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Proliferation inhibition of cisplatin and aquaporin 5 expression in human ovarian cancer cell CAOV3

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Abstract
Aims The purpose of this study is to investigate the association between proliferation inhibition of cisplatin and aquaporin 5 (AQP5) expression and its regulation in ovarian carcinoma cell CAOV3.
Methods Cell growth rate was measured by MTT after CAOV3 cells were incubated with cisplatin or NF-κB inhibitor PDTC. Western blot and RT-PCR were used to detect the expression of AQP5 and NF-κB p65.
Results Our results showed that expression of AQP5, NF-κB in cytoplasm and karyon and IκBα in cytoplasm protein in CAOV3 cells can be induced to decrease by cisplatin with concentration-dependent manner, and there is a positive correlation between AQP5 protein and cell growth rate ($r = 0.607$, $P < 0.05$). When cells were incubated with 10 μg/ml cisplatin, AQP5, NF-κB p65, and IκBα increased rapidly at 6–12 h, but decreased at 24 h, remain on low level until to 72 h. Expression of AQP5 could be induced to decrease by PDTC, and a positive correlation between AQP5 protein expression and NF-κB p65 and IκBα ($r = 0.894, 0.857$; $P < 0.05$).
Conclusions Proliferation inhibition of cisplatin is related with AQP5 expression, and NF-κB may be involved in mechanism of AQP5 regulation. AQP5 will be potential target for therapy of ovarian carcinoma.

Keywords Saquaporin 5 · Cisplatin · NF-kappaB · Ovarian neoplasms

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The aquaporins (AQPs) are a family of integral membrane proteins that selectively transport water and small solutes. Thirteen mammalian AQPs have been molecularly identified and localized to various epithelial, endothelial, and other tissues. Although the role of AQPs in human pathology has been explored extensively, only recently has its role in cancer become an area of interest. Studies have showed that AQPs were over-expressed in malignant tumors of different tissues, and which may be involved in tumor angiogenesis and cell migration [1, 2]. Aquaporin 5(AQP5) was found in various epitheliums including salivary, lancrima l, airway submucosal glands, pancreatic epithelium, etc. [3–6]. Shen et al. reported that AQP5 was over-expressed in lung cancer tissues, and up-regulation of AQP5 expression in lung cancer tissue was associated with poor prognosis [7]. Zhang et al. showed that expression level of AQP5 protein in intestinal type of adenocarcinoma was significantly higher than that in accompanying normal mucosa, and AQP5 expression was associated with lymph node metastasis and lymphovascular invasion in patients [8]. Watanabe et al. [9] also found that up-regulation of AQP5 might be involved in differentiation of human gastric cancer cells. We also have previously found that AQP5 expression in ovarian malignant and borderline tumors was significantly higher than that of benign ovarian tumors and normal ovarian tissue, and increased AQP5 protein level was associated with lymph node metastasis and ascites, which suggested that AQP5 may play an important role in tumorigenesis of epithelial ovarian tumors [10].

Nuclear factor-κB (NF-κB), a eukaryotic transcription factor, takes part in inflammatory reaction, immune response as well as cell proliferation and apoptosis. It is not only related with the genesis of tumors but also influences chemo-sensitivity of tumors and patients prognosis to different degrees. NF-κB inhibitor IB suppresses NF-κB

nuclear localization signals via binding NF-κB, therefore, inhibits the activity of NF-κB [11]. To elucidate the role of AQP5 on cisplatin's cell proliferation of ovarian caner and its mechanism, cisplatin and NF-κB inhibitor PDTC (ammonium pyrrolidine dithiocarbamate) were used to test their effects on expression of AQP5, NF-κB p56, and inhibitor of κBα (IκBα) in ovarian cancer cells CAOV3.

Methods

Cell culture and growth rate experiment

The human ovarian carcinoma (CAOV3) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). CAOV3 cells were cultured in RPMI 1640 medium (Gibco, BRL, US) supplemented with 15% fetal bovine serum and maintained at 37°C and 5% CO_2 in a humid environment. Cells were treated with one of the following solutions: (1) RPMI 1640 medium alone (the control group); (2) 2.5, 5, 10, or 20 μg/ml cisplatin for 24 h, and 10 μg/ml cisplatin for 6, 12, 24, 48, or 72 h; (3) 0.1, 1, 10 mM PDTC for 24 h, and 10 mM for 12, 24, and 48 h.

Cell growth rate was measured by MTT assay. Cells were seeded in 96-well plates for 24 h and then exposed to various concentrations of cisplatin for 24–48 h. The cells were incubated with 10 μl of 2 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma, US) 4 h at 37°C, and the formazan crystals were lysed with 150 μl of dimethyl sulfoxide (DMSO, Sigma, US) for 10 min. Absorbance at 490 nm was then measured with a microplate reader (Bio-Rad, 680, Hercules, CA). Normal cell culture and no cell wells were set as control. All concentrations were tested in triplicates. Absorbance values were translated to growth rate by the equation: (OD of test concentration − OD of no cell wells)/(OD of control − OD of no cell wells).

Western blotting analysis

Following drugs treatments, CAOV3 cells were washed twice with cold phosphate-buffered saline (PBS, pH 7.2) and scraped. Cells were lysed in ice-cold RIPA buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate (SDS), 0.01% phenylmethylsulfonyl fluoride (PMSF), and 0.1 μg/ml leupeptin. Total protein concentrations were measured by the Bio-Rad protein test kit (Bio-Rad Laboratories Inc., Hercules, CA). Equal volume of protein (50 μg) was subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then the proteins were transferred onto nitrocellulose membranes. After 1 h blocking with 5% non-fat milk powder in Tween-20 TBS (TBS-T) at room temperature, the membranes were incubated with 1:400 dilution of AQP5, NF-κB p65, and IκBα as well as 1:1000 dilution of β-actin primary antibody (Santa Cruz, Santa Cruz, CA, USA) at 4°C overnight. After being washed three times for 10 min each with TBS-T, The membranes were incubated 1 h at room temperature with 1:5000 dilution of the second antibody labeled horseradish peroxidase (HRP) (Santa Cruz, Santa Cruz, CA, USA). The immunoreactive band was detected using a western blotting luminol reagent (Santa Cruz, Santa Cruz, CA, USA). The membranes were exposed to X-Ar-2 films (Eastman Kodak Co., Rochester, NY) and scanned by using a GS-800 (Bio-Rad). The density of each protein band was quantified with Bio-Rad one quantity software analysis system. AQP5, NF-κB p65, and IκBα protein expression was calculated as the ratio of each specific band to β-actin (internal protein standard).

RT-PCR

RT-PCR was used to examine the expression of AQP5 mRNA. Total RNA were isolated from CAOV3 cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA), reverse transcription were carried out with 3 μg RNA at 37°C using random primers and M-MLV reserve transcriptase (Fermentas, Amherst, NY) according to the manufacturer's instructions. The PCR for AQP5 were performed in a final volume of 25 μl using 0.125 U TaKaRa Taq™ (TaKaRa Biotechnology, Dalian, China), 0.5 μM each of the primers, and 200 μM dNTP in 10 nM Tris–HCl buffer (pH 8.3) containing 50 nM KCl and 1.5 nM $MgCl_2$. β-Actin as a house keeping gene was used here for endo-reference control. The sequences of the AQP5 primers are as follows: sense, 5′-CTTCCTCAAGGCCG TGTTC-3′ and anti-sense, 5′-GCTGG AAGGTC AGAATCAGC-3′, amplifying a 398 kb product. β-actin: sense, 5′-GTGGGGCGCCC CAGG CACCA-3′, and anti-sense, 5′-CTCCTTAATGT CACG CACGATTTC-3′, amplifying a 534 bp product. PCR was performed in a DNA thermal cycle, the following conditions were used: 35 cycles for 45 s at 94°C, 45 s at 57°C, 45 s at 72°C. 10 μl of PCR products mixed with 2 μl of loading buffer was electrophoretically separated on 2% agarose gel and were visualized with ethidium bromide. The agarose gel image was captured by using Kodak EDAS 290 (Eastman Kodak Company, New Haven, CT). The intensity of each DNA band was quantified with Kodak 1D 3.5 soft analysis systems. The ratio of each band to control band represented the relative quantity of AQP5 mRNA expression.

Statistical analysis

Values are the mean ± SEM of at least three or four independent experiments. Statistics analysis was performed by

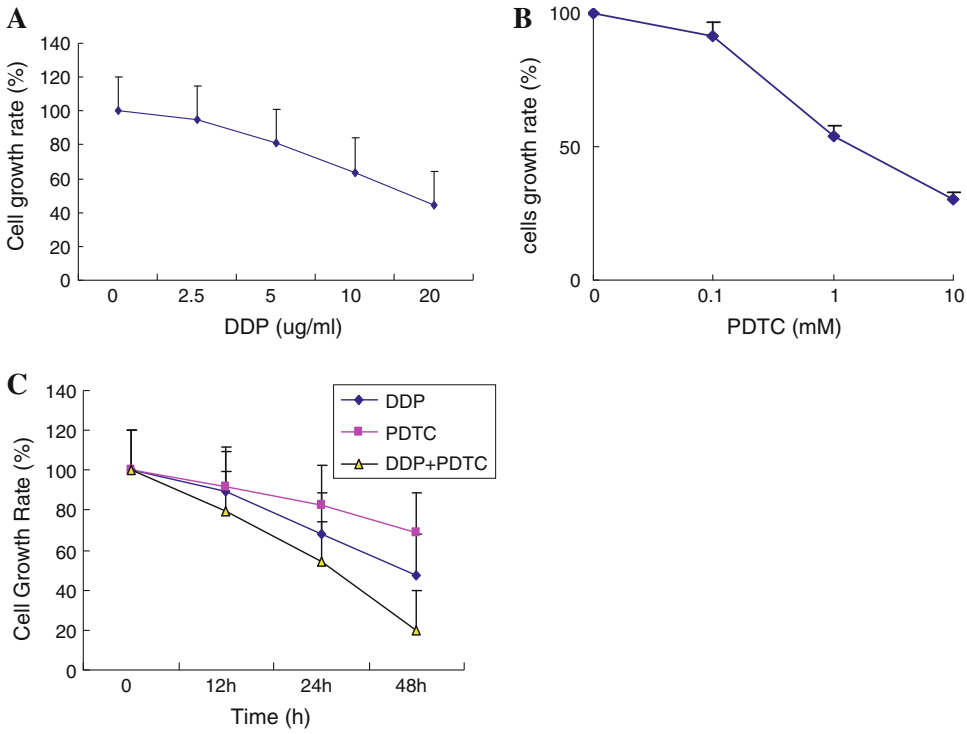


Fig. 1 Growth rate of ovarian cancer cells treated with cisplatin or nuclear factor kappaB(NF-κB) inhibitor PDTC in various concentration and different time. **a** CAOV3 cells were treated with 2.5, 5, 10, or 20 μg/ml cisplatin, respectively, for 24 h. **b** Cells were treated with 0.1, 1, or 10 nM PDTC, respectively. **c** CAOV3 cells were treated with 10 μg/ml cisplatin or 10 nM PDTC or a combination of both two for 6, 12, 24, and 48 h. Cell growth rate was measured by MTT assay

one-way factorial ANOVA combined with Scheffe's test for all comparison pairs or Student's *t* test with equal variance. Differences with *P* values <0.05 were considered significant.

Results

Effect of cisplatin on AQP5 protein expression in ovarian carcinoma CAOV3 cells

CAOV3 cells were treated with various concentrations of cisplatin for various time points to determine whether the response to cisplatin was dose- and time-dependent. Cells were incubated in media supplemented with 2.5, 5, 10, or 20 μg/ml cisplatin for designed time. Results showed that growth rate in CAOV3 cells treated by cisplatin were significantly decreased in concentration and time-dependent manner. APQ5 protein level was decreased significantly with 5, 10, or 20 μg/ml cisplatin in a dose-dependent manner (Fig. 1). APQ5 protein was decreased maximally after treatment with 10 μg/ml cisplatin, therefore this concentration was used for subsequent experiments. To analyze time course of cisplatin-mediated inhibition of AQP5 expression, CAOV3 cells were treated with cisplatin for various time points before isolation of AQP5 protein. AQP5 protein was increased significantly after 6 and 12 h of cisplatin treatment. The maximal increase in AQP5 protein level was observed after 12 h. AQP5 protein was decreased to dramatically to about 40–50% of control level after 24, 48, and 72 h. (Fig. 2)

Effect of cisplatin on NF-κB p65 protein expression in ovarian carcinoma CAOV3 cells

When CAOV3 cells were incubated in media supplemented with various concentration of cisplatin for 24 h, NF-κB p65 protein level in cytoplasm was decreased significantly with 10 or 20 μg/ml cisplatin treatment. The effect of cisplatin on NF-κB p65 protein in karyon was decreased visibly on a dose-dependent manner, which was similar to its cytosol protein. When cells were treated with cisplatin for various time points, NF-κB p65 protein in cytoplasm and karyon was increased significantly after 6 and 12 h. However, NF-κB p65 protein in cytoplasm and karyon was decreased to dramatically to about half of control level with cisplatin treatment after 48 and 72 h (Fig. 3).

Effect of cisplatin on IκBα protein expression in ovarian carcinoma CAOV3 cells

IκBα protein level in cytoplasm and karyon was decreased significantly with 10 or 20 μg/ml cisplatin in a dose-dependent manner. IκBα protein expression in cytoplasm at 2.5 μg/ml cisplatin was increased, but decreased in karyon. The effect of cisplatin on IκBα protein in CAOV3 cells was similar to NF-κB p65 protein. When CAOV3 cells were treated with cisplatin for various times, there was a difference

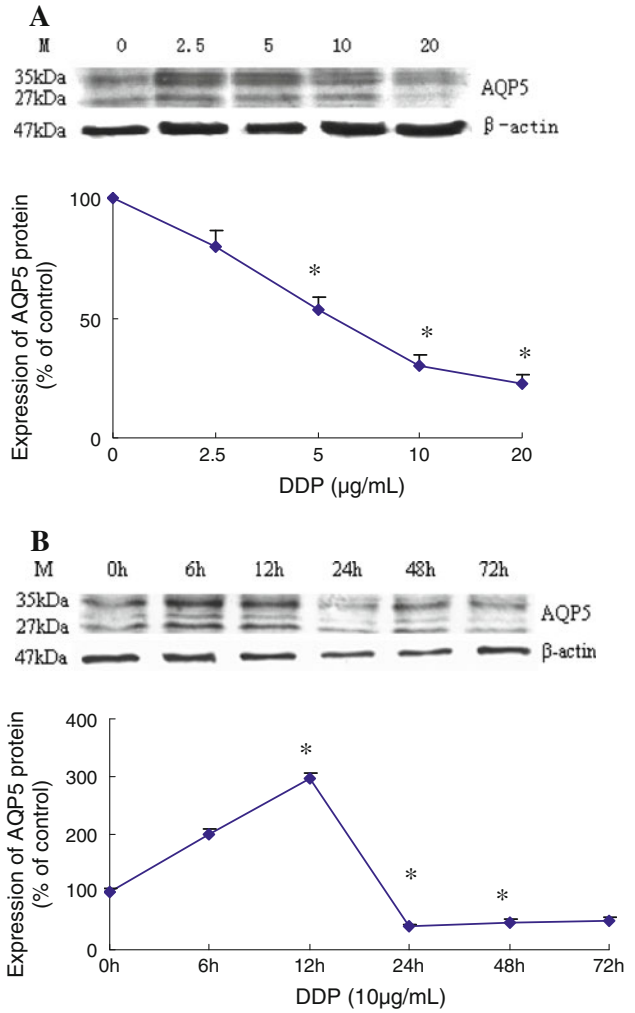


Fig. 2 Aquaporin 5(AQP5) protein expression with cisplatin treatment in ovarian cancer cells. **a** CAOV3 cells were incubated in medium containing in different concentration (0, 2.5, 5, 10, or 20 μg/ml) cisplatin for 24 h. **b** CAOV3 cells were incubated in medium containing in 10 μg/ml cisplatin for designated time (0, 3, 6, 12, 24, 48, and 72 h). Cells were processed for western blot with affinity-purified AQP5 antibody. Western blots ($n = 4$ for each group) were analyzed by densitometry (normalized with respect to β-actin). *$P < 0.05$ versus control (one-way factorial ANOVA combined with Scheffe's test)

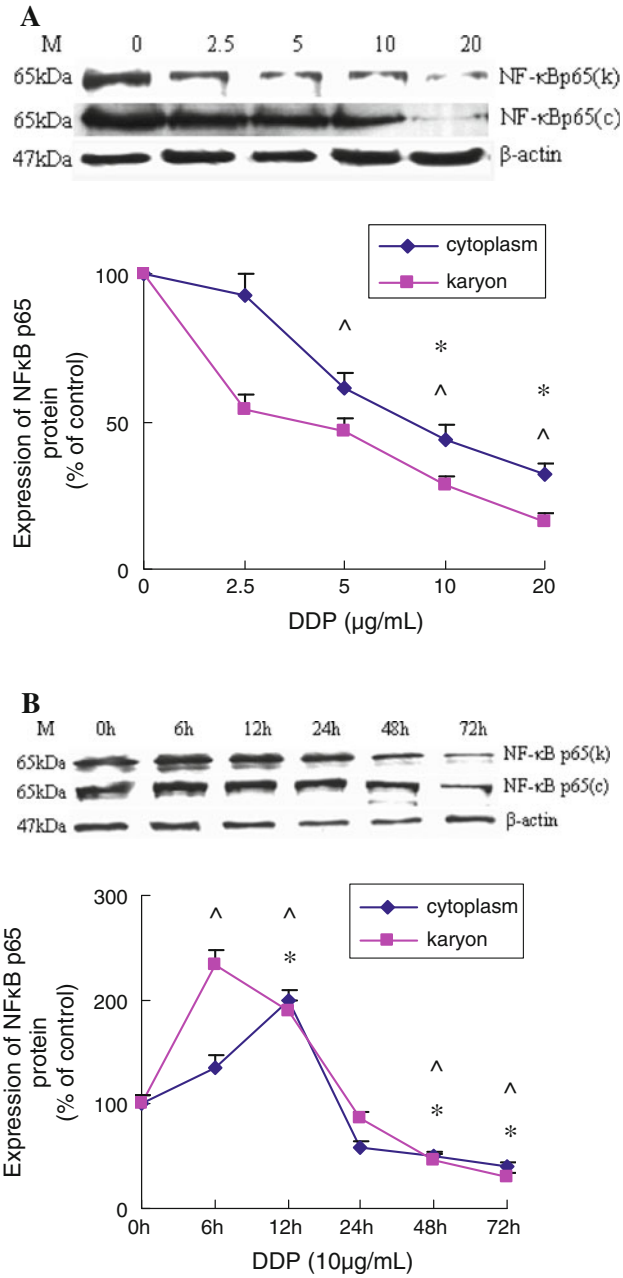


Fig. 3 NF-κB p65 protein expression with cisplatin treatment in ovarian cancer cells. **a** CAOV3 cells were incubated in medium containing in different concentration (0, 2.5, 5, 10, or 20 μg/ml) cisplatin for 24 h. **b** CAOV3 cells were incubated in medium containing in 10 μg/ml cisplatin for designated time (0, 6, 12, 24, 48, and 72 h). Cytosol and nuclear protein were extracted using Nuclear/Cytosol Fractionation Kit. Western blots ($n = 4$ for each group) were analyzed by densitometry (normalized with respect to β-actin), and results expressed as a mean ± SEM percentage of the control value (concentration 0 value). * or ^ $P < 0.05$ versus control (one-way factorial ANOVA combined with Scheffe's test). *Cytoplasm protein (c), ^ karyon protein (k)

of IκBα protein expression in cytoplasm and karyon. IκBα protein in cytoplasm was increased significantly after 6 h with cisplatin treatment. But when cells were treated with cisplatin for 24 h or longer, IκBα protein in cytoplasm was decreased dramatically to about 50%. IκBα protein expression in karyon was decreased at every time point, but significantly decreased at 48 h of treatment (Fig. 4).

Role of the NF-κB on cells proliferation and AQP5 expression

NF-κB inhibitor PDTC was used to determine if the NF-κB pathway was involved in regulation of AQP5 expression. CAOV3 cells were incubated with various concentrations of PDTC for designed time. Results showed that cell growth rate was significantly decreased in concentration

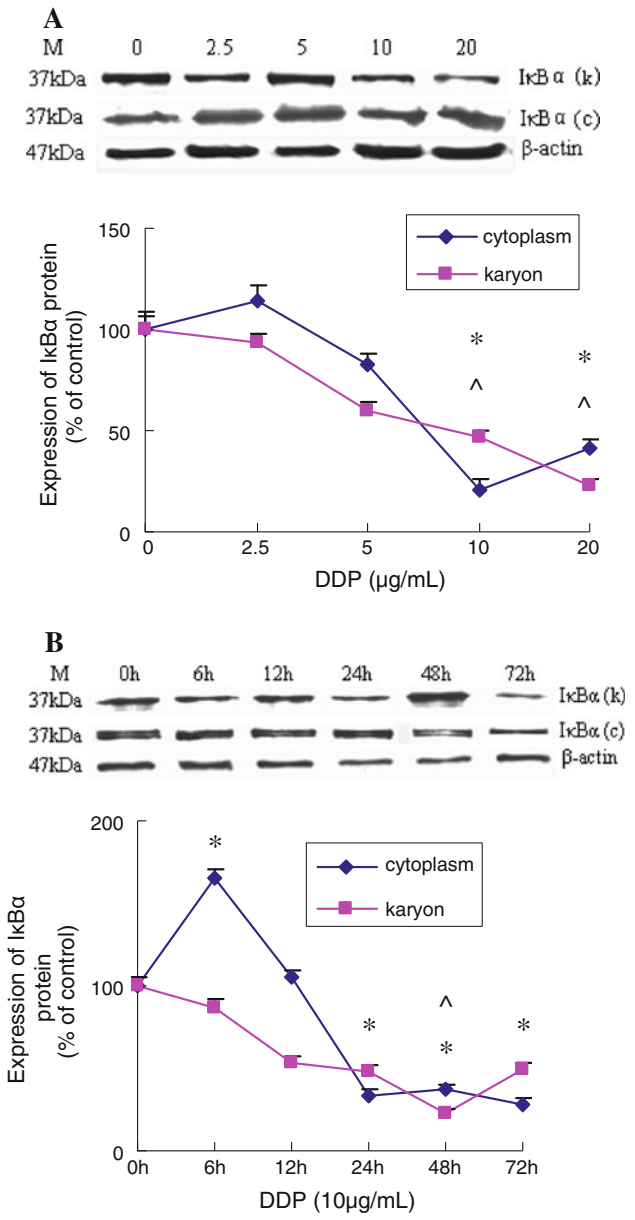


Fig. 4 IκBα protein expression with cisplatin treatment in ovarian cancer cells. **a** CAOV3 cells were incubated in medium containing in various concentrations cisplatin for 24 h. **b** CAOV3 cells were incubated in medium containing in 10 μg/ml cisplatin for designated time. Cytosol and nuclear protein were extracted using Nuclear/Cytosol Fractionation Kit. Western blots ($n = 4$ for each group) were analyzed by densitometry (normalized with respect to β-actin), and results expressed as a mean ± SEM percentage of the control value (concentration 0 value). * or ^ $P < 0.05$ versus control (one-way factorial ANOVA combined with Scheffe's test). *Cytoplasm protein (c), ^ karyon protein (k)

and time-dependent manner (Fig. 1), and PDTC improved the cytotoxic effect of cisplatin on CAOV3 cells (Fig. 1). Western blotting and RT-PCR were used to observe the effect of PDTC on AQP5 expression in CAOV3 cells. Results showed that PDTC decreased the expression of AQP5 mRNA and protein in concentration-dependent manner as well as time-dependent manner. When CAOV3 cells were incubated with 0.1, 1, 10 mM PDTC for 24 h, expression of AQP5 mRNA and protein were significantly decreased with 1 and 10 mM PDTC, and AQP5 mRNA and protein expression were significantly decreased after 24 and 48 h of treatment with1 mM PDTC (Fig. 5).

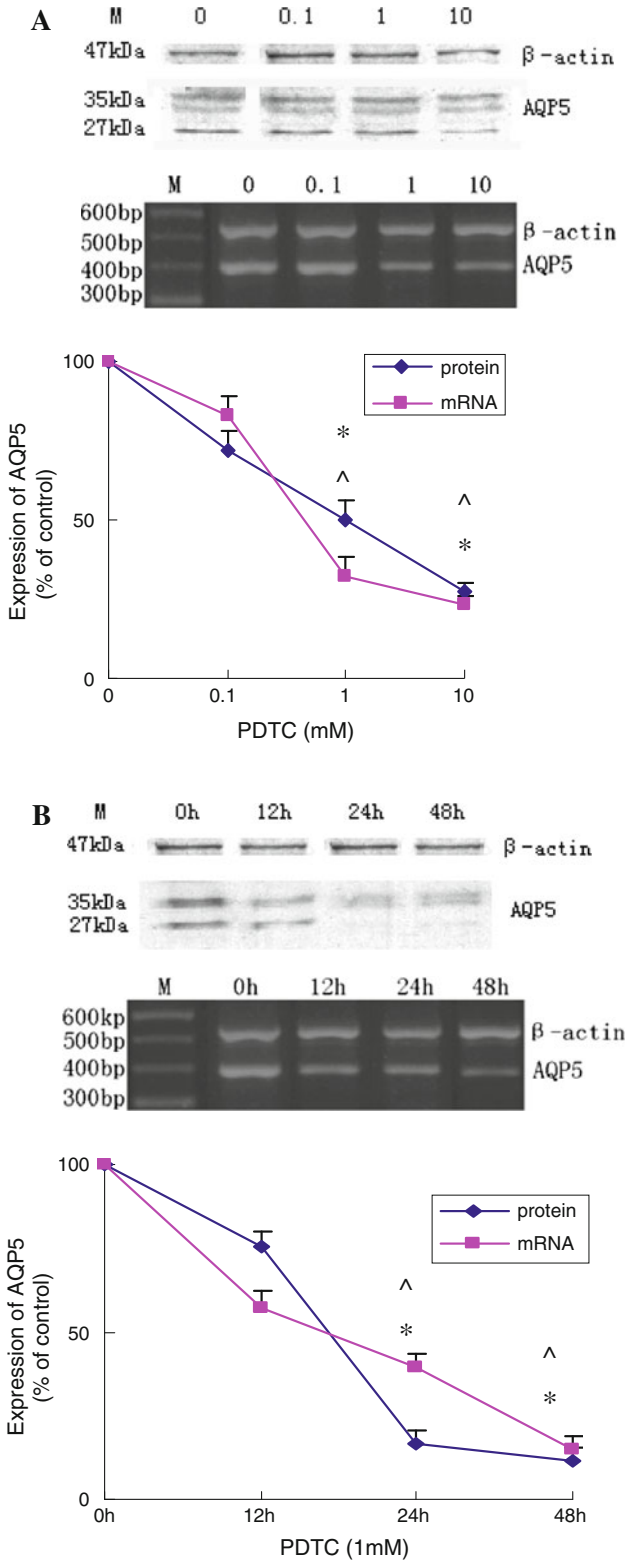
Correlation of AQP5 protein and CAOV3 growth rate

Person analysis indicated that there is a positive correlation between AQP5 protein expression and the growth rate of CAOV3 cells induced by cisplatin ($r = 0.607$, $P = 0.009$). There is a positive correlation between AQP5 protein expression and NF-κB p65 as well as IκBα in CAOV3 cytoplasm induced by cisplatin ($r = 0.894$, 0.857; $P = 0.000$, 0.000). There is a positive correlation between CAOV3 cells growth rate of and NF-κB p65 protein in cytoplasm and karyon induced by cisplatin ($r = 0.901$; $P = 0.000$).

Discussion

Aquaporin water channels are expressed primarily in cell plasma membranes. Recent researches showed that AQPs were associated with tumor cell proliferation, invasion, and metastasis. Controlling the balance of fluid secretion and absorption may play an important role in drug metabolism of tumor. Consequently, AQPs are attractive targets for the development of novel drug therapies for cancer [12]. It has been reported that AQP1 inhibitor acetazolamide could suppress the expression of AQP1 in lung cancer cells and inhibited lung metastasis [13]. Nicchia et al. [14] reported that astrocyte cell growth was impaired by RNAi inhibiting expression of AQP4, and AQP3 deletion in mice prevented skin tumor formation [15]. Above evidences suggest that AQPs may be related to chemosensitivity and drug -resistance of cancer.

Our results showed that AQP5 expression was reduced gradually as the concentration of cisplatin increasing, AQP5 expression was negatively correlated to the proliferation inhibition rate of CAOV3 cells, which suggested that AQP5 expression is important in growth state of CAOV3 cells, and AQP5 is associated with sensitivity to cisplatin. Interestingly, AQP5 showed a transient up-regulation within 12 h, and subsequent down-regulation of its expression within 24 to 72 h after cisplatin treated. We speculated that cells would consume a large amount of water required for synthesis metabolism to repair the cisplatin-induced damage in early. As a compensatory, AQP5 was transferred from intracellular vesicles to the cell membrane, while synthesis and transcription of AQP5 increased. With the



◀ **Fig. 5** Aquaporin5 (AQP5) expression in CAOV3 cells treated with nuclear factor kappaB (NF-κB) inhibitor PDTC. **a** Dose–response relationship of AQP5 protein expression with PDTC treatment. CAOV3 cells were incubated in medium containing in designed concentrations of PDTC for 24 h. Cells were processed for western blot and RT-PCR. **b** Time course of AQP5 protein expression with PDTC treatment. CAOV3 cells were incubated in medium containing in 1 mM PDTC for designated time. * or ^ $P < 0.05$ versus control (one-way factorial ANOVA combined with Scheffe's test)

prolonged drug treatments, cells were irreversible damage, and AQP5 expression increases sharply. Thus, we speculate that AQP5 may be one of the candidates for inducing cytotoxicity effect of cisplatin on ovarian cancer cells.

Currently, regulation mechanism of AQP5 expression is unclear. Kang et al. [16] reported that AQP5 activated the Ras/extracellular signal-regulated kinase/retinoblastoma protein signaling pathway in colon cancer cells, which could be a molecular mechanism for colon cancer development. Yao et al. [17] found that lipopolysaccharide induced down-regulation of expression of AQP5 mRNA in the parotid gland is mediated via a complex of these two classes of transcription factors, NF-κB and p-c-Jun/c-Fos. A series of promoter sequences have been found in upstream of open reading frame of AQP5, including NF-κB, AP1, AP2, SP1, and CREB [18, 19]. As a kind of nuclear factor, NF-κB can combine with specific DNA sequence of promoters to regulate their expression. Some researches indicated that NF-κB is activated persistently in ovarian epithelial cancer, and aggressiveness of ovarian cancer is closely associated with the destruction of the balance between NF-κB and its inhibitor IκB and following changes of series of κB-dependent gene expression [20]. Towne et al. [21] found that TNF-alpha decreases AQP5 mRNA and protein expression and that the molecular pathway for this effect involves TNFR1 and activated NF-κB in mouse pulmonary epithelial cells. Ito et al. [22] proved that IL-1β could up-regulate the expression of AQP4 in rat astrocytes by activating NF-κB signaling pathway. This study confirmed that blocking NF-κB by PDTC can decrease the expression of AQP5 in CAOV3 cells, suggesting that NF-κB may be involved in the regulation of AQP5 expression in ovarian cancer.

Furthermore, our studies showed that expression of NF-κB, IκBα, and AQP5 were inhibited in a similar process by cisplatin, and there is a positive correlation between AQP5 protein expression and NF-κB p65 as well as IκBα in CAOV3 cytoplasm after ciplatin treatment. These results further proved the regulation of AQP5 by NF-κB, and which suggested that AQP5 and NF-κB could be related with cell proliferation inhibition by cisplatin and drug metabolism in cell. AQP5 may play an important role in chemosensitivity and drug resistant of ovarian cancer, but the exact mechanism of AQP5 on drugs needs further study confirmed. In conclusion, the current study demonstrates that AQP5 is associated with the growth state of CAOV3 cells, and cisplatin can downregulate AQP5 expression. NF-κB and IκBα may be involved in the down-regulation

of AQP5 expression in response to cisplatin. Therefore, AQP5 appears to be a promising target for the development of novel drug therapies for ovarian cancer.

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Conflict of interest We declare that we have no conflict of interest.

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