

# Role of Wnt/ $\beta$ -catenin, Wnt/c-Jun N-terminal kinase and Wnt/ $\text{Ca}^{2+}$ pathways in cisplatin-induced chemoresistance in ovarian cancer

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**Abstract.** The aim of the present study was to explore the expression of Wnt signaling proteins  $\beta$ -catenin, c-Jun N-terminal kinase (JNK) and  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) in ovarian cancer cells, and assess the correlation between this expression and cisplatin-induced chemoresistance. SKOV3 ovarian carcinoma cells and SKOV3/DDP (cisplatin resistant) cells were treated with cisplatin in the absence or presence of a Wnt signaling activator (CHIR-99021, glycogen synthase kinase  $3\beta$  inhibitor) or inhibitor (XAV-939, tankyrase inhibitor). Following incubation for 48 h, cell viability, proliferation and cytotoxicity were measured using a sensitive colorimetric cell counting kit. Expression levels of  $\beta$ -catenin, JNK and CaMKII were detected by western blot and immunofluorescence staining. The results of the current study identified that  $\beta$ -catenin and JNK expression levels were significantly higher ( $P < 0.01$  and  $P < 0.05$  respectively), while CaMKII expression was lower ( $P > 0.05$ ), in SKOV3/DDP cells compared with SKOV3 cells. Moreover, following treatment with  $20 \mu\text{M}$  cisplatin, reduced expression of  $\beta$ -catenin and JNK ( $P < 0.05$  and  $P < 0.01$  respectively), and increased expression of CaMKII ( $P < 0.01$ ), was observed in SKOV3 and SKOV3/DDP cell lines. Furthermore, inhibition of  $\beta$ -catenin signaling by XAV-939 effectively reversed cisplatin chemoresistance in SKOV3/DDP cells. Similarly, XAV-939 downregulated JNK expression ( $P < 0.001$ ), but upregulated CaMKII expression ( $P < 0.001$ ), in SKOV3/DDP cells. In conclusion, abnormal activation of

Wnt/ $\beta$ -catenin and Wnt/JNK signaling pathways in ovarian cancer cells promotes cisplatin resistance, while the Wnt/ $\text{Ca}^{2+}$  signaling pathway reduces cisplatin resistance. This indicates that  $\beta$ -catenin, JNK and CaMKII are potential therapeutic targets in chemoresistant ovarian cancers.

## Introduction

Ovarian cancer is the fifth most common cause of death in women worldwide (1). Typical treatment for ovarian cancer involves cytoreductive surgery combined with cisplatin-based chemotherapy (2). Unfortunately, the initial response rate of this treatment is not sustainable and in 70% of women disease reoccurs within months as a result of chemoresistance (3,4). It has been suggested that the high mortality rate in ovarian cancer can be partly attributed to this high frequency of chemoresistance (5).

Cisplatin is a platinum compound that was found to arrest binary fission in *Escherichia coli* in the 1960s, and it is now a crucial chemotherapeutic drug in the treatment of numerous cancers, including ovarian, as a single agent and in combination with other anticancer drugs (6,7). The issue of resistance to cisplatin remains a major obstacle in the successful treatment of ovarian cancer (8). Previous studies have indicated that inhibition of intrinsic cell death signaling pathways, activation of cell survival signaling pathways, and dysregulation of oncogenes, tumor suppressor genes and microRNAs contributes to cisplatin chemoresistance in ovarian cancer cells (9,10). However, the underlying mechanisms by which cisplatin chemoresistance occurs in ovarian cancer cells remain unclear.

Previous studies have identified that abnormal Wnt signaling serves a role in the development of breast (11), gastric (12), lung (13), prostate (14) and endometrial (15) cancers. In ovarian cancer, persistent activation of Wnt signaling was observed to increase cell survival, and several studies have concluded that aberrant regulation of Wnt signaling induces tumor cell chemoresistance (16-18).

The Wnt signaling pathway serves a critical role in embryogenesis and oncogenesis (19). Wnt ligands are a family of secreted proteins comprising of 19 members (10).

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Wnt ligands bind to a Frizzled (Fzd) family receptor and low-density lipoprotein receptor-related protein (LRP)-5/6 to initiate signaling (10). Wnt signaling is divided into canonical Wnt signaling pathway (Wnt/ $\beta$ -catenin) and non-canonical Wnt signaling pathways (19).

In the canonical Wnt signaling pathway, illustrated in Fig. 1A, Wnt ligands engage Fzd and LRP-5/6, which inhibits glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ), leading to stabilization and increasing expression levels of  $\beta$ -catenin in the cytoplasm. Stable  $\beta$ -catenin translocates into the nucleus where it binds to the transcription factor T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) to control the transcription of Wnt target genes (20).

Dysregulation of the Wnt/ $\beta$ -catenin signaling pathway has been identified in numerous cancers, including ovarian (21).  $\beta$ -catenin is the primary component of this signaling pathway, and mutations in the gene encoding  $\beta$ -catenin (*CTNNB1*), leading to alteration of the Wnt/ $\beta$ -catenin signaling pathway, have been found in the endometrioid subtype of ovarian cancer (22,23). Furthermore, aberrant expression of  $\beta$ -catenin and Wnt-5A has been observed in ovarian cancer (22,24). In addition, aberrant accumulation of  $\beta$ -catenin is associated with increasing ovarian cancer grade and poor survival (25,26). However, the use of  $\beta$ -catenin as a prognostic marker for ovarian cancer is disputed. To the best of our knowledge, the impact of  $\beta$ -catenin expression levels on chemoresistance has not been evaluated in ovarian cancer.

The non-canonical Wnt signaling pathways involve signaling that uses downstream effectors, without mediation by  $\beta$ -catenin-TCF/LEF. In contrast to canonical Wnt signaling, these signaling pathways may have transcriptional and non-transcriptional effects (27). In the non-canonical Wnt/ $\text{Ca}^{2+}$  signaling pathway, Wnt ligands binding to Fzd receptors initiates the activation of phospholipase C via G protein-coupled receptor signaling, causing an increase in intracellular  $\text{Ca}^{2+}$ , resulting in activation of  $\text{Ca}^{2+}$ /calmodulin-dependent kinase II (CaMKII) and protein kinase C. Previous studies have identified that deregulation of the Wnt/ $\text{Ca}^{2+}$  signaling pathway mediates cytoskeleton rearrangements, cellular proliferation, cellular motility and epithelial-mesenchymal transition in cancer development and progression (Fig. 1B) (27).

In the non-canonical Wnt/JNK signaling pathway, Wnt-Fzd binding activates a number of small guanosine-5'-triphosphate (GTP)-binding proteins, including c-Jun N-terminal kinase (JNK), which affect a wide range of cellular processes, including cytoskeleton rearrangement, cell polarity, cell migration and gene expression (28). Previous studies have identified that aberrant Wnt/JNK signaling may initiate and stimulate the development of malignant phenotypes through effects on cell proliferation, survival, polarity, invasion and metastasis (Fig. 1C) (29,30).

Aberrant Wnt/ $\beta$ -catenin, Wnt/JNK and Wnt/ $\text{Ca}^{2+}$  signaling has been associated with increasing ovarian cancer grade and poor survival (21,27,29). However, to the best of our knowledge, the relationship between these signaling pathways and ovarian cancer cisplatin chemoresistance has not yet been investigated (23,29).

The present study aims to determine the expression of  $\beta$ -catenin, JNK and CaMKII in a cisplatin-sensitive ovarian

cancer cell line and a cisplatin-resistant variant, and then assess the correlation between expression of these proteins and cisplatin chemoresistance.

## Materials and methods

**Chemicals and reagents.** Cisplatin was purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Cell counting kit-8 (CCK-8; C0037) and 4',6-diamidino-2-phenylindole (DAPI; C1005) were purchased from the Beyotime Institute of Biotechnology (Haimen, China) and stored at  $-20^{\circ}\text{C}$ . Antibodies including non-phospho (active)  $\beta$ -catenin (Ser33/37/Thr41) (D13A1) rabbit mAb (#8814), SAPK/JNK rabbit mAb (#9252) and CaMKII (pan) (D11A10) Rabbit mAb (#4436) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA), and the  $\beta$ -actin mouse mAb (JT9001S) was purchased from Anbo Biotechnology Co., Ltd. (San Francisco, CA, USA). The Wnt signaling inhibitor (XAV-939) and activator (CHIR-99021) were purchased from the Selleck Chemicals (Shanghai, China), and completely dissolved in DMSO at a stock concentration (1 mM). Fluorescein isothiocyanate (FITC)-Conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (ZF-0312) and FITC-Conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (ZF-0315) were from OriGene Technologies, Inc. (Beijing, China).

**Cell culture.** The human ovarian cancer adenocarcinoma cell line SKOV3 and its cisplatin-resistant variant, SKOV3/DDP, were purchased from The Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 media, supplemented with 100 U/ml penicillin/streptomycin and 10% fetal bovine serum (FBS) at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . In addition, the SKOV3/DDP cell media included 2  $\mu\text{M}$  cisplatin to maintain chemoresistance. All of the described reagents were purchased from GE Healthcare Life Sciences (HyClone; Logan, UT, USA).

**Cell viability and cytotoxicity assay.** Cell viability was detected using the CCK-8 kit. Briefly, cells were seeded at  $5 \times 10^3$  cells/well into 96-well plates. Following 48 h incubation as same as previously described conditions, cells were treated with cisplatin, CHIR-99021, XAV-939, cisplatin + CHIR-99021 or cisplatin + XAV-939 for indicated concentrations. There were six replicate wells for each concentration, the control group contained untreated cells and in the blank group (no cells or drugs) only added 10% FBS and RPMI-1640. Cells were incubated for 24, 48 or 72 h prior to the addition of 10  $\mu\text{l}$  CCK-8 reagent and 90  $\mu\text{l}$  RPMI-1640 to each well and further culture for 1 h. The absorbance of each well at 490 nm ( $A_{490}$ ) was then analyzed using a microplate reader (Model 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The effect of the drugs on the cell survival was calculated using the following formula: Survival rate (%) =  $[(A_{490} \text{ of treated group} - A_{490} \text{ of blank group}) / (A_{490} \text{ of control group} - A_{490} \text{ of blank group})] \times 100$ . The inhibition rate (%) was calculated as:  $(100\% - \text{survival rate } \%)$ . For the concentration of cisplatin necessary to result in 50% inhibition ( $\text{IC}_{50}$ ), the data was calculated using the weighted linear regression method with SPSS 22.0 software (IBM SPSS, Armonk, NY, USA). Each experiment was repeated >3 times.

**Western blotting.** Cells were treated with cisplatin (20  $\mu$ M), CHIR-99021 (3  $\mu$ M), XAV-939 (1  $\mu$ M), cisplatin + CHIR-99021 (20 and 3  $\mu$ M, respectively), cisplatin + XAV-939 (20 and 1  $\mu$ M, respectively) or RPMI-1640 alone for 48 h, as previously described. Then, total protein was extracted by treatment with RIPA lysis buffer (P0013B; Beyotime Institute of Biotechnology) for 45 min on ice, centrifugation (10,000  $\times$  g) for 25 min at 4°C and collection of the supernatants, which were stored at -20°C until required. Total protein concentration was determined using the BCA Protein Assay Kit (P0010S; Beyotime Institute of Biotechnology). Equal quantities of protein preparations (50  $\mu$ g) were separated by 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were then blocked with 5% non-fat milk at room temperature for between 1 and 2 h and incubated overnight with primary antibodies against the following proteins:  $\beta$ -catenin (1:1,000), JNK (1:2,000), CaMKII (1:1,000) and  $\beta$ -actin (1:5,000). Next, membranes were incubated with the appropriate fluorescently-labeled secondary antibodies (1:10,000). Between each step, protein bands were washed with 1X PBST. Protein bands were detected using an Odyssey Infrared Imaging System (Gene Company, Ltd., Hong Kong, China). Quantitative analysis was performed using ImageJ analysis software (National Institutes of Health, USA) with  $\beta$ -actin serving as a control.

**Immunofluorescence staining.** Cells were cultured at  $2 \times 10^4$  cells/well into 24-well plates for 48 h and treated with cisplatin, CHIR-99021, XAV-939, cisplatin + CHIR-99021, cisplatin + XAV-939 or RPMI-1640 alone for 48 h, as previously described. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 and blocked with 5% horse serum (Vector Laboratories, Inc., Burlingame, CA, USA) in PBS-Tween for 30 min. Preparations were incubated with primary antibodies [ $\beta$ -catenin (1:200), JNK (1:200) and CaMKII (1:400)] as previously described at 4°C overnight and then with the appropriate fluorescently-labeled secondary antibodies (previously described; 1:1,000) for 60 min at room temperature. In addition, nuclei were stained with DAPI. Between each step, protein bands were washed with 1X PBST. Preparations were visualized and images acquired using the EVOS XL Cell Imaging System (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

**Statistical analysis.** Data are presented as means  $\pm$  the standard deviation. Data was analyzed using SPSS software (version 22.0). Differences between groups were statistically analyzed using the Student's t-test or a paired t-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Cisplatin sensitivity in SKOV3 and SKOV3/DDP cell lines.** To determine the sensitivity of SKOV3 and SKOV3/DDP cells to cisplatin, cells were exposed to a gradient of cisplatin concentrations (0-80  $\mu$ M) for 24, 48 or 72 h and cell viability was measured using the CCK-8 kit. The inhibition rate of SKOV3 and SKOV3/DDP cells with cisplatin was found to increase in a time-dependent and dose-dependent manner (Fig. 2A-C). There was a significant difference between the concentrations of cisplatin required for  $IC_{50}$  after 48 h in SKOV3 and SKOV3/DDP

cells (6.086 $\pm$ 0.38 vs. 26.135 $\pm$ 1.825  $\mu$ M, respectively;  $P < 0.05$ ; Fig. 2D), highlighting that SKOV3/DDP cells were significantly cisplatin-resistant compared with SKOV3 cells. From the analysis of these results, a dose of 20  $\mu$ M cisplatin and an exposure time of 48 h were used in subsequent studies.

**Expression of  $\beta$ -catenin, JNK and CaMKII in SKOV3 and SKOV3/DDP cell lines.** To determine if cisplatin chemoresistance seen in ovarian cancer cells was associated with deregulation of Wnt signaling pathways, the present study evaluated the protein expression levels of  $\beta$ -catenin, JNK and CaMKII in SKOV3 and SKOV3/DDP cell lines. Quantification of western blotting results (Fig. 3A) identified that expression levels of  $\beta$ -catenin and JNK were significantly higher in SKOV3/DDP cells compared with SKOV3 cells ( $^*P < 0.01$ ,  $^{\#}P < 0.05$ ; Fig. 3B). However, CaMKII protein expression levels were lower in SKOV3/DDP cells compared with SKOV3 cells. Following incubation with 20  $\mu$ M cisplatin for 48 h,  $\beta$ -catenin expression was reduced in SKOV3 and SKOV3/DDP cells ( $^*P < 0.01$ ,  $^{\#}P < 0.001$ ; Fig. 3B). Similarly, JNK protein expression levels were significantly decreased in SKOV3 and SKOV3/DDP cells following treatment with 20  $\mu$ M cisplatin ( $^*P < 0.05$ ,  $^{\#}P < 0.001$ ; Fig. 3B). In contrast, cisplatin treatment significantly increased CaMKII protein expression levels in SKOV3 and SKOV3/DDP cells ( $^{\#}P < 0.001$ ,  $^*P < 0.01$ ; Fig. 3B). The difference in CaMKII expression between SKOV3 and SKOV3/DDP cells was significant following cisplatin treatment ( $^*P < 0.05$ ; Fig. 3B). Similar results were observed in immunofluorescence stains (Fig. 3C-E). These results suggest that abnormal activation of Wnt/ $\beta$ -catenin and Wnt/JNK signaling pathways, and the abnormal inactivation of the Wnt/ $Ca^{2+}$  signaling pathway, may be associated with cisplatin chemoresistance in SKOV3/DDP cells.

**Association between Wnt/ $\beta$ -catenin, Wnt/JNK and Wnt/ $Ca^{2+}$  signaling pathways and ovarian cancer cisplatin-resistance.** To confirm the association observed between the deregulation of Wnt signaling pathways and cisplatin-resistance in ovarian cancer cells, CHIR-99021 (glycogen synthase kinase 3 $\beta$  inhibitor, GSK3 $\beta$ ) or XAV-939 (tankyrase inhibitor) were used in addition to cisplatin to specifically activate or inhibit the Wnt/ $\beta$ -catenin signaling pathway, respectively. The effect of these drugs on SKOV3/DDP cell proliferation was then examined using the CCK-8 kit. Compared with the control group without any drugs, the majority of doses of CHIR-99021 or XAV-939 could not induce significant cell death (Fig. 4A and B). However, 3  $\mu$ M CHIR-99021 combined with cisplatin significantly suppressed the cytotoxicity of cisplatin ( $P < 0.05$ ; Fig. 4A) and 1  $\mu$ M XAV-939 combined with cisplatin significantly increased the cytotoxicity of cisplatin ( $P < 0.05$ ; Fig. 4B).

Next, SKOV3/DDP cells were treated with or without cisplatin in the presence or absence of 3  $\mu$ M CHIR-99021 or 1  $\mu$ M XAV-939, and the expression levels of  $\beta$ -catenin, JNK and CaMKII were examined by western blotting and immunofluorescence staining. Compared with the untreated group, the level of  $\beta$ -catenin was increased by CHIR-99021 treatment (Fig. 4C and D). Furthermore, CHIR-99021 significantly reversed the loss of  $\beta$ -catenin seen in cisplatin-treated cells ( $^*P < 0.05$ ; Fig. 4D), reduced cisplatin-induced loss of JNK ( $^*P < 0.05$ ) and significantly reduced cisplatin-induced upregulation of

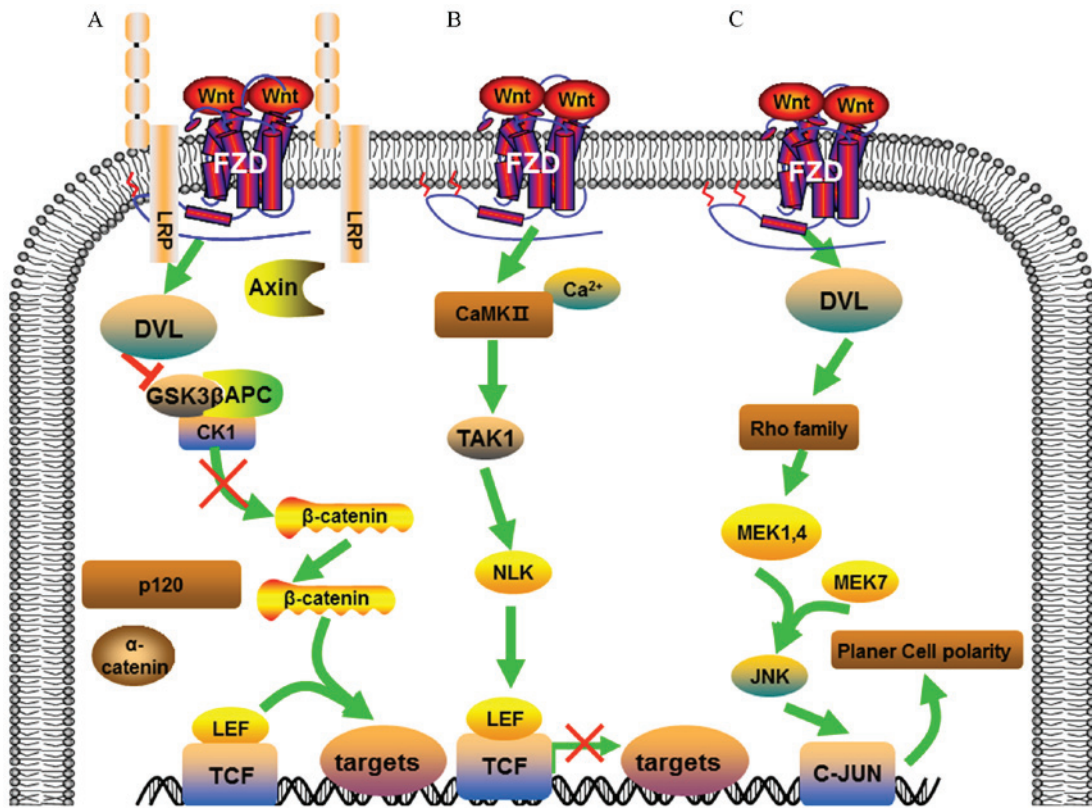


Figure 1. Illustration of the Wnt signaling pathways. (A) Wnt/ $\beta$ -catenin signaling pathway. Wnt ligands bind to Fzd/LRP-5/6, increasing stabilization and accumulation of  $\beta$ -catenin.  $\beta$ -catenin associates with TCF/LEF to control expression of target genes. (B) Wnt/ $\text{Ca}^{2+}$  signaling pathway. Wnt activates intracellular  $\text{Ca}^{2+}$  release and  $\text{Ca}^{2+}$ -dependent protein kinases, such as CaMKII. TAK1 and NLK interfere with  $\beta$ -catenin/TCF signaling pathway. (C) Wnt/JNK signaling pathway. Receptor stimulation activates DVL, resulting in the activation of Rho family GTPases. Rho triggers c-Jun expression through phosphorylation of JNK. FZD, frizzled; LRP, low-density lipoprotein receptor-related protein-5/6; DVL, dishevelled; GSK3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; APC, adenomatous polyposis coli; CK1, casein kinase 1; TCF/LEF, transcription factor/lymphoid enhancer-binding factor; CaMKII,  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II; TAK1, TGF- $\beta$ -activated kinase 1; NLK, serine/threonine protein kinase NLK; MEK MAPK/ERK kinase; JNK, c-Jun N-terminal kinase.

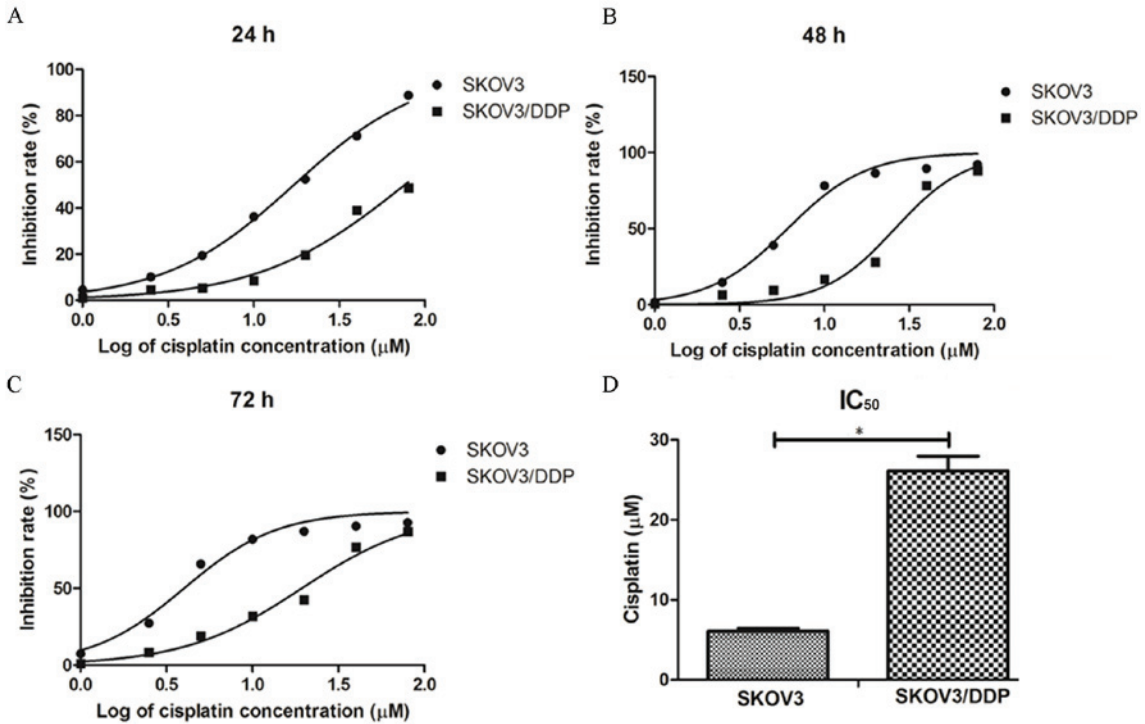


Figure 2. SKOV3 and SKOV3/DDP cell sensitivity to cisplatin. The inhibition rate of SKOV3 and SKOV3/DDP cells was measured following treatment with 0.0, 0.5, 1.0, 1.5 and 2.0  $\mu\text{M}$  cisplatin for (A) 24, (B) 48 and (C) 72 h. (D) SKOV3/DDP and SKOV3 cells were treated with cisplatin for 48 h and the  $\text{IC}_{50}$  of cisplatin in two cell lines was analyzed, with results expressed as the mean  $\pm$  standard deviation. \* $P < 0.05$ .  $\text{IC}_{50}$ , the concentration of the inhibitor required for 50% inhibition.

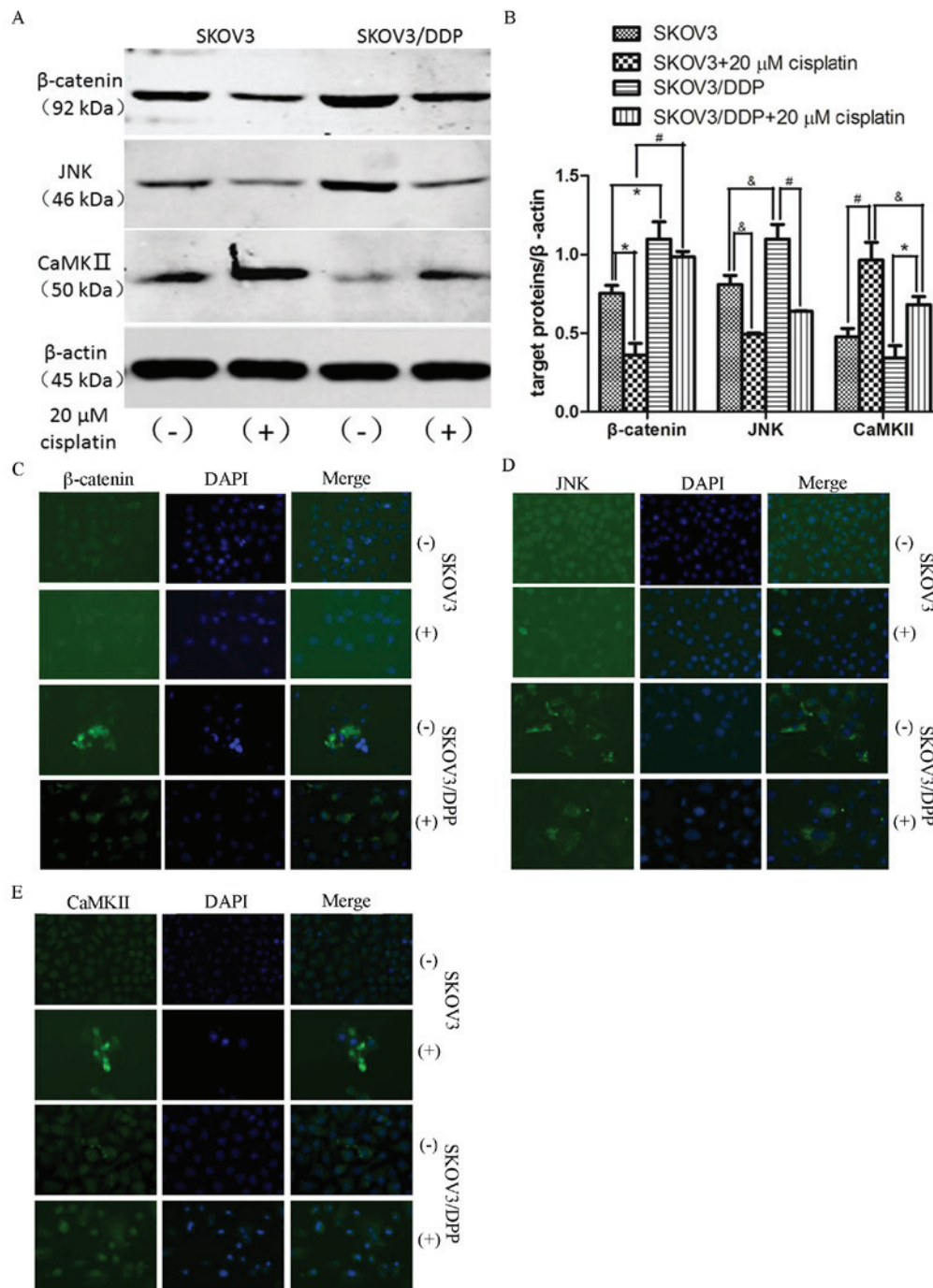


Figure 3. Expression of β-catenin, JNK and CaMKII in SKOV3 and SKOV3/DDP cells with and without the presence of cisplatin. (A) Western blot for β-catenin, JNK and CaMKII proteins from cells treated with cisplatin (20 μM) for 48 h and untreated cells. (B) Quantification of protein expression levels from western blotting. Each protein was normalized to β-actin. \*P<0.05, †P<0.01, ‡P<0.001. In addition, immunofluorescence staining for the expression and localization of (C) β-catenin, (D) JNK and (E) CaMKII proteins with and without cisplatin (20 μg) was performed. JNK, c-Jun N-terminal kinase; CaMKII, Ca<sup>2+</sup>/calmodulin-dependent protein kinase II; DAPI, 4',6-diamidino-2-phenylindole.

CaMKII (†P<0.001; Fig. 4D). In contrast, XAV-939 significantly decreased expression levels of β-catenin and JNK (†P<0.001), and significantly increased expression levels of CaMKII in cells (†P<0.01), with and without co-treatment of cisplatin (Fig. 4E and F). Interestingly, the expression levels of β-catenin and JNK were positively correlated, and the level of CaMKII was negatively correlated with β-catenin and JNK expression levels (Fig. 4E and F). Similar effects of CHIR-99021 and XAV-939 were also observed in immunofluorescence stains (Fig. 5).

These results indicate that the Wnt/β-catenin signaling pathway activation antagonizes cisplatin-induced cell death, while the Wnt/β-catenin signaling pathway inhibition enhances the cytotoxic effect of cisplatin on ovarian cancer cells.

### Discussion

Ovarian cancer is the leading cause of mortality among gynecological cancers (1). In addition to cytoreductive surgery,

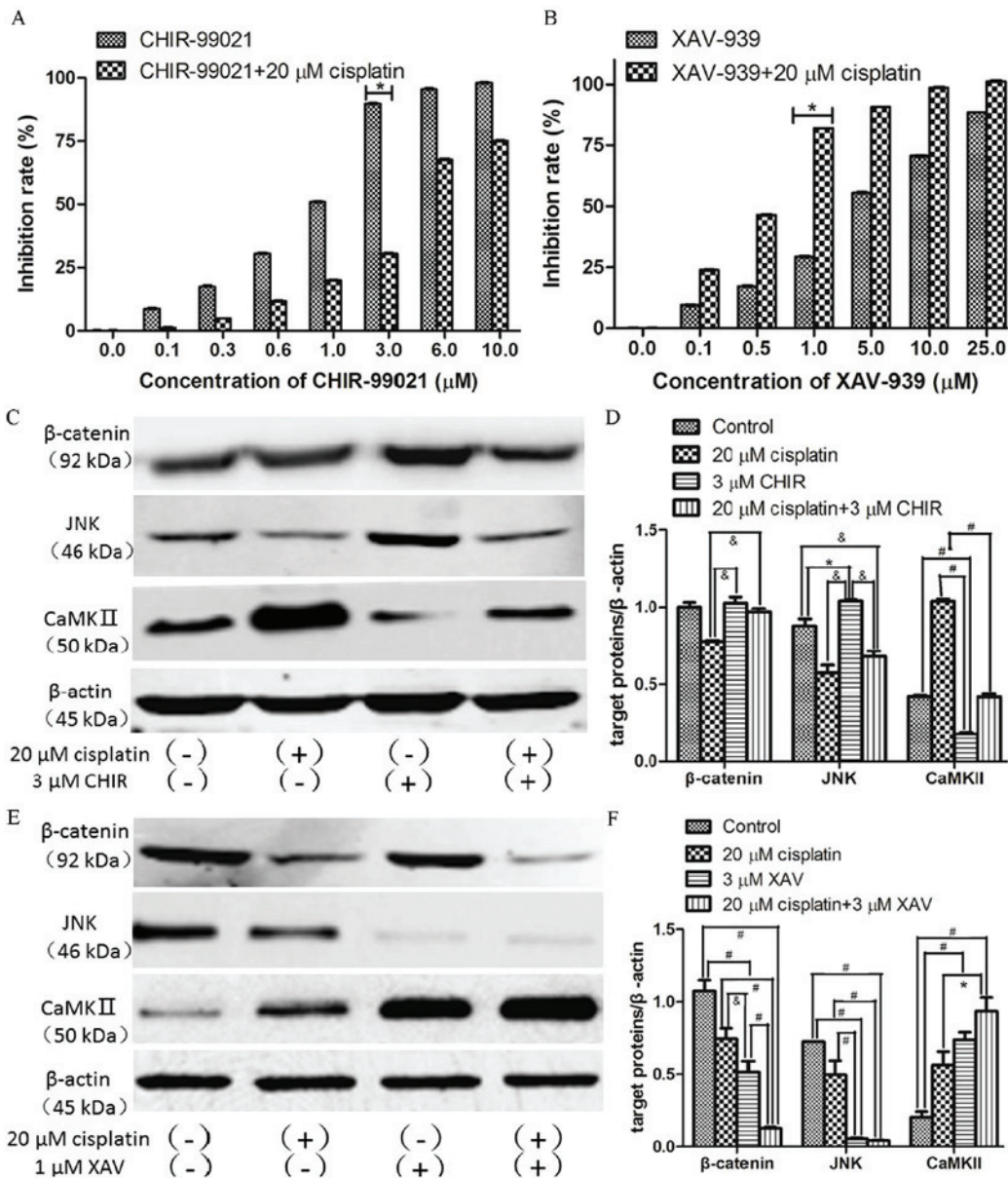


Figure 4. Expression of  $\beta$ -catenin, JNK and CaMKII in SKOV3/DDP cells following treatment with CHIR-99021 or XAV-939 with and without cisplatin. SKOV3/DDP cells were treated with a range of concentration of (A) CHIR-99021 or (B) XAV-939 alone or in combination with 20  $\mu$ M cisplatin for 48 h. (C) Western blot for  $\beta$ -catenin, JNK and CaMKII proteins from SKOV3/DDP cells treated with CHIR-99021 (3  $\mu$ M), cisplatin (20  $\mu$ M) or both for 48 h. (D) Quantification of protein expression levels from previous western blot. Each protein was normalized to  $\beta$ -actin. (E) Western blot for  $\beta$ -catenin, JNK and CaMKII proteins from SKOV3/DDP cells treated with XAV-939 (1  $\mu$ M), cisplatin (20  $\mu$ M) or both for 48 h. (F) Quantification of protein expression levels from previous western blot. Each protein was normalized to  $\beta$ -actin. \* $P$ <0.05,  $^{\#}P$ <0.01,  $^{\&}P$ <0.001. JNK, c-Jun N-terminal kinase; CaMKII, Ca<sup>2+</sup>/calmodulin-dependent protein kinase II; CHIR, CHIR-99021; XAV, XAV-939; DAPI, 4',6-diamidino-2-phenylindole.

cisplatin-based chemotherapy is one of the most important therapeutic strategies for ovarian cancer (2). Previous studies have shown that Wnt signaling pathway deregulation correlates with ovarian cancer cell proliferation, survival, invasion and metastasis (25,26,28,30,31). Furthermore, cisplatin chemoresistance has been an obstacle in the successful treatment of ovarian cancer (8,32,33). Several studies have concluded that aberrant regulation of Wnt signaling induces tumor cell chemoresistance (16-18). However, the molecular mechanisms by which Wnt signaling leads to cisplatin chemoresistance remain poorly understood.

In the present study, a specific Wnt/ $\beta$ -catenin signaling pathway activator (CHIR-99021) and inhibitor (XAV-939) were

used to explore the association between the deregulation of Wnt signaling and cisplatin-resistance in ovarian cancer cells. Firstly, the human ovarian cancer adenocarcinoma cell line SKOV3 and its cisplatin-resistant variant, SKOV3/DDP, were selected as cell models. The IC<sub>50</sub> value of cisplatin in SKOV3/DDP cells was approximately four-fold that of SKOV3 cells, showing that SKOV3/DDP cells were more cisplatin-resistant. Thus, these cell lines are suitable models for the exploring cisplatin chemoresistance.

Previous studies of the endometrioid subtype of ovarian cancer have found mutations of CTNNB1 and abnormal expression of  $\beta$ -catenin (22,23). The present study demonstrated that  $\beta$ -catenin and JNK expression levels were increased in

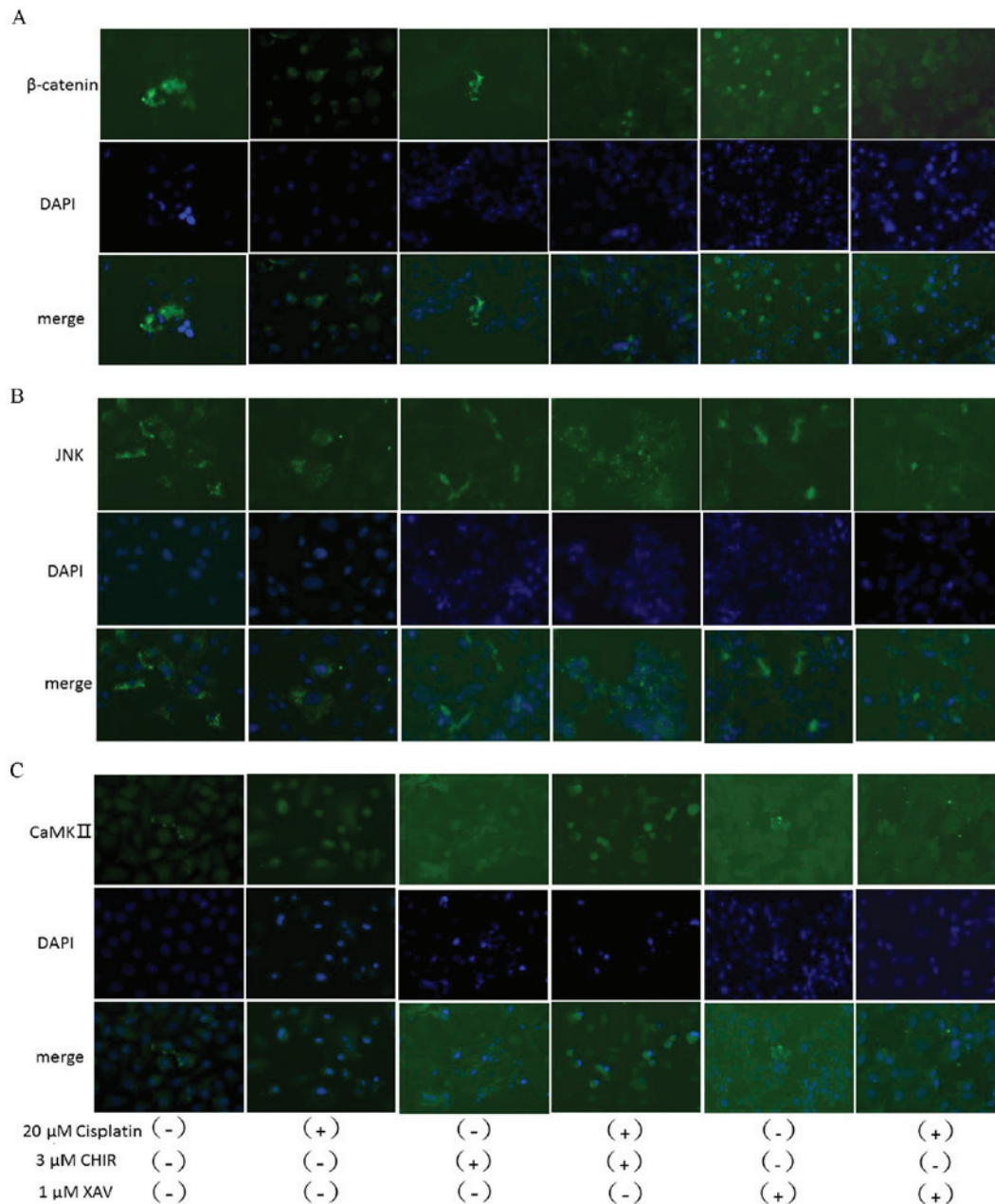


Figure 5. Immunofluorescence staining for the expression and localization of  $\beta$ -catenin, JNK and CaMKII treated with and without cisplatin, CHIR-99021, XAV-939 or a combination. SKOV3/DDP cells were immunofluorescence stained for (A)  $\beta$ -catenin, (B) JNK or (C) CaMKII following treatment with cisplatin (20  $\mu$ M), CHIR-99021 (3  $\mu$ M), XAV-939 (1  $\mu$ M) or a combination for 48 h. JNK, c-Jun N-terminal kinase; CaMKII, Ca<sup>2+</sup>/calmodulin-dependent protein kinase II; CHIR, CHIR-99021; XAV, XAV-939; DAPI, 4',6-diamidino-2-phenylindole.

cisplatin-resistant compared with cisplatin-sensitive ovarian cancer cells. In contrast, CaMKII expression levels were lower in cisplatin-resistant ovarian cancer cells.

The correlation of the Wnt/ $\beta$ -catenin and non-canonical Wnt signaling pathways in cisplatin chemoresistance is controversial. Some investigators believe that both canonical and noncanonical Wnt signaling pathways may promote each other in cisplatin chemoresistance (34). However, other research suggests that the Wnt/ $\beta$ -catenin signaling pathway is crucial to cisplatin chemoresistance and that the non-canonical Wnt signaling pathways obstruct this action (35-37). In the current study, the Wnt/ $\beta$ -catenin signaling pathway agonist CHIR-99021, which specifically inhibits GSK3 $\beta$ , was used to investigate the correlation between the Wnt/ $\beta$ -catenin and

non-canonical Wnt signaling pathways. The SKOV3/DDP cell inhibition rate with CHIR-99021 in combination with cisplatin was lower compared with that of cisplatin alone. The level of  $\beta$ -catenin was increased as a result of CHIR-99021 treatment, due to Wnt/ $\beta$ -catenin signaling pathway activation. In addition, CHIR-99021 in combination with cisplatin enhanced expression of JNK and significantly decreased the expression of CaMKII compared with cisplatin alone. Similarly, Zhao *et al* (21) found an association between high expression levels of  $\beta$ -catenin and cisplatin chemoresistance in A2780/DDP cells. This suggests that interference with  $\beta$ -catenin expression could partly reverse cisplatin chemoresistance in ovarian cancer cells.

SKOV3/DDP cells co-treated with XAV-939, a selective Wnt/ $\beta$ -catenin signaling pathway inhibitor, and cisplatin

produced a greater inhibition of proliferation than when applied alone. Expression of  $\beta$ -catenin and JNK was significantly reduced, and expression of CaMKII was significantly higher, in SKOV3/DPP cells following co-treatment with XAV-939 and cisplatin compared with untreated cells or cells treated with cisplatin alone. Furthermore, the expression levels of  $\beta$ -catenin and JNK were positively correlated, while the level of CaMKII was negatively correlated with of  $\beta$ -catenin and JNK expression levels.

In conclusion, the results of the present study identify that, in SKOV3/DDP cells, the Wnt/ $\beta$ -catenin and Wnt/JNK signaling pathways are positively correlated with cisplatin chemoresistance, while the Wnt/ $\text{Ca}^{2+}$  signaling pathway is negatively correlated with cisplatin chemoresistance. This suggests that inhibiting the Wnt/ $\beta$ -catenin and Wnt/JNK signaling pathways, and activating the Wnt/ $\text{Ca}^{2+}$  signaling pathway, could reverse cisplatin-resistance in ovarian cancer cells. Developing specific inhibitors and activators for these signaling pathways may provide a treatment for cisplatin-resistant ovarian cancer, and the key players of canonical ( $\beta$ -catenin) and non-canonical (JNK and CaMKII) Wnt signaling are potential targets for drug development.

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