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Cytoplasmic p21 is responsible for paclitaxel resistance in ovarian cancer A2780 cells

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Summary

Purpose: P21 which bound to cyclin-dependent kinase complexes was originally described as a suppressor of cancer cell proliferation, while many recent studies have shown p21, when accumulated in the cell cytoplasm, could promote tumor progression. This study was conducted to investigate the role of p21 in the paclitaxel (PTX) resistance of ovarian cancer. *Materials and Methods:* Regulation of cytoplasmic p21 was performed through transfection of Akt2 constitutively active vector, Akt2 shRNA and p21 siRNA in the ovarian cancer cell line A2780. Akt2, p-Akt, and p21 expression were examined by Western blot and cell apoptosis rates were assessed by flow cytometry after treatment with PTX. *Results:* Induction of p21 translocation into the cytoplasm via constitutively active Akt2 transfection in A2780 enhanced the resistance to PTX, while inhibition of p21 translocation into the cytoplasm via Akt2 shRNA transfection in A2780 cells significantly increased PTX treatment sensitivity. Furthermore, knockdown of cytoplasmic p21 by direct p21 siRNA transfection in Akt2 overexpressed A2780 cells notably increased PTX-induced apoptosis. *Conclusion:* Cytoplasmic p21 may represent a potential therapeutic target for ovarian tumors that are resistant to PTX treatment.

Key words: Cytoplasmic p21; PTX resistance; Drug resistance; Ovarian cancer; Akt2.

Introduction

Ovarian cancer represents the sixth most commonly diagnosed cancer among women in the world and it causes more deaths per year than any other cancer of the female reproductive system [1, 2]. The major obstacle to successful therapy is drug resistance which leads to low five-year survival rates [3]. It is widely acknowledged that the sensitivity to chemotherapy is determined by the apoptotic response of cancer cells to chemotherapeutic drugs [4]. In the previous study, many molecules, such as PI3K/Akt [5, 6], PTEN [7], BRCA [8], and MDR [9], have been confirmed to be involved in the regulation of apoptosis and in the complicated signaling network that determines the fate of cancer cells, i.e., either "death" or "survival." More importantly, the present authors' recent study has also found that cytoplasmic p21 was not only a novel biomarker of cisplatin resistance but also represented a potential therapeutic target for ovarian tumors that were resistant to cisplatin treatment [10].

In addition to cisplatin, there is another conventional anticancer agent paclitaxel (PTX), which is widely used for the chemotherapeutic treatment of ovarian cancer patients [11]. Nevertheless, less than 50% of ovarian cancers exhibit a satisfactory response to PTX and effective strategies are needed to enhance its sensitivity [12]. Given that multidrug resistance and high cross-resistance occurred in the ovarian cancer treatment [9, 13], this study was further conducted to investigate whether p21 could act as a similar role in regulating the PTX resistance, just as cisplatin resistance. Here, the authors report that in the ovarian cancer cell line A2780, induction of p21 translocation into the cytoplasm by transfection of constitutively active Akt2 could significantly enhance the resistance to PTX, while inhibition of p21 translocation into the cytoplasm by transfection of Akt2 shRNA notably increased PTX-induced apoptosis. Furthermore, knockdown of cytoplasmic p21 by p21 siRNA transfection in Akt2 overexpressed A2780 cells notably increased PTX induced apoptosis. In summary, cytoplasmic p21 may represent a potential therapeutic target for multidrug resistance in ovarian tumors.

Materials and Methods

Cell lines and cell culture

The A2780 ovarian cancer cell line was obtained from a European manufacturer. Cells were cultured in RPMI-1640 supplemented with two mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2.

Construction of plasmids

A constitutively active Akt expression vector (AAkt2), short hairpin RNA targeting Akt2 (Akt2Sh), small p21 RNA interfering

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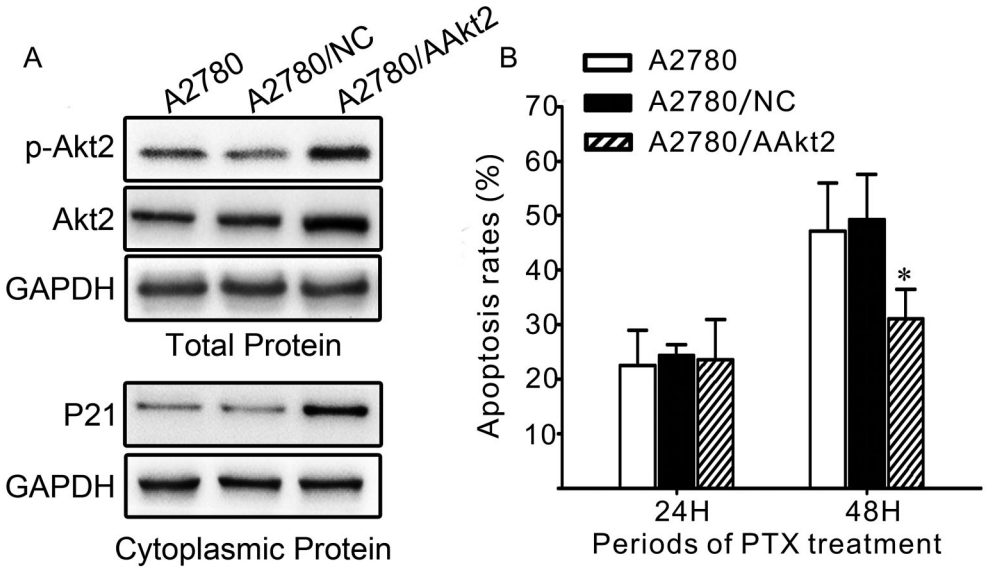

Figure 1. — Induction of p21 translocation into the cytoplasm decreases sensitivity to PTX in A2780. (A) Western blot images representing the expression level of total Akt, p-Akt, and cytoplasmic p21 in A2780 cells of different groups. (B) Apoptosis rates of A2780 cells treated with 100 nmol/L PTX at indicated time points. (*$p < 0.05$).

fragment (p21si), and their corresponding control plasmids were constructed as described previously [5, 10].

Establishment of stable-expression cell lines of AAkt2 and Akt2sh in A2780 cells

A2780 cancer cells were stably transfected with AAkt2 and Akt2sh vectors using lipofectamine 2000. The empty vectors of pcDNA3.1 and pEGFPC1 were transfected as negative controls. The cells were selected with 600 μg/μl G418. After 21 days the G418-resistant cell pools were established and inoculated into 100 mm dishes for further propagation.

Transient transfection for RNAi targeting

The Akt2 stably transfected A2780 cancer cells were transfected with small p21 RNA interfering fragment (p21si) using lipofectamine 2000. After incubation for six hours, the transfection solution was replaced with fresh complete growth medium. Then 48 hours post-transfection, the cells were assayed for the expression of p21, Akt2, and p-akt, and treated with PTX for further experiment.

Western blot analysis

Total proteins were extracted by lysing cells in buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 50 mM NaF, 0.5% NP-40, one mM Na_3VO_4, one mM phenylmethylsulfonyl fluoride, 25 mg/ml leupeptin, and 25 mg/ml aprotinin. The lysates were cleared by centrifugation, and the supernatants were obtained. Cytoplasmic proteins were exacted using the N-PER kit according to manufacturer's instructions. Equal amounts of protein lysate were used for Western blot analyses using the indicated antibodies. Specific signals were visualized with NBT/BCIP.

Analysis of apoptosis

Cells were harvested, washed with PBS, and stained with the annexin-V/PI apoptosis kit according to manufacturer's instructions. Apoptosis rates were evaluated using a flow cytometer, and the data were analyzed using cell fit software.

Statistical analysis

All experiments were repeated three times. Results expressed as mean ± SD were analyzed using the Student *t* test. Differences were considered significant when $p < 0.05$. Data was analyzed using SPSS software version 13.0.

Results

Induction of p21 translocation into the cytoplasm decreases sensitivity to PTX in A2780

The plasmid of Akt2 (A2780/AAkt2) and empty plasmid (A2780/NC) were stably transfected into A2780 cells. Total and cytoplasmic protein were extracted from the cells and assessed by Western blot. As is shown in Figure 1A, the expression levels of Akt and p-Akt were significantly enhanced in A2780/AAkt2 cells when compared to A2780/NC cells and A2780 cells. Moreover, cytoplasmic p21 protein levels were markedly increased in A2780/AAkt2 cells compared to the non-transfected control cells and vector-transfected cells (Figure 1A). Flow cytometric analysis of cells treated with 100 nmol/L PTX for 48 hours showed that A2780/AAkt2 cells exhibited lower levels of apoptosis rate (31% ± 5.5%) than A2780/NC cells (49.2% ± 8.4%) and A2780 cells (47.2 ± 8.8%) ($p < 0.05$, Figure 1B). However, there were no significant differences in the apoptotic rates among the three groups when treated for 24 h ($p > 0.05$, Figure 1B). Based on the aforementioned results, it is demonstrated that accumulation of p21 in cytoplasm through activation of Akt2 impairs the sensitivity of A2780 cells to PTX.

Inhibition of p21 translocation into cytoplasm restores the sensitivity to PTX in A2780 cells

Short hairpin RNA targeting Akt2 and its vector control plasmid Sh-Scr were stably transfected into A2780 cells (A2780/Sh-Akt2 cells and A2780/Sh-Scr cells). Total and cytoplasmic protein was extracted and detected by West-

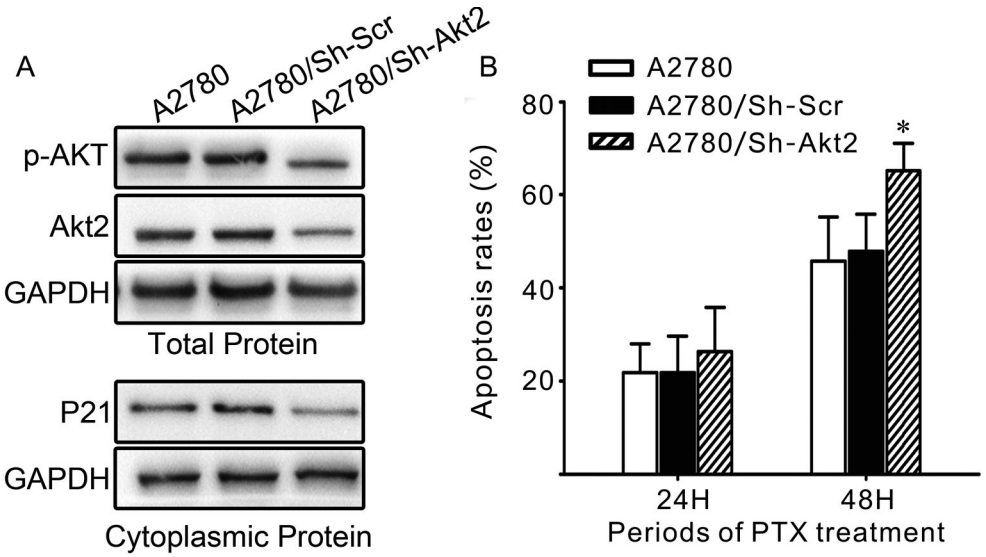

Figure 2. — Inhibition of p21 translocation into cytoplasm restores the sensitivity to PTX in A2780 cells. (A) Western blot images representing the expression level of total Akt, p-Akt, and cytoplasmic p21 in A2780 cells. (B) Apoptosis rates of A2780 cells treated with 100 nmol/L PTX at indicated time points. (*p < 0.05).

ern blot analysis. Compared to the non-transfected control cells and vector-transfected cells, the protein levels of Akt and p-Akt in A2780/Sh-Akt2 cells were significantly decreased (Figure 2A). Additionally, there was also a remarkable decrease in cytoplasmic p21 in A2780/Sh-Akt2 cells when compared with the controls (Figure 2A). As is shown in Figure 2B, flow cytometric analysis of cells treated with 100 nmol/L PTX for 48 hours demonstrated that A2780/Sh-Akt2 cells exhibited 65.1% ± 5.9% apoptosis rate, which was higher than vector-transfected cell (47.9% ± 8.0%) and non-transfected control cells (45.7% ± 9.5%) ($p < 0.05$). However, there were no significant differences in the apoptotic rates among the three groups when treated with 100 nmol/L PTX for 24 hours ($p > 0.05$, Figure 2B). Collectively, these results demonstrate that inhibition of cytoplasmic p21 through inactivation of Akt2 increases the sensitivity of A2780 cells to PTX.

Knockdown of cytoplasmic p21 restores the sensitivity to PTX in A2780/AAkt2 cells

To further clarify whether cytoplasmic p21 contributes to PTX resistance, RNA interference assay in A2780/AAkt2 was applied to decrease p21 that was mainly in the cytoplasm. P21si and its mismatched fragment of p21sm were transiently transfected in A2780/AAkt2 cells. As is shown in Figure 3A, p21si transfection exhibited a notable decrease in cytoplasmic p21 compared with control groups, however there was no significant change in the expression of Akt2 and p-Akt as evaluated by Western blot. After transfection, these cells were exposed to 100 nmol/L PTX for 48 hours. As shown in Figure 3B, the apoptosis rate was 49.6 ± 10.0% in A2780/AAkt2/p21si cells, which was notably higher than A2780/AAkt2/p21sm cells (32.7 ± 5.9%) and A2780/AAkt2 cells (30.2 ± 3.2%) ($p < 0.05$, Figure 3B) as assessed by flow cytometry. No significant differences in the apoptotic rates among the three groups were found to be treated with PTX for 24 hours (data not shown). These results demonstrate that the reduction of cytoplasmic p21 contributes to the increased sensitivity to PTX in A2780/AAkt2 cells.

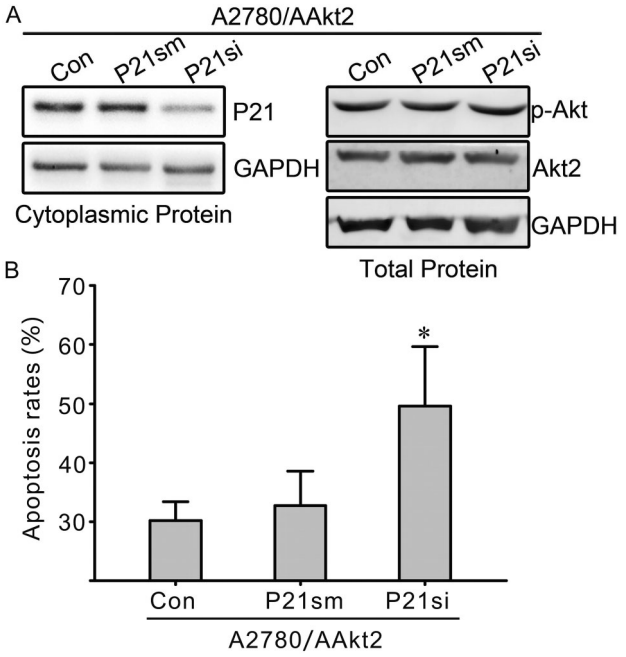

Figure 3. — Knockdown of cytoplasmic p21 restores the sensitivity to PTX in A2780/AAkt2 cells. (A) Western blot showing the changes in total Akt, p-Akt, and cytoplasmic p21 protein levels observed in A2780, A2780/AAkt2/p21si, and A2780/AAkt2/p21sm cells. (B) Apoptosis rates in response to 100 nmol/L PTX for 48 hours in three groups. (*p < 0.05).

Discussion

Previous studies demonstrated that p21 could act as a "tumor suppressor" by binding to cyclin/CDK complexes and proliferating cell nuclear antigen [14]. However, other studies [15] have revealed that p21 can be a paradoxical tumor promoting agent and has been positively related to the poor prognosis in cancer patients due to its accumulation in the cytoplasm. Besson *et al.* [16] reported that control of the p21 subcellular localization could represent an important regulatory switch from nuclear tumor suppressor to cytoplasmic tumor booster. Nevertheless little has been known about the role of p21 in cancer cell chemoresistance until Koster *et al.* reported cytoplasmic p21 expression determined cisplatin resistance in testicular cancer [17]. Recently, the present authors' study has further validated that not only cytoplasmic p21 was a novel clinical biomarker of cisplatin resistance but it represents a potential therapeutic target for ovarian tumors that were refractory to cisplatin-based treatment [10], which supplemented their previous studies [5, 7] and verifies that p21 was a downstream effector in the PI3K/Akt2 pathway that contributes to cisplatin resistance.

Besides cisplatin, there is another regular anticancer agent PTX, which has been widely used for the first-line chemotherapeutic treatment of ovarian cancer patients [11]. However, there were more than 50% ovarian cancer patients who showed a impaired sensitivity to the drug [12]. Effective strategies have been made to investigate the mechanism by which mediated the PTX resistance and many molecules such as kallikrein-related-peptidase 4 (KLK4) [18], multidrug resistance gene MDR1 [9], NFκB [6], and p27 [12] have been found to confer to the drug resistance. Given that multi-drug resistance and high cross-resistance occurred in the ovarian cancer treatment [9, 13], it could be easily speculated whether different chemotherapeutic drugs resistance could be reversed in a similar way. Therefore, this study was conducted to investigate whether PTX shared a similar drug resistance mechanism with cisplatin. In addition, the present authors sought to determine whether interfering with cytoplasmic p21 could enhance the susceptibility of cancer cells to PTX.

It was reported that the activation of phosphate dylinositol 3-kinase (PI3K)/Akt signaling could stimulate the accumulation of p21 in the cytoplasm [19]. There are three isoforms of Akt including Akt1, Akt2, and Akt3. All three isoforms share a high degree of amino acid sequence identity, especially within the kinase domain [20], and are activated by similar pathways. As a member of Akt family, Akt2 has been shown to be increased in approximately 30% of ovarian cancers [21], and it has been acknowledged to be involved as an anti-apoptotic factor in a number of different cell death paradigms. Similar to the previous investigation [10], the modulation of cytoplasmic p21 in this study was accomplished through activation or inactivation of the expression of Akt2. Induction of p21 translocation into the cytoplasm by transfection of constitutively active Akt2 in A2780 led to the increased resistance to PTX, while inhibition of p21 translocation into the cytoplasm by transfection of Akt2 shRNA into A2780 cells significantly increased PTX-induced apoptosis. To further validate this, PTX resistance is directly regulated by cytoplasmic p21, knockdown of cytoplasmic p21 by p21 siRNA transfection in Akt2 overexpressed cells in which p21 localized mainly in the cytoplasm was performed. As anticipated, p21si transfection resulted in a notable decrease in cytoplasmic p21 compared with control groups, while there was no significant change in the expression of Akt2 and p-Akt. Therefore, it can be safely concluded that cytoplasmic p21 represent a novel therapeutic target for PTX resistance in ovarian tumor.

Conclusion

The present study demonstrated that the accumulation of cytoplasmic p21 diminishes the sensitivity of A2780 ovarian cancer cells to PTX. This may provide a new target for reversing resistance to PTX in ovarian cancer in the future clinical practice.

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