

• Clinical Research •

Effect of topotecan on expression of aquaporin protein 5 and nuclear factor- κ B in ovarian cancer SKOV3 cells

Xue-Jun Chen, Jian-Hua Yang and Wei Zheng

Department of Gynecology,
Second Affiliated Hospital,
Zhejiang University School
of Medicine,
Hangzhou, Zhejiang, 310006,
P.R. China

Correspondence to: Wei Zheng
Tel.: 86.571.87783505
Fax: 86.571.87068001
Email: dwzheng06@yahoo.com.cn

This paper was translated into English from
its original publication in Chinese.
Translated by: Xue-Ming Sun and Wei Liu
on 2009-08-08

The original Chinese version of this paper
is published in: *Ai Zheng (Chinese Journal
of Cancer)* 28(8); [http://www.cjcsysu.cn/cn/
article.asp?id=15661](http://www.cjcsysu.cn/cn/article.asp?id=15661))

Submitted: 2008-11-18
Revised: 2009-04-09

[Abstract] **Background and Objective:** Overexpression of aquaporin protein 5 (AQP5) is associated with the metastasis, migration and angiogenesis of ovarian cancer, but its function in cell proliferation has not been described. This study was to explore the effects of topotecan (TPT) on the expression of AQP5, nuclear factor- κ B (NF- κ B) and its receptor I κ B α in ovarian cancer SKOV3 cells. **Methods:** When SKOV3 cells were treated with TPT of various concentrations for different time, cell proliferation was measured by MTT assay, the expression of AQP5, NF- κ B and I κ B α was detected by Western blot. **Results:** After SKOV3 cells were incubated with 0.4 μ g/mL TPT for 24 h, the expression of AQP5, NF- κ B p65 in cytoplasm and nuclei, and I κ B α in cytoplasm were decreased, and remained at low levels till 72 h ($P < 0.05$). When SKOV3 cells were treated with 0.2, 0.4, 0.6 and 0.8 μ g/mL TPT for 24 h, the expression of AQP5 as well as NF- κ B p65 and I κ B α in nuclei were decreased ($P < 0.005$). The protein level of AQP5 was decreased by 57.9% when cells were treated with 0.8 μ g/mL TPT. AQP5 expression was negatively correlated to the proliferation inhibition rate of SKOV3 cells induced by TPT ($r = -0.965$, $P < 0.05$), and positively correlated to NF- κ B p65 and I κ B α expression ($r = 0.903$, 0.896 , $P < 0.05$). **Conclusion:** When inhibiting the proliferation of ovarian cancer cells, TPT may down-regulate the expression of AQP5 and NF- κ B.

Key words: ovarian neoplasm, aquaporin 5, nuclear factor, topotecan

For rapid proliferation, division, invasion to surrounding matrix, and movement through the vascular wall, cancer cells need rapid transmembrane transportation of water more than normal cells. Aquaporins (AQPs) is a kind of transmembrane protein which transports water specifically. Its abnormal expression is associated with the infiltration and metastasis of cancer cells as well as angiogenesis.^{1,2} Overexpression of AQP5 may be associated with the genesis, development and prognosis of ovarian cancer.³

Nuclear factor-B (NF-B), a group of eukaryotic transcription factor, is comprises hetero- and homodimeric complexes of NF-B1 (P50), RelA (P65), NF-B2 (P52) and RelB. Among them, P50/P65 dimers are the most abundant. NF-B inhibitor IB suppresses NF-B nuclear localization signals via binding NF-B, therefore, inhibits the activity of NF-B. NF-B takes part in inflammatory reaction, immune response as well as cell proliferation, apoptosis and differentiation. It is not only associated with the genesis and

development of tumors but also influences chemo-sensitivity of tumors and patients prognosis to different degrees.⁴

Topotecan (TPT), a derivative of hydroxycamptothecin, takes antitumor function by inhibiting topoisomerase- I (topo-I) and has no cross-resistance with platinum-like drugs and taxel. It is one of the main drugs for recurrent or advanced ovarian cancer. This study was to explore the effects of TPT on the expression of AQP5, NF-B p65 and its inhibitor IB in ovarian cancer SKOV3 cells.

Materials and Methods

Cells and main reagents. Ovarian carcinoma cell line SKOV3 was bought from the ATCC. Goat anti-human AQP-5 polyclonal antibody (sc-9890), mouse anti-human NF-B p65 monoclonal antibody (sc-8000), rabbit anti-human IB α polyclonal antibody (sc-371), and goat anti- β -actin polyclonal antibody (sc-1616) were bought from Santa Cruz Biotechnology. NE-PER nuclear and cytoplasmic extraction reagents were bought from Pierce Biotechnology. SP immunohistochemical kit was bought from Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd.

Cell culture and proliferate inhibition experiment. SKOV3 cells were cultured in RPMI-1640 medium containing 10% fetal calf serum at 37°C in an incubator containing 5% CO₂. Cell proliferation was detected by MTT assay. SKOV3 cells at logarithmic growing phase were suspended to prepare cell suspension at 1×10^4 /mL, and seeded into a 96-well plate which was set three parallel wells. The cells were cultured with 0.2, 0.4, 0.6, 0.8, and 1.0 μ g/mL TPT for 24 h or 0.4 μ g/mL TPT for 3, 6, 12, 24, 48, and 72 h, respectively, or cultured in RPMI-1640 medium as control. The absorbance at 570 nm (A_{570}) was measured. Cell proliferation inhibition rate (%) = $(1 - A_{570} \text{ value of TPT group} / A_{570} \text{ value of control group}) \times 100\%$.

SP immunohistochemistry. SKOV3 cells at logarithmic growing phase were suspended to prepare cell suspension at 1×10^4 /mL, and seeded into 24-well plates (2 mL/well). After

treatment of TPT, the cells were fixed by 4% paraform for 30 min, and stained by HE method and immunohistochemical method. The working concentrations of primary antibodies were all 1:50. The cells were cultured at 4°C in a refrigerator overnight, colored by DAB, and counterstained by hematoxylin.

Quantitive detection of protein expression by Western blot. SKOV3 cells at logarithmic growing phase were suspended to prepare cell suspension at 110^5 /mL, and seeded in 50 mL culture flasks. After treatment of TPT, the cells were collected for total protein extraction. Nuclear and cytoplasmic proteins were extracted according to the instructions of NE-PER nuclear and cytoplasmic extraction reagents, then 50 μ g protein was electrophoresed and transferred onto membrane, then cultured with goat anti-AQP5 antibody (1:1000), mouse anti-NF-B p65 antibody (1:1000), rabbit anti-IB antibody (1:1000), and goat anti- β -actin antibody (1:500), separately, at 4°C overnight and visualized with peroxidase horseradish-labelled second antibody (1:5000) at room temperature for 1 h. Gray scales were analyzed by Quantity One software (Bio-Rad Company). The relative expression level of cytoplasmic target protein was represented by the ratio of the gray scale of target protein to that of -actin. The relative expression level of nuclear target protein was represented by the gray scale value of target protein.

Statistical analysis. All data were expressed as mean \pm SD, and analyzed by t test using SPSS 11.5 software package. $p < 0.05$ was considered significant

Results

Inhibitory effect of TPT on proliferation of SKOV3 cells. When cultured with 0.4 μ g/mL TPT, the proliferation of SKOV3 cells was significantly inhibited in a time-dependent manner ($r=0.931$, $P=0.04$) (Table 1); the proliferation inhibition rate reached $(57.2 \pm 2.6)\%$ at 72 h. When cultured with TPT for 24 h, the proliferation of SKOV3 cells was significantly inhibited in a concentration-dependent manner ($r=0.958$, $P=0.$

03); the proliferation inhibition rate reached $(56.0 \pm 2.9)\%$ when cells were treated with $1.0 \mu\text{g/mL}$ TPT for 24 h.

Influence of TPT on expression of AQP5, NF-B p65 and IB in SKOV3 cells. AQP5 was mainly expressed on cell membrane and in cytoplasm. NF-B p65 and IB were mainly expressed in cytoplasm of SKOV3 cells. After treatment of TPT, the expression of AQP, NF-B p6 and IB in SKOV3 cells was down-regulated and AQP5 expressed on nuclear membrane and in nuclei (Table 3). After SKOV3 cells were incubated with $0.4 \mu\text{g/mL}$ TPT for 24 h, the expression of NF-B

p65 and IB in cytoplasm and nuclei were down-regulated, and remained at low levels till 72 h ($P<0.05$). After cells were treated with $0.4 \mu\text{g/mL}$ TPT for 72 h, the expression of AQP5 was down-regulated by 81.4% (Table 1, Fig. 4).

After SKOV3 cells were incubated with TPT at different concentrations for 24 h, the expression of AQP5 was down-regulated gradually as concentration of TPT increasing; meanwhile, the expression of NF-B p65 and IB in nuclei were down-regulated gradually ($P<0.05$), but the changes of their expression in cytoplasm were not obvious ($P>0.05$) (Fig. 5, Table 2).

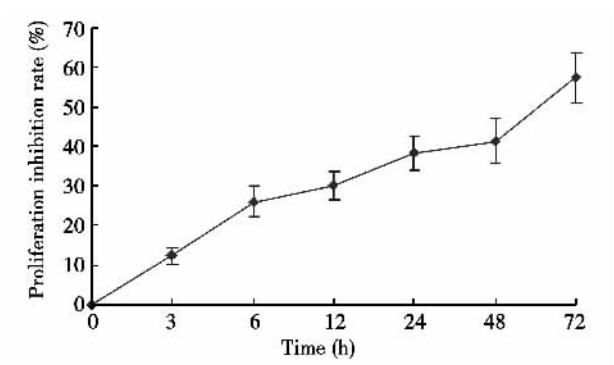


Figure 1 Proliferation inhibition rate of SKOV3 cells treated with 0.4 mg/mL topotecan for different time

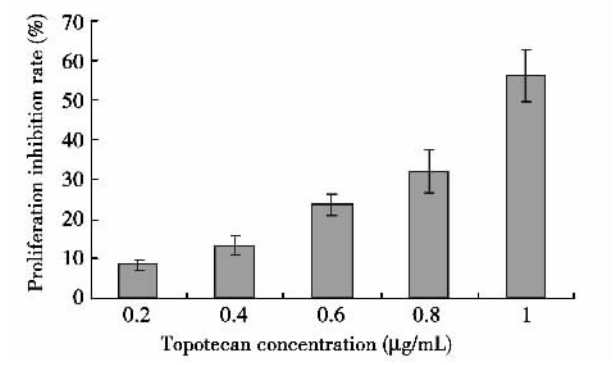


Figure 2 Proliferation inhibition rate of SKOV3 cells treated with different concentrations of topotecan for 24 h

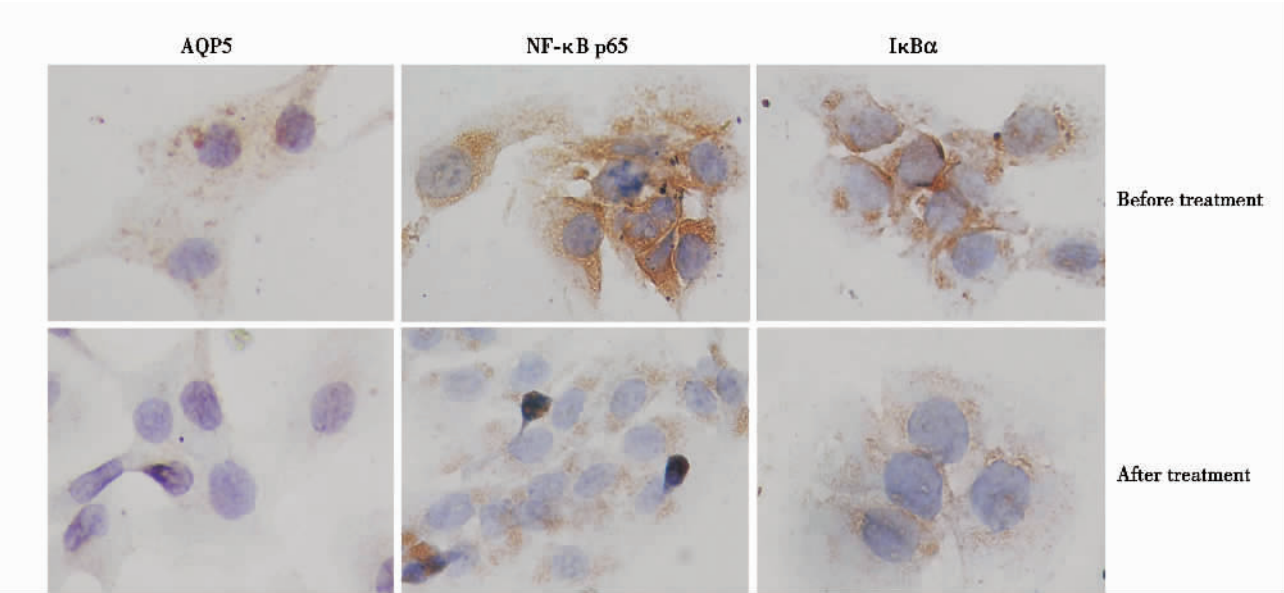


Figure 3 Expression of aquaporin protein 5 (AQP5), nuclear factor-κB (NF-κB p65) and its receptor $\text{I}\kappa\text{B}\alpha$ in SKOV3 cells before and after treatment of 0.4 mg/mL topotecan (TPT) (SP $\times 400$)
After treatment of 0.4 mg/mL TPT, AQP5, NF-κB p65 and $\text{I}\kappa\text{B}\alpha$ expression in cytoplasm and nuclei are weakened.

Table 1 Expression of aquaporin protein 5 (AQP5), nuclear factor-κB (NF-κB p65) and its receptor IκBα in SKOV3 cells treated with 0.4 mg/mL topotecan (TPT)

Group	AQP5	p65 in cytoplasm	IκBα in cytoplasm	p65 in nuclei	IκBα in nuclei
Control	0.59±0.09	1.62±0.24	0.90±0.34	1.76±0.62	4.29±0.61
TPT 12 h	0.34±0.19	1.49±0.41	0.81±0.26	2.90±0.56	2.30±0.35
24 h	0.33±0.06	1.26±0.23	0.67±0.15	1.29±0.25	2.01±0.17
48 h	0.13±0.05	0.49±0.07	0.64±0.16	0.95±0.14	0.94±0.15
72 h	0.11±0.03 ^a	0.14±0.04 ^a	0.47±0.13 ^a	0.94±0.16 ^a	0.65±0.18 ^a

All values are presented as mean ± SD of three experiments. ^a*P*<0.05, vs. control.

Table 2 Expression of AQP5, NF-κB p65 and IκBα in SKOV3 cells treated with different concentrations of TPT

Group	AQP5	p65 in cytoplasm	IκBα in cytoplasm	p65 in nuclei	IκBα in nuclei
Control	0.57±0.04	2.36±0.23	1.88±0.12	1.33±0.21	0.72±0.22
TPT 0.2 mg/mL	0.49±0.05	1.49±0.16	1.65±0.15	0.92±0.09	0.60±0.31
0.4 mg/mL	0.36±0.04	1.73±0.17	1.56±0.21	0.88±0.06	0.53±0.10
0.8 mg/mL	0.24±0.01 ^a	1.45±0.19	1.39±0.05	0.84±0.04 ^a	0.51±0.04 ^a

All values are presented as mean ± SD of three experiments. ^a*P*<0.05, vs. control.

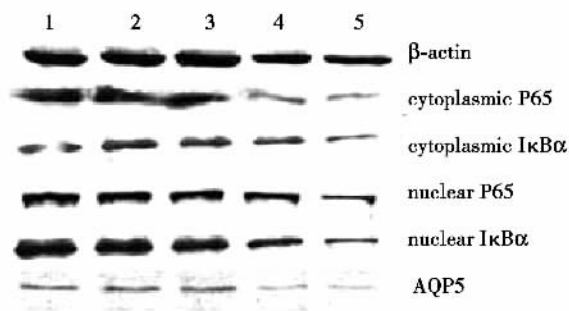


Figure 4 Expression of AQP5, NF-κB p65, and IκBα in SKOV3 cells after treatment of 0.4 mg/mL TPT detected by Western blot

Lane 1: untreated SKOV3 cells; lanes 2–5: SKOV3 cells treated with 0.4 mg/mL TPT for 12, 24, 48, and 72 h, respectively.

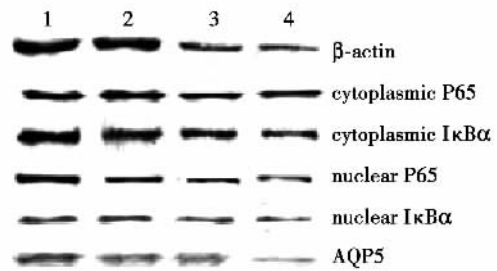


Figure 5 Expression of AQP5, NF-κB p65, and IκBα in SKOV3 cells after treatment of different concentrations of TPT for 24 h

Lane 1: untreated SKOV3 cells; lanes 2–4: SKOV3 cells treated with 0.2, 0.4 and 0.8 mg/mL TPT for 24 h, respectively.

Correlations of AQP5 expression to SKOV3 cell proliferation, NF-B p65 and IB expression. The expression of AQP5 in SKOV3 cells was positively correlated to those of NF-B p65 and IB in nuclei ($r=0.903$, 0.896 , $P<0.05$). The inhibitory effect of TPT on proliferation of SKOV3 cells was negatively correlated to the expression of AQP5 ($r=-0.965$; $P<0.05$).

Discussion

Recent researches showed that AQPs play important roles in tumor growth, invasion and

metastasis. Secretion and absorption of fluid could be balanced by inhibiting or controlling the expression of AQPs, suggesting that AQPs would be new targets of tumor prevention and therapy.^{1, 56} It has been reported that AQP1 inhibitor acetazolamide could suppress the expression of AQP1 in lung cancer cells and efficiently inhibit lung metastasis.⁵ It was showed that AQP4 expression in astrocyte was efficiently inhibited and cell growth was impaired after RNA interference, suggesting that AQPs may associate with chemosensitivity and drug-resistance of recurrent tumor.⁶ Yang et al.^{3,7,8} found that the

overexpression of AQP1 and AQP5 are associated with tumorigenesis, development and ascites formation of ovarian cancer. Their further research showed that ascites of ovarian cancer was associated with the up-regulation of AQP5 expression in ovarian cancer SKOV3 and CAOV3 cells, while chemotherapy reduced AQP5 expression.⁹ Our results showed that the proliferation of SKOV3 cells was inhibited and AQP5 expression was reduced gradually as the concentration of TPT increasing and treatment time prolonging, AQP5 expression was negatively correlated to the proliferation inhibition rate of SKOV3 cells, suggesting that AQP5 expression is associated with growth state of SKOV3 cells.

Currently, regulation mechanism of AQPs expression is unclear. The expression of AQPs is regulated mainly in a long-term pattern and a short-term pattern.^{10,11} In short-term pattern, the activity of AQPs is changed under the regulation of some factors of internal environment (phosphorylation mechanism) or the number of functional water channels is changed (shuttle mechanism). In long-term pattern, the synthesis of AQPs at transcriptional level is enhanced, then its protein synthesis is also enhanced. A series of promoter sequences have been found in upstream of open reading frame of AQP5, for instance, NF- κ B, AP1, AP2, SP1, and CREB.^{12,13} NF- κ B, a kind of nuclear factor, exists in many kinds of cells. It can combine with specific DNA sequences of promoters or enhancers of relative genes to regulate their expression. Some investigations indicated that NF- κ B is activated persistently in ovarian epithelial cancer, the genesis, invasion and metastasis of ovarian cancer are closely associated with the destruction of the normal balance between NF- κ B and its inhibitor I κ B and following changes of series of κ B-dependent gene expression.^{14,15} Towne et al.¹⁵ found that TNF- α could down-regulate the expression of AQP5 protein and mRNA in mouse pulmonary epithelial cells through combining with TNF- α receptor-1 and activating NF- κ B signalling pathway. Ito et al.¹⁶ proved that IL-1 β could up-regulate the expression of AQP4 mRNA in rat astrocytes by activating NF- κ B signalling pathway. Previous studies

showed that AQP5 expression and genetic transcription would be inhibited if NF-B were blocked; DDP can inhibit the expression of NF-B p65 and IB, meanwhile, it can down-regulate AQP5 expression in a similar way; in addition, AQP5 expression is positively correlated to NF-B p65 and IB expression in cytoplasm, suggesting that the down-regulation of AQP5 expression in SKOV3 cells by DDP is associated with NF-B.⁹ This study confirmed that TPT has a good antitumor effect on ovarian cancer in vitro. TPT can down-regulate the expression of AQP5, NF-B p65 and IB α when it inhibit cell proliferation. As an effective chemotherapeutic drug for ovarian cancer, TPT treat ovarian cancer and restrain ascites probably via down-regulating the expression of AQPs in ovarian cancer cells, suggesting the possibility of AQPs as therapeutic targets of ovarian cancer. Above all, AQP5 expression is associated with the growth state of SKOV3 cells. TPT down-regulate AQP5 and NF-B expression; both of them may associate with the antitumorous effect of chemotherapeutic drugs.

References

- [1] Monzani E, Shtil AA, La Porta CA. The water channels, new druggable targets to combat cancer cell survival, invasiveness and metastasis [J]. *Curr Drug Targets*, 2007,8 (10):1132–1137.
- [2] Saadoun S, Papadopoulos MC, Hara-Chikuma M, et al. Impairment of angiogenesis and cell migration by targeted aquaporin-1 gene disruption [J]. *Nature*, 2005,434 (7034): 786–792.
- [3] Yang JH, Shi YF, Cheng Q, et al. Expression and localization of aquaporin-5 in the epithelial ovarian tumors [J]. *Gynecol Oncol*, 2006,100(2):294–299.
- [4] Sebels S, Arlt A, Schäfer H. NF-kappaB as a molecular target in the therapy of pancreatic carcinoma [J]. *Recent Results Cancer Res*, 2008,177:151–164.
- [5] Xiang Y, Ma B, Li T, et al. Acetazolamide inhibits aquaporin-1 protein expression and angiogenesis [J]. *Acta Pharmacol Sin*, 2004,25(6):812–816.
- [6] Nicchia GP, Frigeri A, Liuzzi GM, et al. Inhibition of aquaporin-4 expression in astrocytes by RNAi determines alteration in cell morphology, growth, and water transport and induces changes in ischemia-related genes [J]. *FASEB J*, 2003,17(11):1508–1510.
- [7] Yang JH, Shi YF, Cheng Q, et al. Protein and mRNA

- expression of aquaporin-1 in epithelial ovarian tumors and its clinic significance [J]. *Zhonghua Fu Chan Ke Za Zhi*, 2005,40(9):623-626. [in Chinese]
- [8] Yang JH, Shi YF, Chen XD, et al. The influence of aquaporin-1 and microvessel density on ovarian carcinogenesis and ascites formation [J]. *Int J Gynecol Cancer*, 2006,16(Suppl.):400-405.
- [9] Yang JH, Zheng W, Shi YF, et al. Cisplatin on the effect of aquaporin 5 protein expression of SKOV3 and its regulation [J]. *Chin J Cell Biol*, 2008,36(5):771-775. [in Chinese]
- [10] Papadopoulos MC, Saadoun S, Davies DC, et al. Emerging molecular mechanisms of brain tumor edema [J]. *Br J Neurosurg*, 2001, 15(2):101-108.
- [11] King LS, Yasui M, Agre P. Aquaporins in health and disease [J]. *Mol Med Today*, 2000,6(2):60-65.
- [12] Ishibashi K, Kuwahara M, Gu Y, et al. Cloning an functional expression of a new quaporin abundantly expression in the peripheral leukocytes permeable to water and urea, but not to glycerol [J]. *Biochem Biophys Commun*, 1998,244(1):268-274.
- [13] King LS, Nielsen S, Agre P. Quaporins in complex tissues. I. Developmental patterns in respiratory and glandular tissues of rat [J]. *Am J Physiol*, 1997,273(Pt 1):C1541-1548.
- [14] O'Shea-Greenfield A, Smale ST. Roles of TATA and initiator elements in determining the start site location and direction of RNA polymerase II transcription [J]. *J Biol Chem*, 1992,267(2):1391-1402.
- [15] Towne JE, Krane CM, Bachurski CJ, et al. Tumor necrosis factor-alpha inhibits aquaporin 5 expression in mouse lung epithelial cells [J]. *J Biol Chem*, 2001,276(22):18657-18664.
- [16] Ito H, Yamamoto N, Arima H, et al. Interleukin-1beta induces the expression of aquaporin-4 through a nuclear factor- κ B pathway in rat astrocytes [J]. *J Neurochem*, 2006,99(1):107-118.