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ORIGINAL ARTICLE

Knockdown of cyclophilin A reverses paclitaxel resistance in human endometrial cancer cells via suppression of MAPK kinase pathways

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Abstract
Purpose Paclitaxel resistance remains to be a major obstacle to the chemotherapy of endometrial cancer. Using proteomic-based approach, we used to identify cyclophilin A (CypA) as a potential therapeutic target for endometrial cancer. As a natural continuation, this study aimed to reveal the correlation between CypA and paclitaxel resistance and evaluate the possibility of CypA as a therapeutic target for reversal of resistance.
Methods Two paclitaxel-resistant endometrial cancer cell sublines HEC-1-B/TAX and AN3CA/TAX were generated, and expressions of CypA, P-gp, MRP-2 and survivin were demonstrated by Western blotting. CypA was knocked down by RNA interference, and the subsequent effects on the alteration of paclitaxel resistance were examined by MTT, flow cytometry and migratory/invasive transwell assays. MAPK kinases activities were examined by Western blotting.
Results CypA knockdown led to significant inhibition of cell proliferation, induction of apoptosis and suppression of migratory/invasive capacity in HEC-1-B/TAX and AN3CA/TAX cells when exposed to paclitaxel. CypA knockdown led to reductions in total and phosphorylated MAPK kinases, including Akt, ERK1/2, p38 MAPK and JNK, in HEC-1-B/TAX cells. Furthermore, pretreatment with MAPK kinase inhibitors exhibited a synergistic effect in combination with CypA knockdown.
Conclusions These results demonstrated that CypA expression was up-regulated in paclitaxel-resistant cancer cells, and knockdown of CypA could reverse the paclitaxel resistance through, at least partly, suppression of MAPK kinase pathways, presenting a possibility of CypA serving as a therapeutic target to overcome paclitaxel resistance.

Keywords Endometrial cancer · Paclitaxel resistance · Cyclophilin A · MAPK kinase · RNA interference

Introduction

Endometrial cancer is one of the most common malignancies of the female genital tract with an approximately 80 % 5-year survival for all stage taken together. Presently, surgery combined with adjuvant or postsurgical chemotherapy is the predominant treatment strategy for endometrial cancer. Paclitaxel, obtained from plants and available in clinics from the 1990s, represents one of the most important chemotherapy agents to endometrial cancer. It binds to β-tubulin subunits and inhibits microtubule dynamics, thereby blocking cell cycle progression during mitosis at the metaphase/anaphase transition and activating cell death [1]. In clinic, application of paclitaxel makes a great contribution to the improvement of the life quality and overall survival of patients bearing endometrial cancers [2]. A group of patients also suffered cancer recurrence or metastasis when the standard treatment has been completed or even during the course of postsurgical chemotherapy, suggesting a development of paclitaxel resistance. In addition, a minor of patients even do not respond at the first cycle of chemotherapy. Up to date,

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there have been many reports being proposed to explain the underlying mechanism of paclitaxel resistance, including overexpression of the drug efflux pump P-glycoprotein (P-gp) and differential expression of β-tubulin isotypes or gene mutations [3, 4].

In a previous study [5], we adopted a proteomic approach using two-dimensional electrophoresis (2-DE) to compare endometrial cancer and individual matched normal endometrium. A number of proteins were identified by MALDI-Q-TOF MS (matrix-assisted laser desorption/ionization quadrupole time-of-flight tandem mass spectrometry), and Cyclophilin A (CypA) was one of the most significantly altered proteins. Multivariate analysis indicated that it was an independent prognostic factor for survival of endometrial cancer patients. Knockdown of CypA by RNA interference led to suppression of cancer cell proliferation and induction of apoptosis in vitro and inhibition of xenograft tumor growth in vivo. These results suggested that CypA might serve as a novel therapeutic molecular target for endometrial cancer.

CypA is a peptidylprolyl *cis-trans* isomerase (PPIase), which plays important roles in protein folding, trafficking, assembly, immune-modulator and cell signaling. Recently, intensive studies demonstrate that it displays an elevated expression in various types of cancer and promotes cancer cell proliferation, cell migration/invasion and inhibits apoptosis [6]. Its overexpression correlates with poor outcome of the patients. However, studies focusing on the potential relation between CypA and drug resistance remain limited. Choi et al. [7] compared the resistance of cells with CypA transfectants and CypA knockdown to cisplatin and found that up-regulation of CypA-rendered resistance to cisplatin-induced apoptosis. An oligo-microarray analysis by Chen et al. [8] revealed that CypA could increase the expressions of many cytokine-, drug transport- and drug metabolism-related genes. The drug sensitivities were compared between SK-Hep1-CypA cells and control cells, and increased resistance was observed for anticancer agent doxorubicin (DOX) and vincristine (VCR).

Based on the findings of our study and other reports, we hypothesize that overexpression of CypA should contribute to development of drug resistance in cancer cells, and knockdown of CypA may restore the sensitivity to anticancer agents. Therefore, in this work, two paclitaxel-resistant endometrial cancer cell sublines were generated, and the role of CypA in alteration of the paclitaxel resistance and the possible pathways involved were explored by RNA interference-based approaches. As a natural continuation of our proteomic discovery, this study was designed in an effort to determine whether CypA could serve as a therapeutic target in the chemotherapy to endometrial cancer with paclitaxel resistance.

Materials and methods

Cell line generation

The human endometrial cancer cell lines HEC-1-B and AN3CA were purchased from American Type Culture Collection (ATCC, Manassas, VA). To generate the resistant sublines HEC-1-B/TAX and AN3CA/TAX, HEC-1-B and AN3CA cells were exposed to stepwise increasing paclitaxel concentrations from 1 to 1,500 nM. HEC-1-B and AN3CA cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10 % fetal bovine serum (FCS) in a 5 % CO_2 atmosphere at 37 °C, and HEC-1-B/TAX and AN3CA/TAX cells were continuously cultured in paclitaxel to ensure the drug-resistant phenotype.

siRNA synthesis and cell transfection

Four double strand siRNA oligonucleotides targeting human CypA were designed according to the published sequence of human CypA (GenBank™ accession number NM_021130). The siRNA sequences used were as follows: CypA-siRNA-1: 5′-CcggaaGTGAAAGAAGGCATGAATATTCAAG AGATATTCATGCCTTCTTTCACttTTTTTg-3′; CypA-siRNA-2: 5′-CcggCTGACTGTGGACAACTCGAATTTCA AGAGAATTCGAGTTGTCCACAGTCAGTTTTTg-3′; CypA-siRNA-3: 5′-CcggGAATGGCAAGACCAGCAAGA ATTCAAGAGATTCTTGCTGGTCTTGCCAT TCTTTTTg-3′; and CypA-siRNA-4: 5′-CcggGTTTGCAGA CAAGGTCCCAAATTCAAGAGATTTGGGACCTTGT CTGCAAACTTTTTg-3′. Each siRNA sequence contained a 21-mer target sequence, a 9-mer loop sequence (TTCAAGAGA), a 21-mer complementary target sequence and a transcription terminator (TTTTg). Scrambled siRNA nucleotides (MOCK) were used as a negative control, and siRNA nucleotides targeting GAPDH were used as positive control for transfection evaluation.

For transfection, cells were maintained in RPMI 1640 medium until the cell confluence reached about 40 %. The Lipofectamine™ RNAiMAX (Invitrogen) and siRNA were diluted in antibiotics-free medium, respectively, and then combined at a ratio of 2.5:1. The combinations were transfected into the cells at indicated concentrations according to the manufacturer's recommendation.

Quantitative RT-PCR

Total RNA was extracted using the RNeasy Kit (Qiagen, Valencia, CA), and reverse transcription to cDNA was conducted using the Superscript First Strand synthesis system (Invitrogen, Carlsbad, CA). All PCR reactions were carried out on an ABI PRISM^{R}7500 Sequence Detection System

(Applied Biosystems, Foster City, CA) using the SYBR Green Real-Time PCR Master Mix kit (Toyobo, Osaka, Japan) according to the manufacturer's instruction. The 18srRNA primer was included in each plate to account for sample variations, and the mRNA level of each sample was normalized to that of 18srRNA. Each sample was run independently in triplicate. The relative fold change was calculated based on the 2-ΔΔ Ct method (Livak and Schmittgen 2001). The qRT-PCR primer sequences were 5′-CCTAAAG CATACGGGTCCTG-3′ (sense) and 5′-TTTCACTTTGCC AAACACCA-3′ (antisense) for human CypA (Accession number NM_021130), and 5′-CCTGGATACCGCAG CTAGGA-3′ (sense) and 5′-GCGGCGCAATACGAATGCC CC-3′ (antisense) for 18srRNA, respectively.

Western blotting

Cells were collected and lysed in RIPA lysis buffer. After being quantified by a Bradford assay kit (Bio-Rad, Hercules, CA), the lysates were subjected to 12 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5 % dry milk in TBS-Tween 20, the membranes were incubated with primary antibodies overnight at 4 °C. The blots were labeled with horseradish peroxidase-conjugated secondary antibodies, visualized by chemiluminescent detection. The specific antibodies used were as follows: rabbit anti-CypA (Upstate Biotechnology, VA, USA), mouse anti-P-gp (Abcam, Cambridge, UK), rabbit anti-MRP-2, rabbit anti-survivin, rabbit anti-ERK1/2, rabbit anti-p-ERK1/2, rabbit anti-p38 MAPK, rabbit anti-p-p38 MAPK, rabbit anti-JNK, rabbit anti-p-JNK, rabbit anti-Akt, and rabbit anti-p-Akt antibodies. The equivalent loadings were confirmed using mouse anti-GAPDH antibody. All antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA) if not otherwise specified. Experiments were independently performed in triplicate.

MTT assay

Cells were seeded on 96-well plates for indicated durations. MTT was added for 4-h incubation. The plates were then centrifuged at 1,800 rpm for 10 min, and the supernatant was then removed. The MTT formazan precipitate was then dissolved in DMSO, and the optical density (OD) was measured using an enzyme-linked immunosorbent assay (ELISA) reader at a 490 nm wavelength. Experiments were independently performed in triplicate.

Flow cytometry assay

Cells were harvested and fixed in cold 80 % ethanol overnight at 4 °C and double stained with Annexin V-Fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 30 min at room temperature in dark. The stained samples were analyzed on an EPICS ELITE ESP flow cytometer (Beckman Coulter, USA). DNA-bound PI fluorescence was measured with 15 mW air-cooled argon ion laser at 488 nm as excitation sources. Analyses of apoptosis profiles were performed with Coulter Elite 4.5 Multicycle software (Beckman Coulter, USA). At least 1×10^5 cells were counted in each sample. Experiments were independently performed in triplicate.

Migration and invasion assay

Both assays were performed with 24-well Boyden chamber that had 12 mm polycarbonate membranes of 8.0 μm pore size (Millipore, MA, USA). 24 h after siRNA transfection, cells were treated with mitomycin C for 3 h and subsequently resuspended in serum-free medium containing 1,000 nM paclitaxel. In total, 1×10^5 cells were seeded on the upper chamber, and complete medium containing 10 % fetal bovine serum was placed in the lower chamber as a chemo-attractant. After incubation at 37 °C for 12 (to migration assay) or 48 h (to invasion assay), cells on the upper surface of the membrane were removed using a cotton-wool swab. The cells attached to the lower surface of the membrane were fixed with 4 % formaldehyde and stained with crystal violet. The stained cells were counted in 4 low-power fields (magnification 200×). Experiments were independently performed in triplicate. A percentage of cell migration or invasion was calculated from the ratio of stained cells attached to the lower surface of the membrane to the total number of cells loaded in the upper chamber.

Statistical analysis

All quantitative data were recorded as mean ± SD. Comparisons between two groups were performed by Student's *t* test. SPSS 13.0 (SPSS, Chicago, IL) was used for analyses, and statistical significance was defined as $P < 0.05$.

Results

Paclitaxel-resistant cell sublines, HEC-1-B/TAX and AN3CA/TAX

HEC-1-B/TAX and AN3CA/TAX sublines were both generated from its parent cell line by exposure to paclitaxel in stepwise increase concentrations from 1 to 1,500 nM. Cells were exposed to various concentrations of paclitaxel for 48 h and cell viability was measured by MTT assay. MTT assay showed that HEC-1-B/TAX and AN3CA/TAX

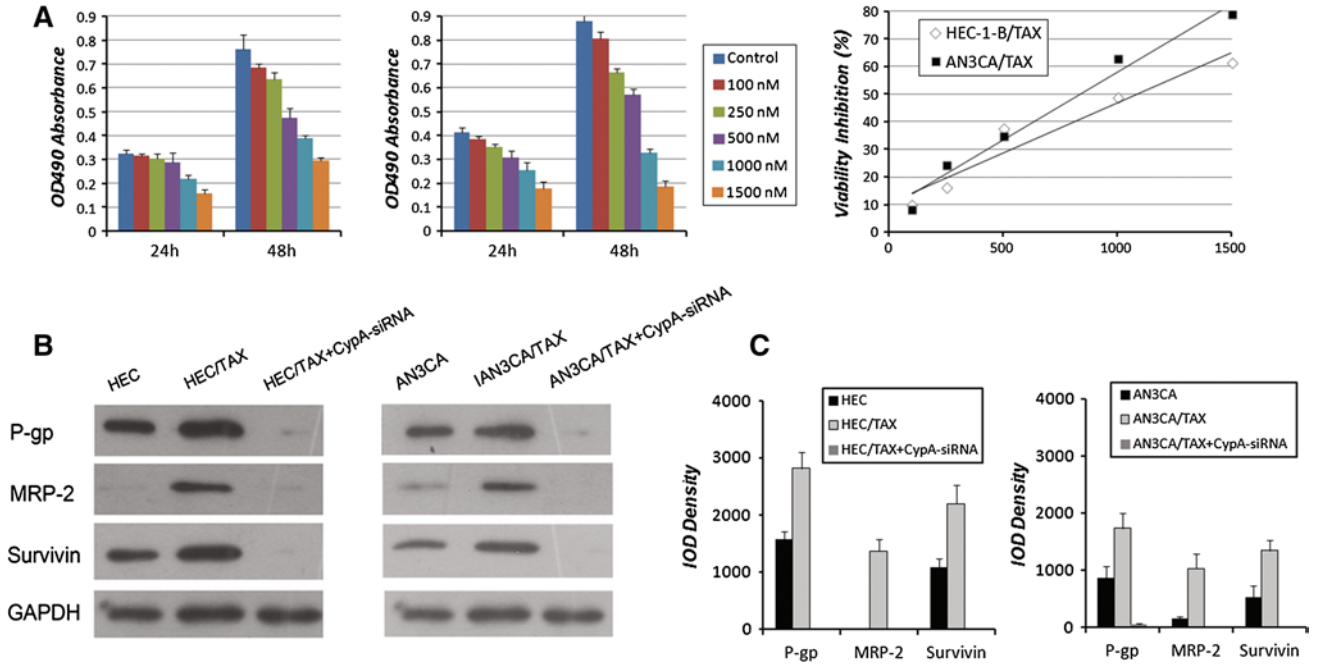


Fig. 1 **a** HEC-1-B/TAX and AN3CA/TAX cells were exposed to paclitaxel at various concentrations for 24 and 48 h, and the cell viability was evaluated by MTT assay (left: HEC-1-B/TAX; right: AN3CA/TAX). The IC_{50}/48 h of HEC-1-B/TAX and AN3CA/TAX cells was calculated accordingly. **b** Western blotting showed that P-gp, MRP-2 and survivin were all up-regulated in both HEC-1-B/TAX and AN3CA/TAX cells in comparison with those in parental cells and significantly decreased when CypA was knocked down. **c** The blotting band was quantified using densitometric analysis and expressed as histogram. Data were recorded as mean ± SD of three independent experiments

cells were about 350- and 250-fold more resistant to paclitaxel as compared with the parental cells, respectively. IC_{50}/48 h was 1,075 and 825 nM for HEC-1-B/TAX and AN3CA/TAX cells, respectively, as shown in Fig. 1a. To further confirm the paclitaxel resistance in HEC-1-B/TAX and AN3CA/TAX cells, expressions of three drug resistance-associated proteins, P-gp, MRP-2 and Survivin, were also examined by Western blotting. As shown in Fig. 1b, P-gp and Survivin were both up-regulated in HEC-1-B/TAX cells by approximately 78 and 103 %, respectively ($P < 0.05$), and both up-regulated in AN3CA/TAX cells by approximately 98 and 152 %, respectively ($P < 0.05$). MRP-2 was detected in both HEC-1-B/TAX and AN3CA/TAX cells, but almost undetectable in their parental cells ($P < 0.05$). The expression of CypA was also examined by Western blotting. As expected, CypA expression was elevated in HEC-1-B/TAX and AN3CA/TAX cells by approximately 100 and 110 % in comparison with that in parental cells, respectively (Fig. 2b, $P < 0.05$).

To further evaluate the possible alteration of paclitaxel resistance by CypA knockdown, expressions of three drug resistance-associated proteins, P-gp, MRP-2 and survivin, were also examined by Western blotting. As shown in Fig. 1b, knockdown of CypA led to significant down-regulation of all these proteins in both HEC-1-B/TAX and AN3CA/TAX cells to nearly undetectable levels, which were even lower than the levels in their parental cells ($P < 0.05$).

Knockdown of CypA by siRNA

To determine the potential activity of elevated expression of CypA in HEC-1-B/TAX cells, siRNA targeting CypA was used to transfect HEC-1-B/TAX cells. As shown in Fig. 2a, four siRNA oligonucleotides yielded 54.77–81.88 and 53.97–83.28 % CypA knockdown in HEC-1-B/TAX and AN3CA/TAX cells as quantified by quantitative RT-PCR, respectively. One siRNA oligonucleotide CypA-siRNA-3, which had the greatest knockdown effect in both lines (81.88 and 83.28 %), was selected for subsequent experiments. Moreover, CypA protein expression was also examined by Western blotting, which showed an approximately 72 and 54 % CypA knockdown by CypA-siRNA-3 in HEC-1-B/TAX and AN3CA/TAX cells, respectively (as shown in Fig 2b). A scrambled siRNA oligonucleotide (MOCK), which showed no effect on CypA expression, was used as negative control for potential non-specific effects of siRNA.

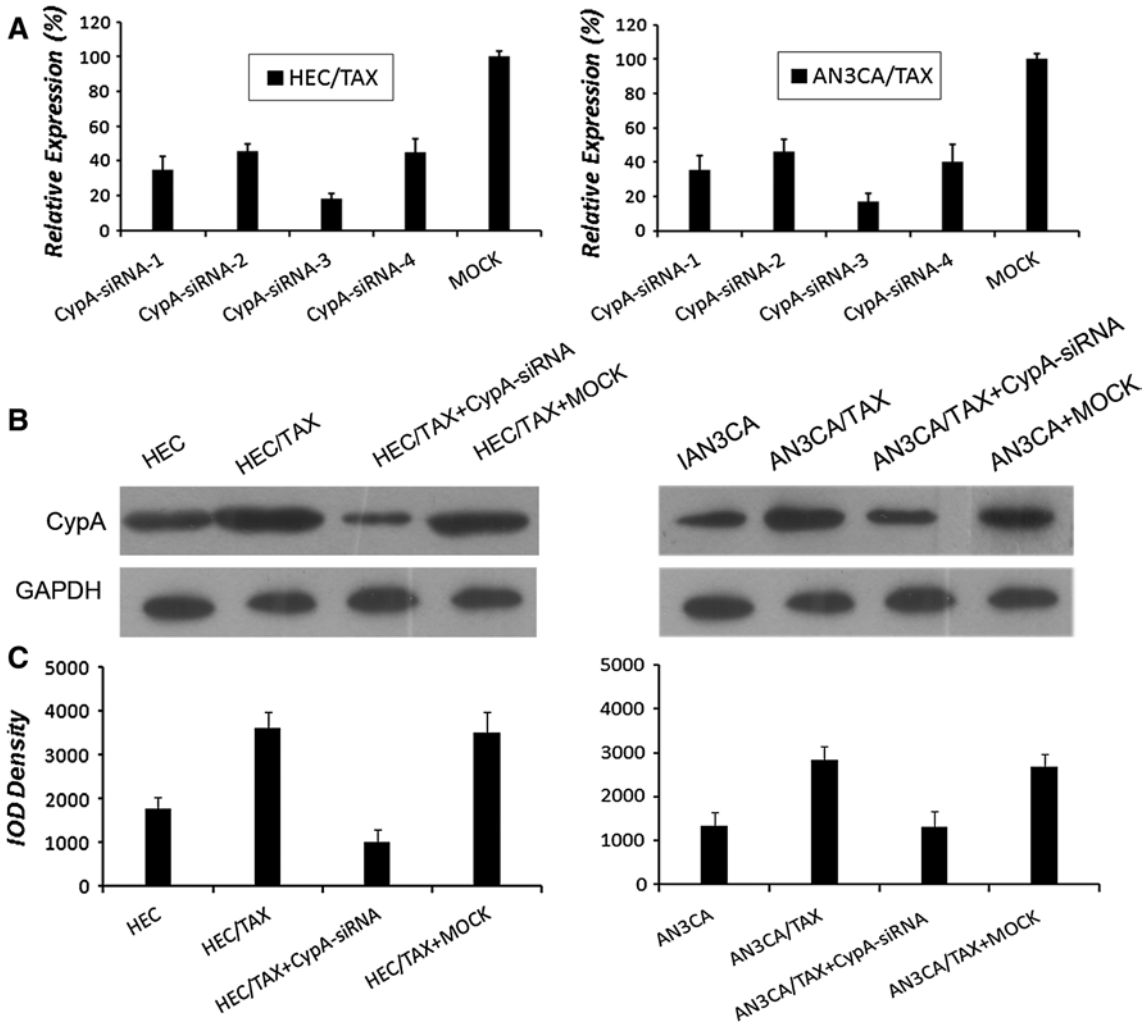
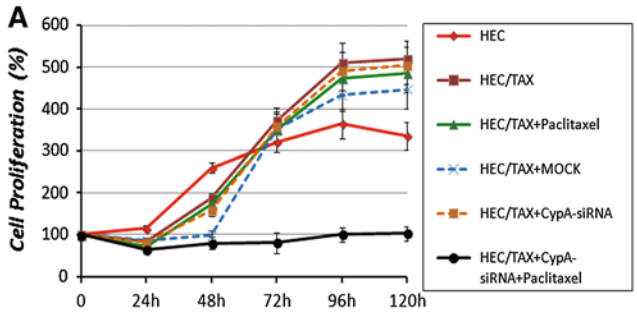


Fig. 2 **a** Knockdown effect of four siRNA oligonucleotides was examined by quantitative RT-PCR. Relative mRNA levels of CypA in HEC-1-B/TAX and AN3CA/TAX cells both significantly decreased, and CypA-siRNA-3 yielded the greatest effect in both lines. **b** Western blotting showed that CypA was up-regulated in both HEC-1-B/TAX and AN3CA/TAX cells in comparison with that in parental cells and knocked down by CypA-siRNA-3. **c** The blotting band was quantified using densitometric analysis and expressed as *histogram*. Data were recorded as mean ± SD of three independent experiments

Reversal of paclitaxel resistance by knockdown of CypA

To determine the potential effects of CypA knockdown on the paclitaxel resistance in HEC-1-B/TAX and AN3CA/TAX cells, MTT assays were performed to evaluate the cell proliferation. The cell proliferation (%) was calculated as OD_{490} Absorbance at a certain time/OD_{490} Absorbance at 0 h. The HEC-1-B/TAX and AN3CA/TAX cells were exposed to 1,000 or 800 nM paclitaxel for 5 days, respectively. As shown in Fig. 3a, no significant difference in cell proliferation was found between the HEC-1-B/TAX cells exposed and not exposed to paclitaxel ($P > 0.05$). While when CypA expression was knocked down, exposure to paclitaxel led to a significant inhibition of HEC-1-B/TAX cell proliferation by approximately 80 %, in comparison with cells without CypA knockdown ($P < 0.05$). No effect on cell proliferation was observed in HEC-1-B/TAX transfected with MOCK-siRNA ($P > 0.05$). Additionally, elevated proliferation capacity was observed in HEC-1-B/TAX cells in comparison with HEC-1-B cells ($P < 0.05$). Similar results were also observed in AN3CA/TAX cells, as shown in Fig. 3b. When CypA expression was knocked down, exposure to paclitaxel led to a significant inhibition of AN3CA/TAX cell proliferation by approximately 75 % ($P < 0.05$).

Flow cytometry assays were further performed to evaluate potential effects of CypA knockdown on the apoptosis and cell cycle arrest. The HEC-1-B/TAX and AN3CA/TAX cells were exposed to 1,000 and 800 nM paclitaxel for 48 h, respectively. As shown in Fig. 4a, no significant difference



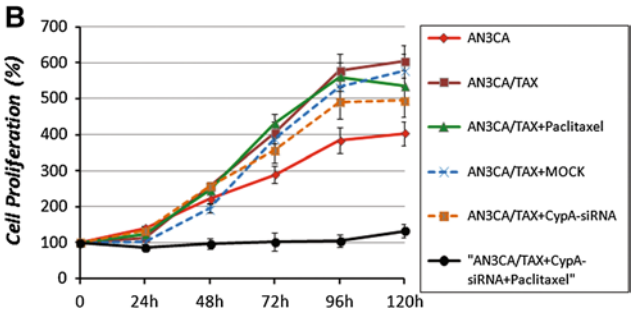


Fig. 3 Effect of CypA knockdown on cell viability. The HEC-1-B/TAX and AN3CA/TAX cells were exposed to 1,000 or 800 nM paclitaxel for 5 days, respectively. MTT assay showed a significant inhibition of cell viability in both HEC-1-B/TAX and AN3CA/TAX cells with CypA knockdown by approximately 80 and 75 %, respectively, in comparison with cells without CypA knockdown. Data were recorded as mean ± SD of three independent experiments

in the proportion of apoptotic cell was observed between the cells exposed and not exposed to paclitaxel (HEC-1-B/TAX: 7.97 ± 2.12 vs. 6.68 ± 2.12 %, $P > 0.05$; AN3CA/TAX: 6.34 ± 3.12 vs. 8.91 ± 4.04 %, $P > 0.05$). However, the apoptosis increased to a proportion of 35.24 ± 6.57 and 42.35 ± 8.41 % in HEC-1-B/TAX and AN3CA/TAX cells, respectively, when CypA was knocked down ($P < 0.05$). In addition, knockdown of CypA alone could also induce a slight increase in apoptosis in HEC-1-B/TAX (13.33 ± 3.43 %, $P < 0.05$) and AN3CA/TAX cells (16.43 ± 4.56 %, $P < 0.05$). As shown in Fig. 4b, no significant difference in the cycle distribution was observed between the cells exposed and not exposed to paclitaxel ($P > 0.05$), indicating their resistance to paclitaxel-induced mitotic arrest. However, when CypA expression was knocked down, significant reduction in cells in G_1 phase and the accumulation of cells in S and G_2/M phases were observed in both HEC-1-B/TAX and AN3CA/TAX cells exposed to paclitaxel ($P < 0.05$).

Migration and invasion assays were performed to evaluate the potential effects of CypA knockdown on the migratory and invasive capacities when exposed to paclitaxel. The HEC-1-B/TAX and AN3CA/TAX cells were exposed to 1,000 or 800 nM paclitaxel in upper chamber for 12 or 48 h for transwell migration or invasion assay, respectively. As shown in Fig. 4c, when exposed to paclitaxel, migration and invasion proportion in HEC-1-B/TAX cells was 8.7 ± 2 and 18.7 ± 4 %, respectively, approximately 3.2- and 4.1-fold higher than that in HEC-1-B cells (2.7 ± 1.5 and 4.6 ± 2 %, $P < 0.05$). Migration and invasion proportion in AN3CA/TAX cells were 11.4 ± 4.5 and 24.3 ± 3.3 %, respectively, approximately 2.5- and 2.9-fold higher than that in AN3CA cells (4.6 ± 2.5 and 8.5 ± 4 %, $P < 0.05$). However, when the CypA was knocked down, both migration and invasion proportions in HEC-1-B/TAX cells exposed to paclitaxel significantly decreased by approximately 60 % (3.8 ± 1.5 and 7 ± 2 %), to the extents similar to HEC-1-B cells. To AN3CA/TAX cells, both migration and invasion proportions decreased by approximately 50 % (5.6 ± 2.6 and 11.6 ± 3.6 %). No effect on migratory or invasive capacity was observed in cells transfected with MOCK-siRNA ($P > 0.05$).

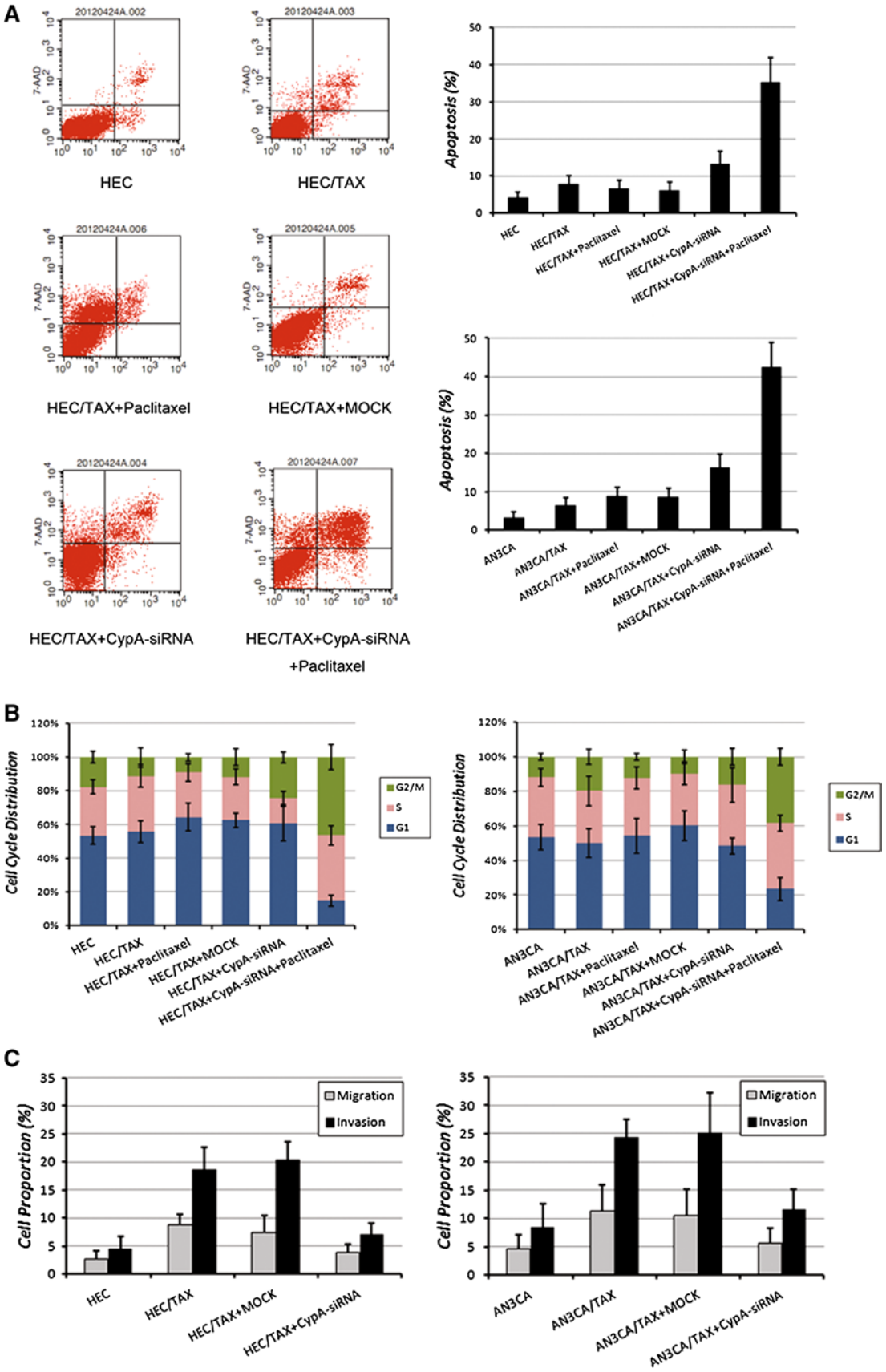
Fig. 4 Effects of CypA knockdown on apoptosis, cell cycle and migratory/invasive capacity. **a** The HEC-1-B/TAX and AN3CA/TAX cells were exposed to 1,000 and 800 nM paclitaxel for 48 h, respectively. Flow cytometry assay showed a significant increase in apoptotic proportion in both sublines with CypA knockdown, in comparison with the cells without CypA knockdown. **b** Flow cytometry assay showed a significant reduction in cells in G_1 phase and accumulation of cells in S and G_2/M phases in both sublines with CypA knockdown when exposed to paclitaxel. **c** The HEC-1-B/TAX and AN3CA/TAX cells were exposed to 1,000 or 800 nM paclitaxel in upper chamber for 12 or 48 h for transwell migration or invasion assay, respectively. Both sublines presented an increased migratory and invasive proportion in comparison with the parental cells when exposed to paclitaxel. When CypA was knocked down, both migratory and invasive proportions in HEC-1-B/TAX and AN3CA/TAX cells significantly decreased by approximately 60 and 50 %, respectively. Data were recorded as mean ± SD of three independent experiments ▶

Suppression of MAPK kinase pathways by CypA knockdown

To explore the potential involvement of MAPK kinase pathways in the CypA knockdown-induced resistance reversal, the expressions of total and phosphorylated Akt, ERK1/2, p38 MAPK, and JNK in HEC-1-B/TAX cells were determined by Western blotting. As shown in Fig. 5, knockdown of CypA led to remarkable reduction in total Akt, ERK1/2, and JNK ($P < 0.05$), but did not apparently influence p38 MAPK, even with marginal significance ($P = 0.05$). Furthermore, knockdown of CypA also led to remarkable reduction in phosphorylations of all these protein kinases.

Synergistic effects of MAPK inhibitors on resistance reversal induced by CypA knockdown

The correlation between CypA knockdown and suppression of MAPK kinase pathways prompted us to investigate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AN3CA/TAX
AN3CA/TAX+Paclitaxel
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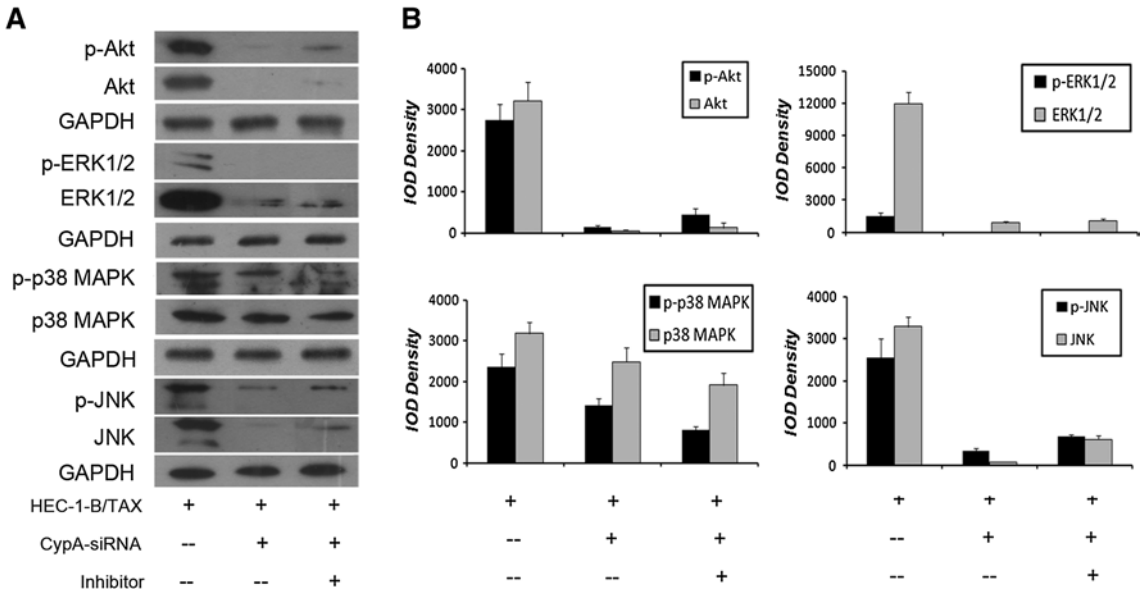

Fig. 5 Suppression of MAPK kinases by CypA knockdown. **a** Western blotting assay showed that expressions of total and phosphorylated Akt, ERK1/2, p38 MAPK, and JNK were significantly down-regulated in HEC-1-B/TAX cells with CypA knockdown. Pretreatment of MAPK kinase inhibitors (LY294002, PD98059, SB203580 and SP600125 to Akt, ERK1/2, p38 MAPK and JNK, respectively) to the cells with CypA knockdown presented no apparent influence on the total and phosphorylated MAPK kinases. **b** The blotting band was quantified using densitometric analysis and expressed as histogram, and data were recorded as mean ± SD of three independent experiments

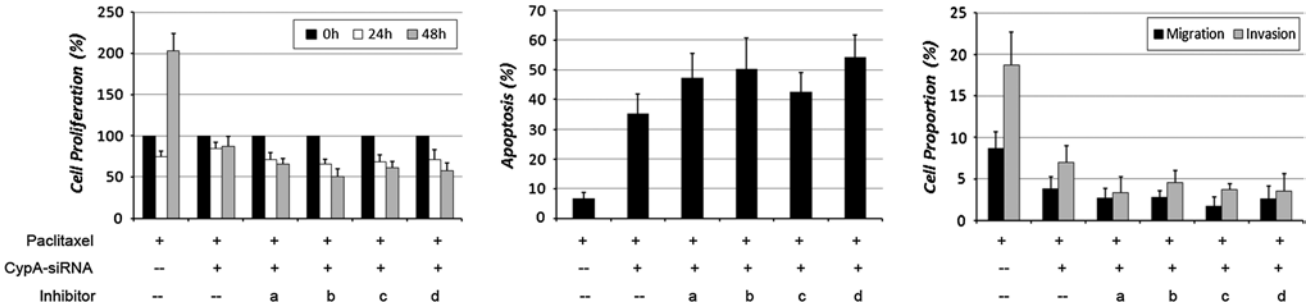

Fig. 6 Effects of MAPK kinase inhibitors on cell proliferation, apoptosis and migratory/invasive capacity. The HEC-1-B/TAX cells with CypA knockdown were pretreated with MAPK kinase inhibitors (*a*: LY294002; *b*: PD98059; *c*: SB203580; and *d*: SP600125) before exposed to paclitaxel. In cells with inhibitors pretreatment, MTT assay showed an inhibition of cell proliferation, and flow cytometry showed an increase in apoptosis proportion and migratory/invasive transwell assay showed a decrease in migratory/invasive proportion in comparison with the cells without inhibitors pretreatment. Data were recorded as mean ± SD of three independent experiments

whether the MAPK kinase pathways should be involved in CypA knockdown-associated resistance reversal to paclitaxel. Then LY294002, PD98059, SB203580 and SP600125 (Cell Signaling Technology, Beverly, MA, USA), specific and potent pharmacological inhibitors of Akt, ERK1/2, p38 MAPK and JNK, respectively, were then used in this experiment. The HEC-1-B/TAX cells, transfected with CypA-siRNA-3, were pretreated with these inhibitors for 60 min (20 μM for LY294002, PD98059, SB203580 and SP600125, respectively) before exposed to 1,000 nM paclitaxel for indicated duration. As shown in Fig. 6, MTT showed that pretreatment with PD98059 before exposure to paclitaxel, a well-established inhibitor of ERK1/2, inhibited cell proliferation by approximately 42 % by MTT assay, in comparison with the cells with CypA knockdown but without PD98059 treatment ($P < 0.05$). Flow cytometry showed that pretreatment with PD98059 increased apoptosis proportion by approximately 43 % (50.37 ± 10.32 vs. 35.24 ± 6.57 %, $P < 0.05$). Transwell assay showed that pretreatment with PD98059 decreased migration and invasion proportions by approximately 26 % (2.8 ± 0.8 vs. 3.8 ± 1.5 %) and 34 % (4.6 ± 1.4 vs. 7 ± 2 %, respectively

($P < 0.05$). Similarly, pretreatment with LY294002, SB203580 and SP600125, inhibitors of Akt, p38 MAPK and JNK, also resulted in decreased cell proliferation, increased apoptosis and decreased migratory/invasive capacity in HEC-1-B/TAX cells with CypA knockdown when exposed to paclitaxel ($P < 0.05$). However, for the HEC-1-B/TAX cells without CypA knockdown, treatment with LY294002, PD98059, SB203580 or SP600125 alone had no apparent effect. These results showed that MAPK kinase inhibitors could promote the effects on reversal of paclitaxel resistance induced by CypA knockdown. Furthermore, it suggested that suppression of MAPK kinase pathways should be involved in the paclitaxel resistance reversal of HEC-1-B/TAX cells induced by CypA knockdown.

Discussion

Paclitaxel is a microtubule-stabilizing agent, and decreased microtubule stability is responsible for its resistance development. Recent studies have identified at least three potential mechanisms by which paclitaxel resistance can develop. Overexpression of P-gp can confer paclitaxel resistance based on its function as a xenobiotic pump that pumps paclitaxel out of cells [9]. However, extensive clinical trials focusing on various kinds of P-gp inhibitors as chemosensitizing agents failed to produce promising outcomes in relapsing solid tumors and hematological malignancies [10–14]. The other two mechanisms are overexpression of β-tubulin isotypes [15, 16] and mutations of β-tubulin [17, 18]. Overexpression of β-tubulin isotype has been described in a restricted number of ovarian cancer patients resistant to paclitaxel [19] and in larger clinical settings in breast cancer [20]. Targeting β-tubulin by seco-taxane suppressed overexpression of β-tubulin and led to overcome of paclitaxel resistance [21]. However, knockdown of β-tubulin by RNA interference showed no effect on sensitivity of resistant ovarian cancer cells to paclitaxel, even partially improved sensitivity to other non-taxoid site microtubule-stabilizing agents [22]. A number of studies reported that mutations of β-tubulin at the paclitaxel binding sites were associated with drug resistance [17, 23]. However, some other studies did not find tubulin mutations in ovarian and lung cancers exhibiting paclitaxel resistance [24–27]. Generally, up to date, none of these has conclusively been shown to be a major cause of paclitaxel resistance, and the potential contribution of overexpression of tubulin isotypes and mutations of β-tubulin in paclitaxel resistance remains debated. Additional clinical studies should be required to ascertain the frequency of β-tubulin mutations in patients exhibiting paclitaxel resistance. Thiele et al. [28] reported that parvulin 17, a kind of peptidyl-prolyl cis/trans isomerases (PPIases), could interact with tubulin and thereby promote the formation of microtubules, which seemed to depend on its PPIase activity. CypA is also a kind of PPIase with activity stabilizing the *cis-trans* transition state and accelerating isomerization of peptide bonds preceding amino acid proline [29]. Considering that paclitaxel is a microtubule-stabilizing agent, we believe it is a promising issue to investigate whether or not the PPIase activity of CypA influences the microtubule assembly and stability, and further produce the resistance to paclitaxel in cancer cells.

Herein, as an alternative strategy, we hypothesized that some certain proteins with differential expression in comprehensive protein profile should be associated with the development of drug resistance, and serve as potential therapeutic target to overcome drug resistance. In our previous study [5], we demonstrated that CypA should serve as a potential prognostic biomarker and therapeutic target to endometrial cancer. Even the reports focusing on CypA and drug resistance remain limited and preliminary, some correlations between overexpression of CypA and drug resistance [7, 8, 30] encouraged us to presume that CypA should be involved in the development of drug resistance. As expected, our results demonstrated the overexpression of CypA in both HEC-1-B/TAX and AN3CA/TAX cells. When CypA was knocked down by RNA interference, we observed inhibition of cell proliferation, increase in apoptosis and suppression in migratory/invasive capacity in cells with exposure to paclitaxel, indicating a reversal of paclitaxel resistance by CypA knockdown. P-gp is an ATP-binding cassette transporter and considered to be a major mechanism of paclitaxel resistance [31]. MRP-2 is also an ATP-binding cassette transporter functioning as organic anion pumps involved in the transport of agents [32]. Survivin plays a great role in paclitaxel-induced microtubular stabilization and mitotic arrest. [33]. We found that P-gp, MRP-2 and Survivin were up-regulated in both HEC-1-B/TAX and AN3CA/TAX cells. All these proteins then decreased to nearly undetectable levels when CypA was knocked down, suggesting that the reversal of paclitaxel resistance by CypA knockdown should be associated with these proteins.

To the best of our knowledge, this study for the first time revealed the expression patterns of CypA in paclitaxel-resistant cancer cells and the correlation with reversal of resistance. Furthermore, we made some effort to explore the possible mechanisms, even preliminary but promising. The common overexpression in various cancer types suggests cytoprotective roles of CypA in diverse pathological processes, behaving as a molecular chaperone for other cellular proteins [34]. Alteration of certain signaling pathways during the anticancer agent treatment might occur as a result of protein misfolding, or disruption of regulatory

network. The chaperon activity of CypA might restore the balance and maintain protein homeostasis, being critical for cancer cell survival and drug resistance development. CD147 is a widely expressed plasma membrane protein, serving as a receptor for CypA. CypA interacts with a proline-containing peptide in the transmembrane domain and initiates activation of MAPK kinase pathways [6, 35, 36], the most common signal pathways shared by other growth factors. Some studies revealed that exogenous CypA stimulated cell proliferation through CD147 by activating MAPK kinase pathways, including ERK1/2 and p38 MAPK, in diverse cancer types [37–40]. In a recent cDNA microarray study focusing on the effects of CypA-siRNA on HEC-1-B cells [41], we found that knockdown of CypA led to significant decrease in migratory/invasive capacity. 3533 and 2772 genes were up-regulated and down-regulated in CypA-knockdown cells, respectively. Functional analysis showed that focal adhesion signaling was one of the most enriched down-regulated pathways, and the expression patterns of 16 genes in focal adhesion signaling, of which encoded MAPK kinases, and focal adhesion kinase (FAK), etc., were validated by qRT-PCR.

Because promoted proliferative capacity is a prominent phonotype of drug-resistant cells, we presumed that suppression of MAPK kinase pathways should be involved in the reversal of paclitaxel resistance by CypA knockdown. We examined the expression profiles of total and phosphorylated Akt, ERK1/2, p38 MAPK, and JNK by Western blotting. Consistent with our presumption, knockdown of CypA led to remarkable reduction in total and phosphorylated Akt, ERK1/2 and JNK in HEC-1-B/TAX cells, indicating suppression of MAPK kinase pathways. Even the total p38 MAPK was not apparently influenced, its phosphorylation was also remarkably reduced. Furthermore, we found that pretreatment with MAPK kinase inhibitors could enhance the effects induced by CypA knockdown, including proliferation inhibition, increased apoptosis induction and migratory/invasive capacity suppression. The presenting of synergistic effect by MAPK inhibitors combined with CypA knockdown indicated that the effects on resistance reversal by CypA knockdown should be, as least partly, associated with suppression of MAPK kinase pathways.

In this study, we demonstrated that CypA expression was up-regulated in paclitaxel-resistant cancer cells and that knockdown of CypA could reverse the paclitaxel resistance through, at least partly, suppression of MAPK kinase pathways. These results suggest a possibility of CypA serving as a therapeutic target to overcome anticancer drug resistance. However, a potential therapeutic target can dramatically improve optimization of treatment regimens, and patients with drug resistance would benefit significantly from the effectiveness of chemotherapy. Thus, translating the CypA identified in protein expression profiles into clinical application as a therapeutic target to reverse paclitaxel resistance remains a complex process requiring multidisciplinary approaches. Whether or not there are other signal transduction pathways involved and elucidating, the underlying mechanisms are warranted for further investigation.

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Conflict of interest None.

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