 4 (See legend on next page.)

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Fig. 4 Lnc-SNHG1 acted as a ceRNA through the sequestration of miR-216b-5p, thereby regulating the expression of PAK2. (**A-D**) PAK2 was a direct target of miR-216b-5p. (**A**) The Luciferase reporter assay demonstrated that miR-216b-5p significantly decreased the luciferase activity of the PAK2-WT construct as opposed to the PAK2-MUT construct. (**B**) Sequencing analysis confirmed the successful construction of the PAK2 and PAK2-MUT plasmids for subsequent utilization in the luciferase reporter assay. (**C-D**) A2780/Taxol cells were transfected with MS2-tagged PAK2-WT and MS2-tagged PAK2- MUT, and then were assayed by RIP. The RIP-qPCR assay demonstrated a significant enrichment of miR-216b-5p in the immunoprecipitation (IP) group, suggesting a binding interaction between AGO2 protein and miR-216b-5p. Additionally, the detection of PAK2 showed a notable enrichment in the IP group, providing further evidence of a potential correlation between PAK2 and miR-216b-5p. (**E**) The RT-qPCR analysis demonstrated a reduction in PAK2 gene expression subsequent to treatment with SNHG1-siRNA-2 and miR-216b-5p mimics in comparison to the control group. (**F-G**). The expression levels of lnc-SNHG1 and miR-216b-5p genes were quantified via RT-qPCR in the seven cell groups mentioned above. (**I**) The interference of lnc-SNHG1 and over-expression of miR-216b-5p both resulted in significant reductions in PAK2 protein levels. (**H**, **J**) The RNA and protein levels of the PAK2 gene were evaluated using western blot analysis and RT-qPCR in the aforementioned seven cell groups. A comparison between the second and third groups demonstrated a notable upregulation in the expression of the PAK2 gene subsequent to the knockdown of miR-216b-5p. Similarly, a comparison between the second and fourth groups indicated a significant downregulation in the expression of the PAK2 gene following the simultaneous knockdown of miR-216b-5p and lnc-SNHG1. A notable reduction in the expression level of the target gene PAK2 was observed in the second and sixth groups upon concurrent knockdown of miR-216b-5p and PAK2. Analysis was conducted using GraphPad Prism software, with RT-qPCR data analyzed using One-way ANOVA. Statistical significance was indicated as follows: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$

findings suggest that lnc-SNHG1 acts as a ceRNA with miR-216b-5p, thereby influencing the sensitivity of ovarian cancer cells to paclitaxel [18].

In this study, we confirm that PAK2 is a direct target of miR-216b-5p. Utilizing the starBase v2.0 database, we observed that miR-216b-5p has a binding site within the 3' untranslated region (3'UTR) of PAK2. To further validate this finding, we conducted luciferase reporter assays employing A2780/Taxol cells. The results indicate a notable reduction in fluorescence intensity following co-transfection of miR-216b-5p-mimics and PAK2-WT, in comparison to co-transfection of miR-216b-5p-mimics and PAK2-MUT. Conversely, no significant alteration was observed when miR-216b-5p-mimics were co-transfected with PAK2-MUT, as opposed to NC-mimics + PAK2-MUT (Fig. 4A-B). These findings suggest a potential interaction between miR-216b-5p-mimics and PAK2-WT. In order to confirm the direct interaction between miR-216b-5p and PAK2, a RIP analysis was conducted. The results of the RIP-qPCR demonstrated a notable increase in the presence of miR-216b-5p in A2780/Taxol cells when MS2-tagged wild-type PAK2 (PAK2-WT MS2) was present, as opposed to the mimic NC (Fig. 4C-D). Taken together, these results offer support for the notion that miR-216b-5p has the ability to directly interact with PAK2.

Furthermore, A2780/Taxol cells were transfected with lnc-SNHG1-siRNA (NC siRNA), and the expression levels of PAK2 were assessed at both the RNA and protein levels. The results indicated that a decrease in lnc-SNHG1 expression was associated with a reduction in PAK2 expression. Additionally, overexpression of miR-216b-5p through transfection with miR-216b-5p mimics resulted in the downregulation of PAK2 expression at both the RNA and protein levels. (Fig. 4E, I).

To investigate the regulatory role of lnc-SNHG1 as a ceRNA through its interaction with miR-216b-5p and subsequent modulation of PAK2 expression, a series of experiments were conducted using A2780/Taxol cells. Experimental groups were designed as follows: A2780/Taxol wild-type as the control (group 1), A2780/Taxol cells transfected with NC-siRNA + miR-216b-5p inhibitor (group 2), NC-siRNA + NC inhibitor (group 3), lnc-SNHG1-siRNA-2 + miR-216b-5p inhibitor (group 4), lnc-SNHG1-siRNA-2 + NC inhibitor (group 5), PAK2 siRNA-3 + miR-216b-5p inhibitor (group 6), and PAK2 siRNA-3 + NC inhibitor (group 7). The validation of lnc-SNHG1 and miR-216b-5p expression levels was confirmed through experimental analysis (Fig. 4F, G). Upon comparing Group 2 and Group 3, a notable upregulation of the target gene PAK2 was observed following the inhibition of miR-216b-5p. Similarly, a significant decrease in PAK2 expression was noted when both miR-216b-5p and lnc-SNHG1 were simultaneously knocked down in Group 4. Additionally, the combined knockdown of miR-216b-5p and PAK2 in Group 6 resulted in a substantial reduction in PAK2 expression at both the RNA and protein levels (Fig. 4H, J).

The lnc-SNHG1/miR-216b-5p/PAK2 axis contributes to the development of chemo-resistance in A2780/Taxol cells to paclitaxel

The rescue experiment demonstrated that, in comparison to the control group administered with miR-216b-5p-mimics + pcDNA3.1 empty vector, the expression levels of the PAK2 gene were markedly elevated in the miR-216b-5p-mimics + PAK2-pcDNA3.1 group. Similarly, a significant upregulation in PAK2 gene expression was observed in the SNHG1-siRNA-2 + PAK2-pcDNA3.1 group when contrasted with the control group treated with SNHG1-siRNA-2 + pcDNA3.1 empty vector(refer to Fig. 5A). Additionally, A2780/Taxol cells displayed enhanced cell proliferation (Fig. 5B), reduced apoptosis (Fig. 5C), improved colony formation (Fig. 5D) and enhanced invasion capability. (Fig. 5E), post transfection with miR-216b-5p-mimics + PAK2-pcDNA3.1, as opposed to miR-216b-5p-mimics + pcDNA3.1 vector, or following transfection with

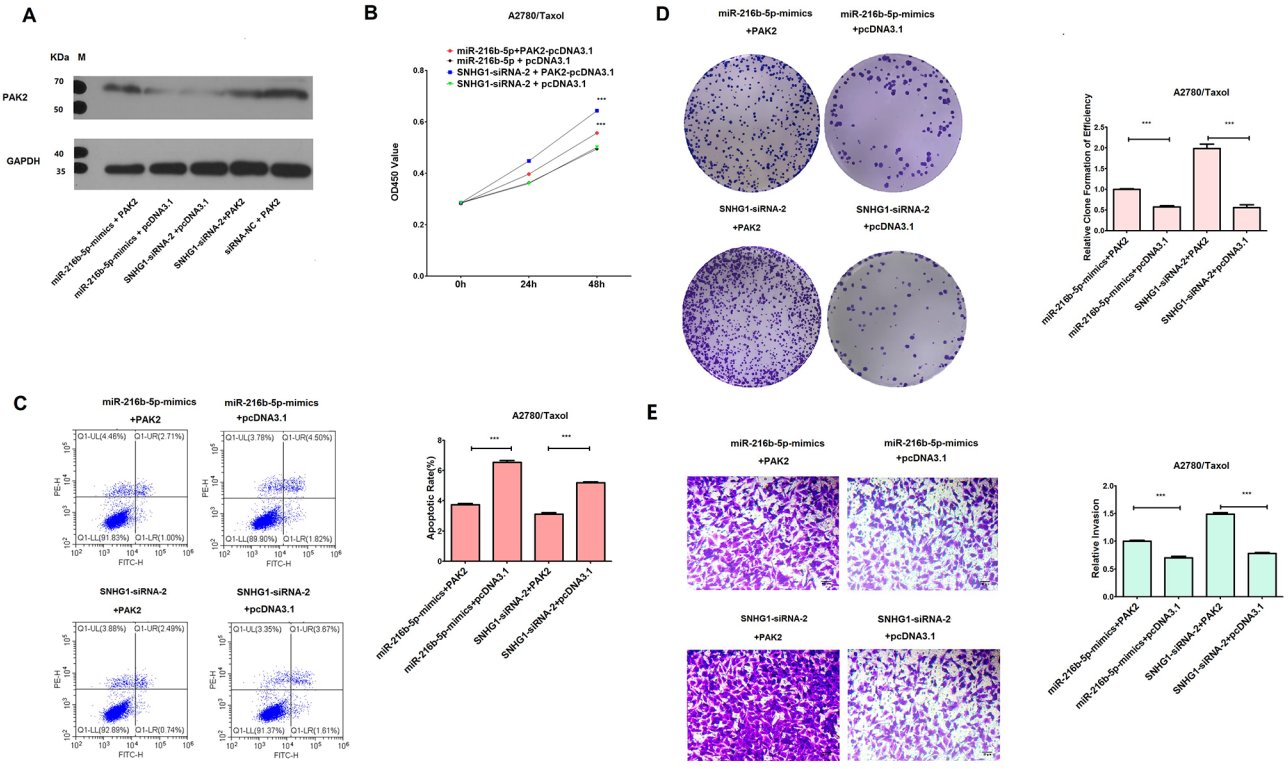

Fig. 5 The rescue experiment confirmed that the lnc-SNHG1/miR-216b-5p/PAK2 axis plays a role in developing chemo-resistance to paclitaxel in A2780/Taxol cells. (**A**) The rescue experiment demonstrated that cells transfected with miR-216b-5p-mimics in conjunction with PAK2-pcDNA3.1, as well as those transfected with SNHG1-siRNA-2 in conjunction with PAK2-pcDNA3.1, exhibited significantly increased PAK2 expression levels compared to cells transfected with miR-216b-5p-mimics plus pcDNA3.1 or SNHG1-siRNA-2 plus pcDNA3.1, respectively. (**B**) A2780/Taxol cells showed significantly higher proliferation in the miR-216b-5p-mimics + PAK2-pcDNA3.1 group compared to the miR-216b-5p-mimics + pcDNA3.1 group. Similarly, cell proliferation was notably increased in the SNHG1-siRNA-2 + PAK2-pcDNA3.1 group compared to the SNHG1-siRNA-2 + pcDNA3.1 group. (**C**) Cells with miR-216b-5p mimics or SNHG1-siRNA-2 and PAK2 overexpression plasmid exhibited reduced apoptosis compared to control groups. (**D**) The rate of clone formation exhibited a significant increase in both the miR-216b-5p-mimics + PAK2-pcDNA3.1 and SNHG1-siRNA-2 + PAK2-pcDNA3.1 experimental groups when compared to their respective control groups($P = 0.0004$). (**E**) Cells transfected with miR-216b-5p mimics and PAK2-pcDNA3.1, as well as those transfected with SNHG1-siRNA-2 and PAK2-pcDNA3.1, exhibited significantly enhanced invasion capability relative to their corresponding control groups. GraphPad Prism software was utilized for data analysis, with Student's t-test employed for colony formation assessments, apoptosis and transwell. Two-way ANOVA utilized for CCK8 and at different time points. Statistical significance was indicated by a p-value less than 0.05 (***$P < 0.001$)

lnc-SNHG1-siRNA-2 + PAK2-pcDNA3.1, as opposed to lnc-SNHG1-siRNA-2 + pcDNA3.1. These results collectively suggest that increased PAK2 levels may lead to the restoration of paclitaxel resistance in A2780/Taxol cells compared to the group showing upregulation of miR-216b-5p or downregulation of lnc-SNHG1.

Discussion

Presently, the predominant strategies for the management of ovarian cancer encompass surgical resection and chemotherapy. Surgical resection is the preferred therapeutic modality, aiming to achieve maximal removal of tumor tissue. Nevertheless, the inherent characteristics of ovarian cancer, particularly its propensity for metastasis, pose substantial challenges to the complete eradication of tumor tissues via surgical resection. This results in an elevated risk of recurrence and further metastatic spread. Moreover, while chemotherapy can effectively control tumor proliferation, the occurrence of drug resistance frequently diminishes the overall efficacy of the treatment. Despite advancements in the field of anti-cancer drug developments [19, 20], the problem of chemotherapy resistance in ovarian cancer remains a formidable challenge in the present context. Consequently, there has been an increasing emphasis on investigating the mechanisms underlying chemotherapy resistance in ovarian cancer and on developing novel therapeutic strategies within this area of research.

The aberrant expression and activity of PAKs in neoplasms have been thoroughly examined and are intricately linked to the onset, advancement, and prognosis of tumors [21–23]. Research has shown that the upregulation of PAKs in various tumor types is associated with heightened proliferation and invasion of tumor cells. PAKs have the capability to promote cell cycle advancement and enhance tumor cell proliferation through the modulation of cyclins and signaling pathways related to cell proliferation. Additionally, PAKs can govern

cytoskeletal reorganization and cell migration, thereby fostering tumor cell invasion and metastasis [24].

PAK1 has been extensively researched within the PAK family across various types of cancer [25, 26]. PAK1 was also found to be over-expressed and activated in ovarian cancer [8, 9, 27] and endometrial cancer [28]. However, there is a scarcity of research examining the involvement of PAK2 in ovarian cancer. Siu MK et al. found that diminishing levels of Pak1 and PAK2 led to a reduction in the migratory and invasive capabilities of ovarian cancer cells [9]. Another study determined that PAK2 plays a role in the mobility of ovarian cancer cells [14]. Recently, high expression of apoptosis-related genes, particularly PAK2, has been identified in high-grade serous ovarian cancer cells through single-cell RNA sequencing [15]. However, the potential association between PAK2 expression and clinical characteristics or prognosis of ovarian cancer patients remains unexplored. Li X's research findings indicate that elevated levels of PAK2 play a significant role in conferring resistance to chemotherapy in cases of human breast invasive ductal carcinoma, primarily through the downregulation of caspase-7 activity [29]. Cho HJ et al. conducted a study elucidating the impact of RhoGDI2 on tumor growth, malignant progression, and chemo-resistance in gastric cancer, as well as the role of PAK2 in mediating these effects within gastric cancer cells [30]. Nevertheless, the precise mechanism by which PAK2 up-regulation contributes to chemo-resistance in ovarian cancer has yet to be elucidated.

In this study, analysis of the RNASEQ dataset revealed a significant increase in PAK2 expression in ovarian cancer tissues compared to adjacent tissues. Additionally, a statistically significant difference in PAK2 expression levels was observed between stage I-II and stage III-IV ovarian cancer. These findings are in line with a previous study that reported elevated PAK2 expression in oral squamous cell carcinoma (OSCC) compared to normal mucosa. Moreover, it was observed that the expression of PAK2 was increased in advanced stages of OSCC. Subsequently, after treatment with TPF (docetaxel, cisplatin, and fluorouracil), there was a notable rise in PAK2 expression in OSCC [31]. Furthermore, Liu R's research indicates a significant discrepancy in PAK2 expression levels between breast cancer cells and normal mammary epithelial cells. Moreover, the study found a strong correlation between the presence of PAK2 and the likelihood of breast cancer metastasis and mortality [32].

Xiao Zhang's recent observations revealed that apoptosis-related genes, including URI1, PAK2, PARP1, CLU, and TIMP3, exhibited higher expression levels in ovarian cancer cells compared to healthy ovarian cells as determined through the application of single-cell RNA sequencing (ScRNA-seq), a finding consistent with the results of our own study [15]. However, they did not conduct additional validation studies to confirm the association between high expression of the gene and clinical pathological features in tissue samples from clinical patients. Our research findings indicate that PAK2 displays elevated expression levels in chemo-resistant serous EOC patients, contrasting with lower levels observed in chemo-sensitive individuals. Furthermore, the upregulation of PAK2 is strongly correlated with chemo-resistance in serous EOC patients, suggesting a negative impact on OS and DFS outcomes. This discovery aligns with a prior study that noted the predominant cytoplasmic localization of both PAK2 and pSer20PAK2 immunostainings in tumor cells within gastric cancer tissues, demonstrating significantly elevated expression levels in comparison to normal gastric mucosa. Additionally, individuals exhibiting heightened expression of PAK2 and pSer20PAK2 proteins were found to be closely associated with adverse clinicopathologic factors [33]. Furthermore, an additional study reported a notable upregulation of PAK2 expression in gastric cancer tissues in comparison to adjacent tissues, suggesting a correlation between elevated PAK2 levels, cellular proliferation, and the advancement of the disease [34]. Moreover, PAK2 was identified as being markedly upregulated in cases of head and neck cancer (HNC), leading to an unfavorable prognosis for patients. Additionally, heightened PAK2 expression was shown to promote cellular proliferation, aerobic glycolysis, and resistance to chemotherapy via the PAK2-c-Myc-PKM2 pathway [35].

Our in vitro analysis demonstrated that the downregulation of PAK2 expression in paclitaxel-resistant ovarian cancer cells (A2780/Taxol) resulted in diminished colony formation, enhanced apoptosis, and impaired cell invasion. These results are consistent with a study by Eleonora Sementino, which showed that PAK2 gene inhibition significantly decreased the occurrence and delayed progression of pleural and peritoneal malignant mesotheliomas in mice. In vitro experiments also demonstrated that the depletion of the PAK2 gene resulted in diminished viability, migration, clonogenic potential, and spheroid formation in malignant mesothelioma cells [36]. Furthermore, a separate investigation revealed that PAK2 can interact with caspase-7 and phosphorylate it, resulting in the suppression of cellular apoptosis and the promotion of chemotherapeutic resistance in human breast invasive ductal carcinoma [29]. Moreover, studies have shown that breast cancer cells with decreased levels of PAK2 demonstrate heightened susceptibility to apoptosis triggered by anticancer medications, whereas elevated PAK2 activity hinders the apoptotic reaction, resulting in anchorage-independent proliferation, expansion, and resistance to drug-induced apoptosis [37]. An intriguing study indicates that exosomes released by leukemic cells

contain PAK2, and the transmission of these exosomes facilitates the proliferation of endothelial cells [38].

In recent studies, non-coding RNAs (ncRNAs) have been suggested as potential regulators of carcinogenesis, metabolism, and drug resistance [39]. One study has proposed that the long non-coding RNA ZEB1-AS1 promotes the progression of colon adenocarcinoma via the miR-455-3p/PAK2 pathway [40]. Furthermore, the exosomal circular RNA circ_0008717, secreted by cancer cells, promotes cell tumorigenicity in non-small cell lung cancer through modulation of the microRNA-1287-5p/PAK2 axis [41]. Additionally, a study has indicated that circular RNA circ_0000119 facilitates the growth and migration of cervical cancer cells through the miR-433-3p/PAK2 axis [42]. Our research substantiates the regulatory function of lnc-SNHG1 as a ceRNA via its interaction with miR-216b-5p, which subsequently modulates PAK2 expression. Through functional analysis involving the transfection of A2780/Taxol cells with miR-216b-5p mimic and PAK2-pcDNA3.1 (pcDNA 3.1 empty plasmid), as well as with SNHG1-siRNA-2 and PAK2-pcDNA3.1 (pcDNA 3.1 empty plasmid), we have substantiated the role of lnc-SNHG1 as a ceRNA. This interaction with miR-216b-5p subsequently modulates the expression of PAK2, thereby potentially affecting the sensitivity of ovarian cancer cells to the chemotherapeutic agent paclitaxel.

Conclusions

The results of this study indicate a significant upregulation of PAK2 mRNA in ovarian cancer tissues compared to adjacent tissues, with this upregulation being correlated with increased mortality rates in patients. Additionally, the overexpression of PAK2 protein is associated with chemo-resistance in serous epithelial ovarian cancer and is predictive of unfavorable OS and DFS outcomes. In vitro experiments revealed that the downregulation of PAK2 expression in paclitaxel-resistant ovarian cancer cells resulted in a reduction in colony formation, an increase in apoptosis, and the inhibition of cell invasion. Furthermore, it was found that lnc-SNHG1 functions as a ceRNA by interacting with miR-216b-5p and subsequently regulating PAK2 expression. Subsequent rescue experiments substantiated the conclusion that lnc-SNHG1 induces paclitaxel resistance in A2780/Taxol cells via the miR-216b-5p/PAK2 axis. To sum up, our findings indicate that PAK2 may serve as a predictive marker for chemotherapy resistance and adverse outcomes in ovarian cancer, thereby highlighting its potential as a therapeutic target.

Abbreviations

EOC	Epithelial ovarian cancer
ceRNA	competing endogenous RNA
OS	Overall survival
DFS	Disease-free survival
RIP	RNA Binding Protein Immunoprecipitation
FIGO	International Federation of Gynecology and Obstetrics
RT-qPCR	Quantitative reverse transcription PCR
TCGA	The Cancer Genome Atlas
GEO	The Gene Expression Omnibus

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-024-12969-1.

Supplementary Material 1

Author contributions

Ting Shuang was involved in study design, execution, and manuscript drafting. Meili Pei was involved in study design and acquisition of data. Shiyun Wu, Yifei Zhao, and Yanqi Yang contributed to the analysis of data. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability

The datasets produced and analyzed in the present study can be obtained from the corresponding author upon request.

Declarations

Ethics approval

The study was approved by the ethics committee of the First Affiliated Hospital of the Xi'an Jiaotong University (approval number: 2020-G143) and the ethics committee of Shanghai Outdo Biotech Company (approval number: YB M-05-02).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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