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PTEN overexpression improves cisplatin-resistance of human ovarian cancer cells through upregulating KRT10 expression

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ABSTRACT

Multi-drug resistance (MDR) is a common cause of the failure of chemotherapy in ovarian cancer. PTEN, a tumor suppressor gene, has been demonstrated to be able to reverse cisplatin-resistance in ovarian cancer cell line C13K. However, the downstream molecules of PTEN involved in the resistance-reversing effect have not been completely clarified. Therefore, we screened the downstream molecules of PTEN and studied their interactions in C13K ovarian cancer cells using a 3D culture model. Firstly, we constructed an ovarian cancer cell line stably expressing PTEN, C13K/PTEN. MTT assay showed that overexpression of PTEN enhanced the sensitivity of C13K cells to cisplatin, but not to paclitaxel. Then we examined the differently expressed proteins that interacted with PTEN in C13K/PTEN cells with or without cisplatin treatment by co-immunoprecipitation. KRT10 was identified as a differently expressed protein in cisplatin-treated C13K/PTEN cells. Further study confirmed that cisplatin could induce upregulation of KRT10 mRNA and protein in C13K/PTEN cells and there was a directly interaction between KRT10 and PTEN. Forced expression of KRT10 in C13K cells also enhanced cisplatin-induced proliferation inhibition and apoptosis of C13K cells. In addition, KRT10 siRNA blocked cisplatin-induced proliferation inhibition of C13K/PTEN cells. In conclusion, our data demonstrate that KRT10 is a downstream molecule of PTEN which improves cisplatin-resistance of ovarian cancer and forced KRT10 overexpression may also act as a therapeutic method for overcoming MDR in ovarian cancer.

1. Introduction

Ovarian cancer is one of the most common gynecologic malignancies. The mortality rate of ovarian cancer ranks first among all the gynecologic malignancies. Although it is curable in the early stages, most women are in advanced end-stage at the time of diagnosis because it does not show any symptoms in its early stages. At present, surgery and platinum and paclitaxel-based chemotherapy are still the main treatment methods for ovarian cancer. Although 75∼80% of the ovarian cancer patients can respond to chemotherapy initially, more than 80% of the patients that underwent chemotherapy may display drug resistance even multi-drug resistance (MDR) ultimately, leading to the five year survival rate is only 30%, suggesting MDR is a common cause of the failure of chemotherapy in ovarian cancer [1]. Therefore, exploring the mechanism of MDR in ovarian cancer is not only beneficial for the understanding of the disease progression, also helpful for developing targeted treatment method to reverse MDR.

PTEN is the first reported tumor suppressor gene with phosphatase activity, which plays a crucial role in the cell apoptosis, cell cycle arrest and cell migration [2,3]. Recent studies showed that chemotherapy drugs, such as cisplatin, doxorubicin and paclitaxel, could induce tumor cells apoptosis through enhanced PTEN activity [4–7]. However, the PTEN activity decreased along with the increase of chemotherapy drugs concentration and thereby inhibited the tumor apoptosis-related signaling pathways and reduced the effect of chemotherapy drugs [8]. These studies suggest that PTEN inactivation plays an important role in tumor MDR [5,9,10]. Accordingly, overexpression of PTEN is able to enhance the anti-tumor effect of chemotherapy drugs in many malignant tumors [11,12]. In our previous study, we also showed that overexpression of PTEN is able to reverse the cisplatin-resistance in the ovarian cancer cell line C13K [10]. Although the inactivation of PI3K/AKT cell survival pathway has been indicated playing a role in the effect of PTEN, the downstream molecules of PTEN involved in the resistance-reversing effect have not been completely clarified.

In recent years, three-dimensional (3D) culture model has been developed to imitate the in vivo microenvironment of tumor

Abbreviations: MDR, multi-drug resistance; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PVDF, Polyvinylidene fluoride.

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growth. The mechanism of tumor microenvironment mediated MDR is correlated with the resistance of tumor cells to chemotherapy-drugs induced apoptosis, suggesting the 3D culture model is an optimal choice of tumor MDR investigation. Therefore, in the present study, we screened the downstream molecules of PTEN and studied their interactions in C13K ovarian cancer cells using a 3D culture model.

2. Materials and methods

2.1. Cell culture

2.1.1. Normal culture

Cisplatin-resistant ovarian cancer cell C13K was cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 units/ml of streptomycin, at 37 °C, 5% CO_2 and 95% humidity.

2.1.2. 3D cultures

The 3D ovarian cancer culture model was performed according to previously reported [13].

2.2. Construction of stable C13K cell line overexpressing PTEN or KRT10

Twenty-four hours before transfection, C13K cells were plated at 70–90% confluence in 6-well plates. Human wild type PTEN expressing plasmid PEGFP-C1/PTEN, KRT10 expressing plasmid PCDNA3-HA/KRT10, as well as control pEGFP and pcDNA3 vectors were transfected using lipofectamine 2000 according to the manufacturer's procedure. To establish cell lines, 400 μg/ml G418 (Life Technologies) was added to the culture medium 48 h after transfection. After 7–10 days of G418 treatment, cells transfected with the plasmids or vectors were trypsinised and plated at a clonal density in a 96-well dish and expanded. The resulting stable transfectants were screened by Western blot and positive colonies were maintained in G418 selection.

2.3. Identification of differently expressed proteins in C13K/PTEN cells after cisplatin treatment

After cisplatin treated for 24 h, cells were washed three times in phosphate-buffered saline (PBS), harvested by scraping, and centrifuged for 5 min at 1000 rpm. The pelleted cells were lysed at 4 °C in 300 μl of cell lysis buffer (50 mM Tris HCl pH = 8, 120 mM NaCl, 1% Nonidet P-40 (NP-40), 2 mM EDTA, Protease Inhibitor Cocktail Tablets) for 30 min. The lysate was centrifuged at 14,000 rpm at 4 °C for 30 min, and the supernatants were incubated with PTEN antibody and 10 μl protein A/G-beads and mixed overnight at 4 °C. The immunoadsorbents were collected by centrifugation for 3 min at 3000 rpm and washed three times by resuspension and centrifugation (3 min at 3000 rpm) in cell lysis buffer. The samples were eluted into 15 μl of SDS loading buffer and heated for 5 min at 100 °C and subjected to SDS–PAGE electrophoresis. Then the gel was subjected to silver nitrate staining.

2.4. MTT assay

Ovarian cancer cells were seeded into 96-well plates at a density of 1×10^4 cells/well in RPMI-1640 supplemented with 10% FBS. Then the cells were treated with or without indicated concentrations of cisplatin. Twenty-four hours later, 10 μl of 5 mg/ml MTT solution was added into each well. The plates were incubated at 37 °C for 4 h, and then the supernatant was discarded and 100 μl DMSO was added into each well and mixed thoroughly before reading on a microplate reader.

2.5. Real time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from ovarian cancer cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. 5 μg of total RNA was reverse-transcribed into cDNA using TIANScript kit (Beijing TIANGEN, Beijing, China). The amplification of the cDNA was accomplished in triplicate using an ABI7000 real-time polymerase chain reaction (PCR) instrument in the presence of the commercially available SYBR Green PCR Master Mix (Beijing TIANGEN). The cDNA was amplified under the following conditions: 95 °C for 5 min for denaturation and subjected to 40 cycles of 95 °C for 45 s, 60 °C for 30 s, and 72 °C for 30 s. The relative expression of KRT10 was calculated based on $2^{-\Delta\Delta Ct}$ method.

2.6. Western blotting

Total protein was extracted as previously described [14]. Briefly, the cells were lyzed with RIPA lysis buffer (Beyotime, Haimen, Jiangsu, China) and then centrifuged at 12,000*g* for 15 min at 4 °C. The supernatants were collected for the next Western blot. Protein concentration was determined with BCA method. Protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to the Polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, US). The blot was then probed with primary antibody followed by reaction with horseradish peroxidase-conjugated secondary antibody. The signal was detected using enhanced chemiluminescence and recorded on X-ray film.

2.7. Co-immunoprecipitation

Forty-eight hours after transfection, cells were washed three times in PBS, harvested by scraping, and centrifuged for 5 min at 1000 rpm. The pelleted cells were lysed at 4 °C in 300 μl of cell lysis buffer (50 mM Tris HCl pH = 8, 120 mM NaCl, 1% Nonidet P-40 (NP-40), 2 mM EDTA, Protease Inhibitor Cocktail Tablets) for 30 min. The lysate was centrifuged at 14,000 rpm at 4 °C for 30 min, and the supernatants were combined with 10 μl of anti-CMYC agarose beads and mixed overnight at 4 °C. The immunoadsorbents were collected by centrifugation for 3 min at 3000 rpm and washed three times by resuspension and centrifugation (3 min at 3000 rpm) in cell lysis buffer. The samples were eluted into 15 μl of SDS loading buffer and heated for 5 min at 100 °C and subjected to SDS–PAGE electrophoresis. After electrophoretic transfer onto PVDF membranes as described above, the membranes were incubated with HA antibody and then with peroxidase-conjugated secondary antibody for detection of immunoreactive bands.

2.8. TUNEL assay

After cisplatin treated for 24 h, the cells were fixed with 4% paraformaldehyde and embedded with paraffin using standard methods. TUNEL assay was performed according to the method reported previously. The nuclei were stained by DAPI. Four different fields of each section were analyzed. The number of TUNEL-positive cells was divided by the total number of ovarian cancer cells to determine the apoptosis rate.

2.9. Statistical analysis

All the results are expressed as the means ± S.D. Statistical significance was determined using SPSS 17.0 for Windows. One-way anova was performed for multiple comparisons followed by Fisher

LSD post hoc comparisons. Differences were deemed significant if $P < 0.05$.

3. Results

3.1. Construction of stable C13K cell line overexpressing PTEN

After PTEN transfection, the monoclone C13K cell survived in the medium containing G418 (400 μg/ml), C13K/PTEN, was picked and cultured in the incubator. Protein was extracted from C13K/PTEN cells and subjected to Western blot analysis. The result showed that PTEN was highly expressed in the C13K/PTEN cells compared with that in normal C13K cells (Fig. 1A).

3.2. Overexpression of PTEN enhanced the sensitivity of C13K cells to cisplatin

To imitate the in vivo microenvironment, we used a 3D culture model in the testing of drug sensitivity of C13K/PTEN cells. MTT assay showed that cisplatin treatment significantly inhibited the proliferation of 3D cultured C13K/PTEN cells in a dose-dependent manner (Fig. 1B). But no significant difference was noted between the control cells and C13K cells transfected with control vector. Interestingly, overexpression of PTEN did not enhance the sensitivity of C13K to paclitaxel (Fig. 1C).

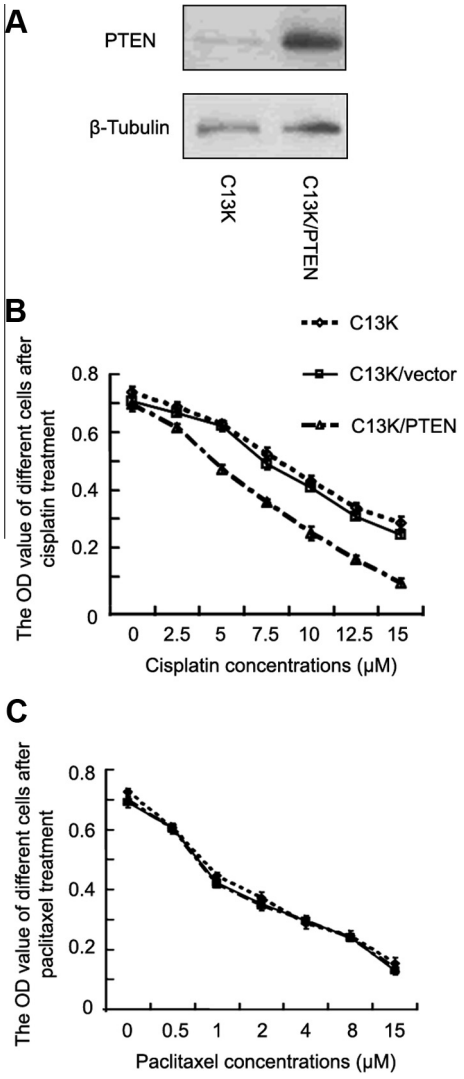

Fig. 1. PTEN overexpression enhanced the chemosensitivity of C13K cells to cisplatin. (A) PTEN was overexpressed in C13K/PTEN cells determined by Western blot. (B) The effect of cisplatin or paclitaxel (C) on the proliferation of ovarian cancer cells. C13K, C13K/Vector and C13K/PTEN cells were cultured in the 3D microenvironment in 96-well plate. When the cells grew to 80% confluence, indicated concentrations of cisplatin (B) or paclitaxel (C) were added into the wells for 48 h. Then MTT assay was performed and the OD value at 490 nm was determined. The data are expressed as means of OD ± S.D. ($n = 3$).

3.3. Identification of KRT10 as a downstream molecule of PTEN

To elucidate the mechanisms involved in the improvement of cisplatin-sensitivity of C13K cells by PTEN overexpression, co-immunoprecipitation was performed to explore the potential proteins that interacted with PTEN. As shown in Fig. 2, compared with control C13K/PTEN cells, an additional band was detected in the extraction of cisplatin-treated C13K/PTEN cells. Mass spectrometric analysis showed that it was KRT10.

3.4. Cisplatin upregulated KRT10 expression in C13K/PTEN cells

To further confirm the expression of KRT10 is induced by the treatment of cisplatin in C13K/PTEN cells, Western blot and real time RT-PCR were performed to determine the expression of KRT10 after 24 h treatment with cisplatin or paclitaxel. Cisplatin treatment significantly increased the KRT10 protein and mRNA levels in C13K/PTEN cells compared with control (Fig. 3A and B). However, paclitaxel did not alter the expression of KRT10. To further verify the interaction of PTEN and KRT10, co-immunoprecipitation was performed (Fig. 3C). The result showed that the level of KRT10 was obviously higher in the cisplatin-treated C13K/PTEN cells than vehicle-treated cells. These results indicate that cisplatin treatment may induce the overexpression of KRT10 in C13K/PTEN cells and there is a direct interaction between PTEN and KRT10 in C13K/PTEN cells.

3.5. Overexpression of KRT10 enhanced the chemosensitivity of C13K cells to cisplatin

In the above, we have demonstrated that overexpression of PTEN could improve the chemo-sensitivity of C13K cells to

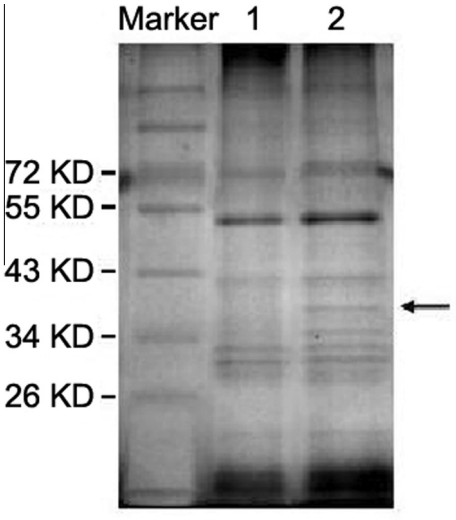

Fig. 2. Co-immunoprecipitation assay of the differently expressed proteins that interacted with PTEN in C13K/PTEN cells. Lane 1: C13K/PTEN cells; Lane 2: cisplatin-treated C13K/PTEN cell. The peptides sequences leading to the identification of the novel band are as follows: 1: AETECQNTEYQQLLDIK; 2: TIDDLKNQILNL TTDNANILLQIDNAR; 3: VLDELTLTKADLEMQIESLTEELAYLKK; 4: NVQALEIELQSQLALK; 5: ELTTEIDNNIEQISSYK; 6: SQYEQLAEQNRKDAEAWFNEK; 7: GSSGGGCFGGS SGGYGGLGGFGGGSFR; 8: ALEESNYELEGK; 9: DAEAWFNEK; 10: QSLEASLAETEGR.

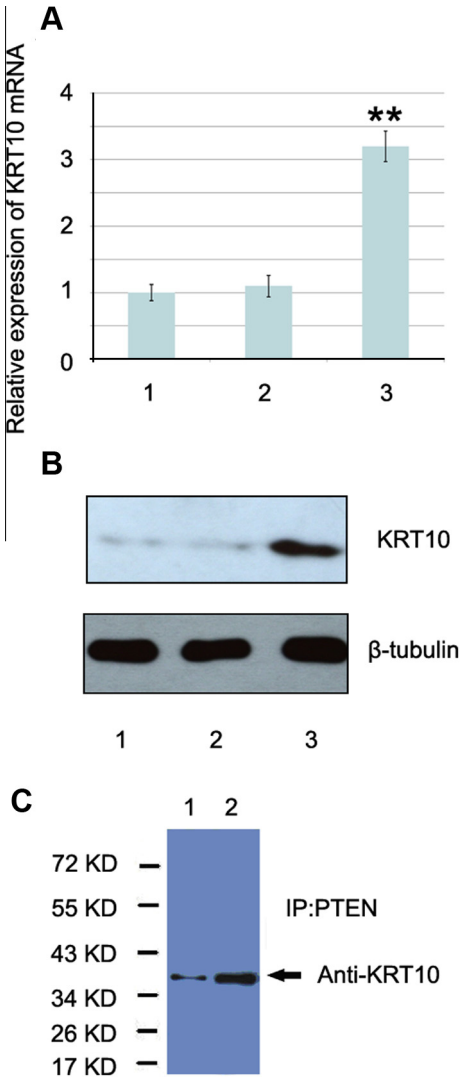

Fig. 3. Cisplatin induced KRT10 expression in C13K/PTEN cells. C13K/PTEN cells were treated with cisplatin (7.5 μM) or paclitaxel (2 μM) for 24 h. Then real time RT-PCR (A) and Western blot (B) were performed to determine the mRNA and protein levels of KRT10. Lane 1: C13K/PTEN cells; Lane 2: paclitaxel treated C13K/PTEN cells; Lane 3: cisplatin-treated C13K/PTEN cell. Each bar represents the mean ± S.D. ($n = 3$). ** $P < 0.01$, compared with C13K/PTEN cells. (C) Co-immunoprecipitation assay of the interaction of PTEN and KRT10 in C13K/PTEN cells. C13K/PTEN cells were treated with cisplatin or vehicle for 48 h, the interaction of PTEN and KRT10 was determined by co-immunoprecipitation assay. Lane 1, vehicle; lane 2, cisplatin.

cisplatin, but not to paclitaxel. Further study showed that cisplatin, but not paclitaxel, induced the overexpression of KRT10 in C13K/PTEN cells. Therefore, we wondered that whether the improvement of the chemo-sensitivity of C13K/PTEN cells to cisplatin was mediated by KRT10. Therefore, we investigated the role of KRT10 in the increase of cisplatin-sensitivity in C13K cells. After KRT10 expression plasmid was stably transfected into C13K cells (C13K/KRT10), cisplatin-sensitivity of C13K/KRT10 was tested. MTT assay showed that overexpression of KRT10 significantly enhanced the inhibitory effect of cisplatin on the growth of C13K cells (Fig. 4A). Overexpression of KRT10 also increased the apoptosis rate of C13K cells induced by cisplatin (Fig. 4B). These results suggest that overexpression of KRT10 enhances the chemosensitivity of C13K cells to cisplatin. Furthermore, we also examined the role of KRT10 siRNA in the increase of cisplatin-sensitivity in C13K/PTEN cells. As shown in Fig. 4C, KRT10 siRNA knocked down the KRT10 expression in cisplatin-treated C13K/PTEN cells. MTT assay showed that KRT10 siRNA blocked cisplatin-induced proliferation inhibition of C13K/PTEN cells, whereas the control siRNA did not affect the proliferation inhibition of cisplatin in C13K/PTEN cells.

4. Discussion

Resistance to platinum-based chemotherapy is a major cause of treatment failure in human ovarian cancer. Although overexpression of PTEN has been shown to be able to increase the sensitivity of human ovarian cancer cells to cisplatin, the mechanisms involved in the resistance-reversing effect of PTEN have not been clearly understood. In the present study, KRT10 was firstly identified as a downstream molecule of PTEN in C13K cells with overexpressed PTEN. Our results also showed that cisplatin, but not paclitaxel could induce the overexpression of KRT10 in C13K/PTEN cells and there was a direct interaction between PTEN and KRT10. Finally, we demonstrated that overexpression of KRT10 enhanced the chemosensitivity of C13K cells to cisplatin and knockdown of KRT10 blocked the proliferation inhibition effect of cisplatin on C13K/PTEN cells. These results suggest that KRT10 is a downstream molecule of PTEN and cisplatin-induced overexpression of KRT10 is involved in the resistance-reversing effect of PTEN in ovarian cancer cells.

The tumor suppressor PTEN has been implicated in a variety of human cancers [15], including gastric cancer [16], ovarian cancer [5] and hepatocellular carcinoma [17], etc. It is known that PTEN plays important roles not only in cell cycle detention and apoptosis, also in the regulation of cell adherence, migration and differentiation [18]. Recent years, PTEN was also reported to play an important role in the reversal of MDR [19,20]. In addition, gene therapy with PTEN has been found to be an effective way on enhancing chemosensitivity of multiple cancer cells [12,21] and PTEN has become a new potential target in sensitizing cancer cells to chemotherapy [22]. Consistently, our results showed that stable overexpression of PTEN in C13K ovarian cancer cells increased the sensitivity to cisplatin, but not paclitaxel, indicating there should be other molecules involved in the reversal of cisplatin-resistance of PTEN in C13K cells. Therefore, we further analyzed the difference in the proteins that interacted with PTEN in C13K/PTEN cells after vehicle or cisplatin treatment. Interestingly, an additional band was detected in the proteins extracted from cisplatin-treated cells compared with that from control cells. Further analysis showed that it was KRT10, which has been identified in numerous epithelial cells and tissues [23,24].

In previous studies, KRT10 has been reported to be involved in the inhibition of cell-cycle progression, keratinocyte proliferation [25,26,28], and keratinocyte turnover [27,28]. However, the role of KRT10 in the MDR of ovarian cancer has not been reported. Therefore, we next examined the role of KRT10 in the MDR reversal effect in ovarian cancer cells. Our results showed that overexpression of KRT10 increased the cytotoxicity and the apoptosis-induction capacity of cisplatin in C13K cells, suggesting that overexpression of KRT10 sensitizes the drug-resistant ovarian cancer cells C13K to cisplatin. In addition, knockdown of KRT10 with siRNA blocked the proliferation inhibition effect of cisplatin on C13K/PTEN cells. These data implicate that the effect of PTEN on the improvement of chemosensitivity in multi-drug resistant ovarian cancer cells is mediated by KRT10.

In summary, our data demonstrate that KRT10 is a downstream molecule of PTEN which improves cisplatin-resistance of ovarian cancer. Our study may provide a new insight to understand the mechanisms of the reversal effect of PTEN in multi-drug resistant ovarian cancer cells. Forced overexpression of KRT10 may also act as a therapeutic method for overcoming MDR in ovarian cancer.

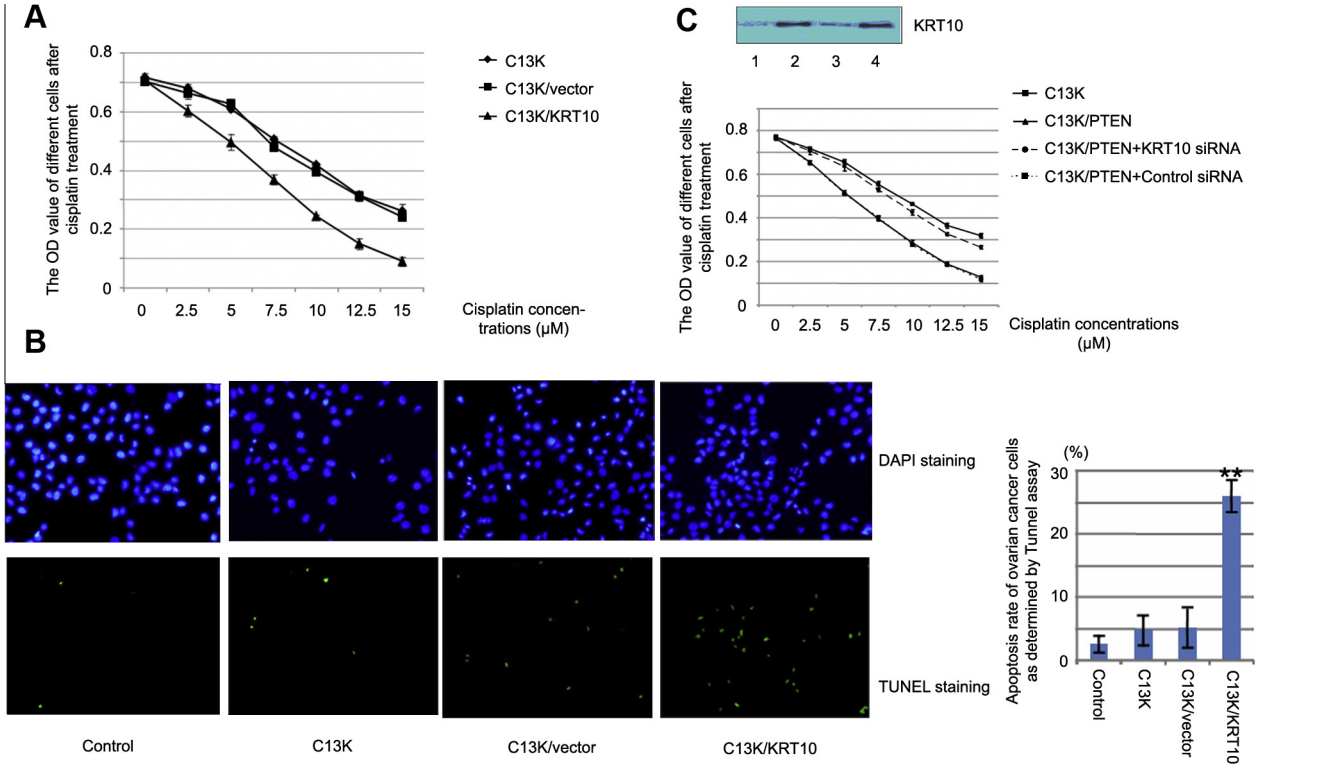

Fig. 4. Forced KRT10 expression enhanced the sensitivity of C13K cells to cisplatin. (A) KRT10 overexpression enhanced the proliferation inhibition of C13K cells induced by cisplatin. C13K cells were stably transfected with PCDNA3-HA/KRT10 or control vector. Then C13K, C13K/KRT10 or C13K/vector cells were seeded in 96-well plates in 3D microenvironment. After treated with indicated concentrations of cisplatin for 48 h, MTT assay was performed to determine the proliferation. Data are expressed as mean ± S.D. ($n = 3$). (B) KRT10 overexpression increased cisplatin-induced apoptosis rate of C13K cells. C13K cells were stably transfected with PCDNA3-HA/KRT10 or control vector. Then C13K, C13K/KRT10 or C13K/vector cells were seeded in 6-well plates in 3D microenvironment. After treated with or without indicated concentrations of cisplatin for 24 h, TUNEL assay was performed to determine the apoptosis of cells. (×100) Data are expressed as mean ± S.D. ($n = 3$). ** $P < 0.01$, compared with C13K/vector cells. (C) KRT10 siRNA blocked cisplatin-induced proliferation inhibition of C13K/PTEN cells. After C13K/PTEN cells were transfected with KRT10 siRNA or control siRNA, cells were seeded in 6-well and 96-well plates in 3D microenvironment. After treated with indicated concentrations of cisplatin for 48 h, the expression of KRT10 was determined by Western blot analysis and proliferation of cells was examined by MTT assay. Western blot: lane 1, C13K cells; lane 2, C13K/PTEN cells; lane 3, C13K/PTEN cells + KRT10 siRNA; lane 4, C13K/PTEN cells + control siRNA. The OD values are expressed as mean ± S.D. ($n = 3$).

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